Reduction in Microbial Manifestation on *In Vitro* Culture of *Eucalyptus Microcorys*

Laura Ribeiro Atala (✉ lauu_ra@hotmail.com)

Federal University of Lavras: Universidade Federal de Lavras <https://orcid.org/0000-0002-6243-3229>

Júlio César Tannure Faria

Federal University of Espírito Santo: Universidade Federal do Espírito Santo

Letícia V Molinari

Federal University of Lavras: Universidade Federal de Lavras <https://orcid.org/0000-0002-2543-4628>

Maria Lopes Martins Avelar

Federal University of Lavras: Universidade Federal de Lavras <https://orcid.org/0000-0001-6790-685X>

Gilvano Ebling Brondani

Federal University of Lavras: Universidade Federal de Lavras <https://orcid.org/0000-0001-8640-5719>

Research Article

Keywords: Eucalypts, Micropropagation, Streptomycin, Sodium hypochlorite.

Posted Date: May 6th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-445563/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Micropropagation is one of the main applications of tissue culture of plants. It allows rejuvenation and, consequently, improves explant rooting of woody species. However, one of its main problems is the risk of microbial manifestation. Here, we aimed to evaluate the survival and the reduction in microbial manifestations on *in vitro* cultures of *Eucalyptus microcorys* with the addition of chemical agents (sodium hypochlorite or streptomycin) to WPM culture medium. We used *in vitro* cultivated explants from adventitious shoots from two *E. microcorys* mother plants, over at 44 years-old. We drew from the multiplication phase explants with clumps of 8 to 12 shoots that displayed bacterial manifestation and submitted them to microbe control, adding different chemical agents at different concentrations to the culture medium: sodium hypochlorite (NaClO) and the antibiotic streptomycin. The results indicated that the best concentrations to reduce the manifestation of microorganisms on *in vitro* cultivation were 100 mg L⁻¹ of streptomycin and sodium hypochlorite with 0.003% of active chlorine, since these concentrations maintained 100% of the explants alive and reduced microbial manifestation more efficiently.

Introduction

Trees from the species-rich genera *Eucalyptus* and *Corymbia* are among the most cultivated in the world, due to their adaptation to different climate and soil conditions, fast growth rates and a large variety of products, such as wood, cellulose, fodder, biofuel, essential oils and bioactive compounds [1]. Among the several species within these genera, *Eucalyptus microcorys* F. Muell has favorable traits for silvicultural applications, including moderate resistance to frost, tolerance to fire, good regeneration capacity through resprouting, and resistance to wood rot caused by the fungus *Gloeophyllum trabeum* [2].

However, despite its potential for wood production, *E. microcorys* has been less employed in forest composition than other eucalypts species, and few scientific reports are found on the topic [3]. One of the main barriers for propagation is the low regenerative capacity caused by the ontogenetic aging of tissues [4], which reduces regeneration capacity and hinders adventitious rooting [5].

An alternative to overcome the issues related to woody species propagation is micropropagation, which is one of the main applications of plant tissue culture [6]. In addition to its practicality and greater impact for the mass multiplication of tree species [7], micropropagation is an alternative for tissue rejuvenation and, consequently, for the improvement of adventitious rooting in the clones production of *Eucalyptus* spp. and *Corymbia* spp. [8, 9].

However, microbial manifestation of the cultures, especially by fungi and bacteria, hamper explant multiplication and pose the main limitation for the micropropagation of woody species [10]. Microbial manifestations may arise from different sources, such as infected plant material (endophytic manifestation), inadequate handling of the explants and/or instruments, and precarious preparation of the culture medium or laboratory conditions [11].

Microbial manifestation is one of the main causes of economic loss in commercial and scientific laboratories devoted to tissue culture; therefore, controlling is essential for successful *in vitro* cultivation [12]. Accordingly, the prevention or elimination of microbial manifestation from *in vitro* cultivations has been the focus of several studies, ranging from the development of asepsis protocols and handling of mother plants, to the use of antimicrobial products in the culture medium [13].

A few disinfecting compounds have been used aiming to reduce or eliminate microorganisms from *in vitro* cultures, including chlorine-based compounds, such as sodium hypochlorite and calcium hypochlorite [14], and antibiotics [15]. Sodium hypochlorite (NaClO) has been widely used as a chemical sterilizer, both for explant asepsis before inoculation and as a supplement in the culture medium of herbaceous and woody species [16, 17]. In addition to its low cost, easy acquisition and efficiency, sodium hypochlorite is considered less toxic to plant tissue than other compounds under certain conditions [16, 18]. Chemical sterilization with sodium hypochlorite was initially proposed in studies in 2005 [19, 20] and, later, tested on *in vitro* cultures of other plant species including *Ananas comosus* L. cv *Smooth Cayenne* [18], *Sequoia sempervirens* L. [21], *Eucalyptus pellita* L. [22], *Eucalyptus benthamii* Maiden & Cabbage [16], *Hyptis leucocephala* Mart. ex Benth. and *Hyptis platanifolia* Mart. ex Benth [23], *Gerbera hybrida* cv. Essandre [24], *Cochlospermum regium* [25], *Bambusa vulgaris* [26, 27] and *Eucalyptus grandis* × *Eucalyptus urophylla* [28].

Like sodium hypochlorite, antibiotics can also be incorporated into culture media or used as a pre-treatment before explant inoculation *in vitro* [29, 30]. The antibiotic streptomycin, belonging to the group of aminoglycosides, has bactericidal effect for a broad range of gram-negative and gram-positive bacteria and is able to stop the onset of protein synthesis and/or reduce ongoing syntheses [31]. Streptomycin has been tested for controlling the manifestation of microorganisms on *in vitro* cultures of several species, such as *Helianthus tuberosus* [32], *Persea americana* [33], *Solanum tuberosum* [34], *Rauwolfia serpentina* [35], *Guadua angustifolia* Kunth [36], *Vitis vinifera* [37], *Butea monosperma* (Lam.) Kuntze var. *lutea* (Witt.) Maheswari [38] and *Bambusa vulgaris* [27].

Thus, given the importance of culture medium asepsis and the losses entailed by microbial manifestation in *in vitro* cultures, we aimed to evaluate the survival and reduction in microbial manifestation in *Eucalyptus microcorys* explants cultivated *in vitro* through the addition of sodium hypochlorite and streptomycin to the culture medium.

Material And Methods

Source and origin of explants

For the experiments, we used *in vitro* cultivated explants from *Eucalyptus microcorys* adventitious shoots. For the multiplication phase, the explants were kept in woody-plant medium (WPM) [39] supplied with 0.5 mg L⁻¹ of BAP (6-benzylaminopurine) and 0.05 mg L⁻¹ of NAA (α-naphthaleneacetic acid). The explants were cultivated *in vitro* for 18 months, subcultured in intervals of 25 days and kept in a growth room.

These explants cultivated *in vitro* originated from epicormic shoots from two *E. microcorys* mother plants, over at 44 years-old. These trees were sourced from a forest stand comprising different origins of *Eucalyptus* and *Corymbia* species, established in 1974 on the campus of the Federal University of Lavras (21°22'75" S, 44°96'98" W), in the municipality of Lavras – MG, Brazil.

Preparation of the culture medium for multiplication and *in vitro* conditions

We prepared the culture medium with distilled water, 6 g L⁻¹ of agar and 20 g L⁻¹ of sucrose (C₁₂H₂₂O₁₁/342.30 g). Before adding the agar, we adjusted the pH of the culture medium to 5.8 with HCl and/or NaOH. The solution was autoclaved at 121°C (~1.0 kgf cm⁻²) for 20 minutes. We added BAP and NAA to the culture medium before autoclaving. We cultivated the explants in a growth room at 24°C (±1°C), with a 16-hour photoperiod and light intensity of 40 µmol m⁻² s⁻¹.

Reduction in microbial manifestation

We drew from the multiplication phase explants with clumps of 8 to 12 shoots that displayed bacterial manifestation and submitted them to microbe control, adding different chemical agents at different concentrations to the culture medium: sodium hypochlorite (NaClO) and the antibiotic streptomycin (Table 1).

Table 1. Description of chemical agent concentrations added to the culture media to reduce microbial manifestation in *Eucalyptus microcorys* explants

Treatment	Chemical agent	Concentration of active chlorine
1	Sodium hypochlorite	0.001%
2	Sodium hypochlorite	0.003%
3	Sodium hypochlorite	0.005%
4	Streptomycin	100 mg L ⁻¹
5	Streptomycin	250 mg L ⁻¹
6	Streptomycin	500 mg L ⁻¹

Before their addition to the autoclaved culture media, we exposed the streptomycin and sodium hypochlorite to ultraviolet light for 15 minutes in a laminar flow chamber. The streptomycin was added to the culture medium with a syringe coupled to a Millipore® filter of 0.22 µm of pore size. The sodium hypochlorite was added with a pipette. All utensils were previously autoclaved. After adding the chemical agents, we homogenized the culture media and poured them into test tubes where the explants were cultivated *in vitro*. Each test tube (2.0 × 10.0 cm) contained 12 mL of WPM supplemented with 0.5 mg L⁻¹ of BAP and 0.05 mg L⁻¹ of NAA.

Each treatment had 20 replications and followed a completely randomised design in a factorial arrangement (2×6) evaluating the interaction between two individuals of *Eucalyptus microcorys* and six concentrations of the chemical agents targeted at reducing the manifestation of microorganisms. The explants were kept in a growth room and subcultured every 25 days. 75 days following each treatment, we evaluated the percentage of explant survival and the percentage of reduction in microbial manifestation in the culture medium. The methodological sequence is described in Figure 1.

Statistical analysis

We submitted the collected data to a Hartley test ($p > 0.05$) to assess variance homogeneity among treatments and to a Shapiro-Wilk test to ($p > 0.05$) to assess data normality. When needed, the data were Box-Cox transformed to meet normality assumptions. We then performed an analysis of variance (ANOVA, $p < 0.05$) and, accordingly to its significance, compared the data from the qualitative factors through a Scott-Knott test ($p < 0.05$). We performed these analyses in the environment R version 3.5.2 using the package ExpDes [40].

Results And Discussion

Survival of explants

According to the analysis of variance, there was no significant effect between the factor individual and survival percentage. Survival only varied significantly with the chemical agent used. Additionally, no interaction between individuals and chemical agents was found.

The highest survival percentages of *Eucalyptus microcorys* (above 87%) were achieved with the addition of sodium hypochlorite at the three concentrations (0.001%; 0.003% or 0.005% of active chlorine) and with the addition of streptomycin at 100 mg L⁻¹ to the culture media. However, when streptomycin was added at 250 mg L⁻¹ and 500 mg L⁻¹, the percentage of explant survival decreased (Table 2).

In vitro plant production is successful when contamination-related losses do not exceed 2.0% *per* subculture during micropropagation. According to this, we found satisfactory results with sodium hypochlorite at 0.001% and 0.003%, and streptomycin at 100 mg L⁻¹ [41]. These treatments resulted in explant survival percentages between 95% and 100% after the third subculture.

The chemical sterilization of *in vitro* culture media with sodium hypochlorite has been tested for several species. The most commonly used concentration is 0.003% of active chlorine. Higher concentrations may be phytotoxic to the cultures, as shown in *in vitro* cultures of *Eucalyptus benthamii* and *Prunus mume* [42]. In *Eucalyptus benthamii*, active chlorine concentrations above 0.005% reduced the percentage of viable shoots [16]; and in *Prunus mume*, the addition of 0.001% of active chlorine reduced explant survival percentage [43]. Our results for *Eucalyptus microcorys* agree with those reported in the literature: the addition of sodium hypochlorite to the culture medium at 0.001% and 0.003% maintained 100% of explants alive, whereas its addition at 0.005% resulted in an explant survival percentage of 87.5%.

In the literature, evaluating the effect of chemical sterilization on the culture medium of *in vitro* *Cochlospermum regium* multiplication, we found the highest percentages of explant survival at 0.001% and 0.005% of active chlorine concentration: 75% and 65% of survival, respectively [25]. In *Bambusa vulgaris*, the highest survival percentage (68%) was found when sodium hypochlorite was added at 0.004% [26]. In this study, we found similar results to those from the literature, with an explant survival percentage of 87.5% after the addition to the culture medium of sodium hypochlorite at 0.005% of active chlorine. In our study, increasing the concentration of active chlorine above 0.003% on the culture medium increased explant mortality, although not significantly (Table 2).

Many authors have reported the use of antibiotics to control and/or eliminate microorganisms from *in vitro* cultures [13, 44, 45]. But depending on antibiotic concentration and the tolerance of the focal species, antibiotics may also have phytotoxic effects on the culture and alter *in vitro* plant growth [46, 47]. In our study, the use of streptomycin at 250 and 500 mg L⁻¹ reduced the survival percentage of *Eucalyptus microcorys* explants.

In the literature, we find reports of a decrease in *Solanum tuberosum* explant survival with increasing addition of streptomycin in the nutrient medium [34]. At 64 mg L⁻¹ and 128 mg L⁻¹, the use of streptomycin was followed by explant survival percentages around 90% and 80%; whereas at 1,024 mg L⁻¹, survival was around 50%. Our results, of decreasing explant survival with increasing streptomycin concentration, agree with the literature. At 100 mg L⁻¹, the addition of streptomycin was followed by 95% of explant survival; whereas at 250 mg L⁻¹ and 500 mg L⁻¹, explant survival was 82.5% and 85%, respectively.

The use of streptomycin was also tested in *in vitro* cultures of *Butea monosperma* (Lam.) Kuntze var. *lutea* (Witt.) Maheswari. In this case, the highest explant survival (90.8%) was obtained in a liquid WPM culture medium supplied with 400 µl.L⁻¹ of Plant Preservative Mixture (PPM) together with streptomycin at 250 mg L⁻¹ [38]. In our focal species, *Eucalyptus microcorys*, streptomycin at 250 mg L⁻¹ maintained 82.5% of explants alive.

On *in vitro* cultures of *Persea americana*, the addition of streptomycin at concentrations between 12.5 to 200 mg L⁻¹ was shown to have a toxic effect [33]. Therefore, the use of streptomycin would only be recommended in extreme cases, depending on the susceptibility of the manifesting microorganism and the concentration needed to control it. In the micropropagation of *Helianthus tuberosus* the use of streptomycin above 50 mg L⁻¹ inhibits shoot emission [32]. Conversely, our results showed a satisfactory response of *Eucalyptus microcorys* to the addition of streptomycin at 100 mg L⁻¹, which resulted in an average explant survival of 95%.

Table 2. Survival percentage of *Eucalyptus microcorys* explants 75 days after the addition of each chemical agent to the culture medium.

Chemical agent		Survival (%)
Sodium hypochlorite [0.001%] Active chlorine		100.0 ^a
Sodium hypochlorite [0.003%] Active chlorine		100.0 ^a
Sodium hypochlorite [0.005%] Active chlorine		87.5 ^a
Streptomycin	[100 mg L ⁻¹]	95.0 ^a
Streptomycin	[250 mg L ⁻¹]	82.5 ^b
Streptomycin	[500 mg L ⁻¹]	85.0 ^b

Averages followed by the same letter in the *Survival* (%) column do not differ statistically from each other, according to a Scott-Knott test

Reduction of microbial manifestation

The analysis of variance revealed a significant effect between individuals and the reduction in microbial manifestation. The chemical agent used had a significant effect on the reduction of microbial manifestation, and a significant interaction was found between individual and chemical agent. Individual 1 displayed statistically undistinguishable responses to all chemical agents and concentrations in terms of reduction in microbial manifestation. But individual 2 displayed statistically different responses depending on the chemical agent and its concentration (Table 3).

The culture medium supplied with streptomycin at 100 mg L⁻¹ displayed the best result: it reduced microbial manifestation in 65% of individual 2 explants and 15% of individual 1 explants. The addition of sodium hypochlorite at 0.003% of active chlorine reduced microbial manifestation in 25% of individual 2 explants and 5% of individual 1 explants. The other treatments did not contribute to reduce microbial manifestation in either individual (Table 3).

In addition to their better responses to the use of chemical agents for reducing microbial manifestation, explants from individual 2 were also more vigorous. In general, the success of *in vitro* cultures may vary depending on the genetic material, growing conditions, type of explant, asepsis, phytotoxicity and physiological aspects of the mother plant [16]. The latter aspect was likely responsible for the difference in vigor that we found in the explants of the two individuals (or mother plants). The different physiological conditions of the mother plants probably influenced the physical aspects of the explants and their responses in terms of the reduction in microbial manifestation.

In *in vitro* cultures of *Butea monosperma* (Lam.) Kuntze var. *lutea* (Witt.) Maheswari, the addition of streptomycin at 250 mg L⁻¹ (along with 400 µl L⁻¹ of Plant Preservative Mixture (PPM) in a WPM liquid

medium) was shown to be efficient, resulting in the lowest percentage of microbial manifestation (22.2%) [38]. In *Vitis vinifera*, the addition of streptomycin at 100 mg L⁻¹ did not control the manifestation of bacterial canker, and after 40 days of *in vitro* cultivation, 100% of the explants remained with the bacterium [37]. Unlike these reports, our results for *Eucalyptus microcorys* showed that the addition of streptomycin at 250 mg L⁻¹ did not reduce microbial manifestation in the culture medium; whereas at 100 mg L⁻¹, microbial manifestation decreased in 65% of the explants of individual 2.

Streptomycin at the concentration of 100 mg L⁻¹ has been considered efficient to control microbial manifestation in other *in vitro* plant cultures: in *Musa* spp. (banana tree), it reduced microbial manifestation [48]; in *Solanum tuberosum* (potato), it inhibited the growth of bacterial manifestation [34]; and in *Rauwolfia serpentina*, it completely removed apex shoot microbial manifestations [35]. These reports align with our findings for *in vitro* cultures of *Eucalyptus microcorys*, whereby streptomycin at 100 mg L⁻¹ showed the best result by eliminating microbial manifestations in 65% of all explants of individual 2.

In the genus *Aglaonema*, there is a direct relationship between streptomycin concentration and microbial reduction [49]. Increasing the concentration of streptomycin from 25 to 400 mg L⁻¹ decreased microbial manifestations percentages from 83.3% to 0%. Here, we found an inverse relationship between these variables: increasing concentrations of streptomycin were followed by decreasing efficiency in terms of reduction of microorganism manifestation.

Regarding sodium hypochlorite, several studies have reported its effects on the reduction of microbial manifestation in culture media. At low active chlorine concentrations (up to 0.005%), sodium hypochlorite has proven viable in controlling bacterial manifestation in *in vitro* cultures of *Eucalyptus benthamii* at the establishment phase [16], *Eucalyptus pellita* at the multiplication phase [22], *Sequoia sempervirens* L. [21], the hybrid *Gerbera hybrida* cv. Essandre [24], *Cochlospermum regium* [25] and *Bambusa vulgaris* [26]. Our results for *Eucalyptus mycrocoris* agree with the literature: sodium hypochlorite at 0.003% produced the best response by eliminating microbial manifestations in 25% of individual 2 explants and 5% of individual 1 explants.

Table 3. Percentages of reduction in microbial manifestation (RMM) in *Eucalyptus microcorys* explants relative to chemical agent added to the culture medium and individual assessed; data collected 75 days after the experiment

Chemical agent	RMM (%)	
	Individual 1	Individual 2
[0,001%] Active chlorine	0 ^{Aa}	0 ^{Ac}
[0,003%] Active chlorine	5 ^{Ba}	25 ^{Ab}
[0,005%] Active chlorine	0 ^{Aa}	0 ^{Ac}
[100 mg L ⁻¹] Streptomycin	15 ^{Ba}	65 ^{Aa}
[250 mg L ⁻¹] Streptomycin	0 ^{Aa}	0 ^{Ac}
[500 mg L ⁻¹] Streptomycin	0 ^{Aa}	0 ^{Ac}

Averages followed by the same lower-case letter along the column and the same upper-case letter along the row do not differ statistically according to a Scott-Knott test

The addition of chemical agents to *in vitro* cultures is a viable alternative to avoid culture loss and discard. But it is essential to choose a suitable concentration and carefully handle these products to avoid phytotoxicity to the plants and achieve success [50], considering the possibility that the microorganisms build up resistance against these chemical agents [31]. Our results pointed to sodium hypochlorite as a consistent option for its low cost, for its efficiency and for lacking phytotoxic effects on *in vitro* cultures of *Eucalyptus microcorys*. The antibiotic streptomycin, at 100 mg L⁻¹, was also efficient as it was able to reduce microbial manifestation without interfering in the viability or survival of explants.

Conclusion

The addition of sodium hypochlorite and streptomycin to the culture media was efficient for the survival of explants and for the reduction in microbial manifestation in *in vitro* cultures of *Eucalyptus microcorys*.

Individuals 1 and 2 displayed similar responses in terms of survival. Regarding reductions in microbial manifestation, explants of individual 2 displayed a better response to the chemical agents than individual 1.

The best result was achieved with the addition of streptomycin at 100 mg L⁻¹ to the culture medium, which led to 95% of explant survival and 65% of reduction in microbial manifestation in individual 2.

The addition of sodium hypochlorite at 0.003% to the culture medium resulted in an explant survival percentage of 100%, but was less efficient than streptomycin, which eliminated microbial manifestation in 25% of the explants of individual 2.

Declarations

Funding- We thank the National Council for Scientific and Technological Development, Brazil (Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq), Coordination for Improvement of Higher Education Personnel, Brazil (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES – Código de Financiamento 001), and Foundation for Research of the State of Minas Gerais, Brazil (Fundação de Amparo a Pesquisa do Estado de Minas Gerais - FAPEMIG).

Conflicts of interest/Competing interests- Not applicable

Availability of data and material- Not applicable

Code availability- Not applicable

Authors' contributions – Laura Ribeiro Atala and Dr. Júlio César Tannure Faria- contributed to the design and development of the study, establishment of the experiment, data collection, processing and statistical analysis, writing and construction of graphs and tables; M.Sc. Letícia Vaz Molinari and M.Sc. Maria Lopes Martins Avelar- analyzed and interpreted the data, contributed to the discussion of the results and text revision; Prof. Gilvano Ebling Brondani – supervisor, analyses, review, discussion of theme.

Ethics approval- Not applicable

Consent to participate- Not applicable

Consent for publication- Not applicable

References

1. Trueman, S. J., Hung, C. D., & Wendling, I. (2018). Tissue culture of *Corymbia* and *Eucalyptus*. *Forests*, 9(2), 1–42. <https://doi.org/10.3390/f9020084>.
2. Marchesan, R., de Mattos, P. P., & Shimizu, J. (2005). Caracterização física, química e anatômica da madeira de *Eucalyptus microcorys* F. Muell. *Embrapa Florestas-Comunicado Técnico (INFOTECA-E)*, 133.
3. Oliveira, F. N., Fortes, G. A., Paula, J. R., Ferri, P. H., & Santos, S. C. (2014). Seasonal influence on the essential oil of *Eucalyptus microcorys*. *Natural product communications*, 9(4), 575–580. <https://doi.org/10.1177/1934578X1400900439>.
4. Davies, F. T., Geneve, R. L., Wilson, S. B., Hartmann, H. T., & Kester, D. E. (2017). Hartmann & Kester's Plant propagation: principles and practices. Ed. Pearson, 9^a edition, 1024 p.
5. Tormen, G. C. R., de Figueiredo, A. J. R., Ribeiro, A. D. S., dos Santos, L. F., Araujo, J. F., Brondani, G. E., & da Silva, A. L. L (2018). Carbohydrate sources, alanine and calcium for *in vitro* multiplication of *Eucalyptus cloeziana* F. Muell. *Iheringia, Série Botânica*, 73(3), 329–335. <https://doi.org/10.21826/2446-8231201873309>.

6. Paiva, H. N., & Gomes, J. M. (2011). *Propagação vegetativa de espécies florestais* (1. ed., 52 p). Viçosa: UFV.
7. Brondani, G. E., Dutra, L. F., Grossi, F., Wendling, I., & Hornig, J. (2009). Estabelecimento, multiplicação e alongamento *in vitro* de *Eucalyptus benthamii* Maiden & Cabbage × *Eucalyptus dunnii* Maiden. *Revista Árvore*, 33(1), 11–19. <https://doi.org/10.1590/S0100-67622009000100002>.
8. de Oliveira, L. S., Dias, P. C., & Brondani, G. E. (2013). Micropropagação de espécies florestais brasileiras. *Pesquisa florestal brasileira*, 33(76), 439–453. <https://doi.org/10.4336/2013.pfb.33.76.481>.
9. Brondani, G. E., Araujo, M. A. D., Alcântara, B. K. D., Carvalho, J. G. D., Gonçalves, A. N., & Almeida, M. D. (2012). *In vitro* organogenesis of *Eucalyptus grandis*: effects of boron and calcium. *Acta Scientiarum. Agronomy*, 34(4), 403–411. <https://doi.org/10.4025/actasciagron.v34i4.15143>.
10. Ray, S. S., & Ali, N. (2017). Biotic Contamination and Possible Ways of Sterilization: A Review with Reference to Bamboo Micropropagation. *Brazilian Archives of Biology and Technology*, 60. <https://doi.org/10.1590/1678-4324-2016160485>.
11. Bavaresco, L. G., Pasquali, R., & Fluminhan, A. (2017). Cultivo *in vitro* de explantes removidos de plantas cultivadas a campo visando à micropropagação de *Eucalyptus citriodora*. *Periódico Eletrônico Fórum Ambiental da Alta Paulista*, 13(6). <http://dx.doi.org/10.17271/1980082713620171714>.
12. Khan, T., Abbasi, B. H., Iqar, I., Khan, M. A., & Shinwari, Z. K. (2018). Molecular identification and control of endophytic contamination during *in vitro* plantlet development of *Fagonia indica*. *Acta Physiologiae Plantarum*, 40(8), 1–9. <https://doi.org/10.1007/s11738-018-2727-3>.
13. Palú, E. G., Corrêa, L. D. S., Suzuki, A. N., & Boliani, A. C. (2011). Uso de antibióticos para o controle de bactérias endógenas visando à micropropagação da figueira. *Revista Brasileira de Fruticultura*, 33(2), 587–592. <https://doi.org/10.1590/S0100-29452011000200031>.
14. Santos, J. D., Pinheiro, M. V. M., Fontana, D. C., Schmidt, D., & Pretto, M. M. (2019). Estabelecimento *in vitro* de oliveira 'Arbequina' e 'Koroneiki'. *Ciência Florestal*, 29(2), 508–518. <https://doi.org/10.5902/1980509831305>.
15. Pereira, G. A., Ribeiro, B. V., Marcilio, H. C., & Santaella, M. B. (2009). Desinfestação e estabelecimento *in vitro* de explantes de bananeira IAC 2001 em diferentes concentrações de hipoclorito de sódio. *Tecnologia & Ciência Agropecuária*, 3(2), 43–46.
16. Brondani, G. E., de Oliveira, L. S., Bergonci, T., Brondani, A. E., França, F. A. M., da Silva, A. L. L., & Gonçalves, A. N. (2013). Chemical sterilization of culture medium: a low cost alternative to *in vitro* establishment of plants. *Scientia Forestalis*, 41(98), 257–264.
17. Brondani, G. E., OLIVEIRA, L. S., Konzen, E. R., Silva, A. L. D., & Costa, J. L. (2018). Mini-incubators improve the adventitious rooting performance of *Corymbia* and *Eucalyptus* microcuttings according to the environment in which they are conditioned. *Anais da Academia Brasileira de Ciências*, 90(2), 2409–2423. <https://doi.org/10.1590/0001-3765201720170284>.

18. Teixeira, S. L., Ribeiro, J. M., & Teixeira, M. T. (2006). Influence of NaClO on nutrient medium sterilization and on pineapple (*Ananas comosus* cv *Smooth cayenne*) behavior. *Plant Cell, Tissue and Organ Culture*, 86(3), 375–378. <https://doi.org/10.1007/s11240-006-9121-3>.
19. Teixeira, S. L., Teixeira, M. T., & Ribeiro, J. M. (2005). Chemical sterilization of culture medium: 1. culture flasks and covers—rinsing with chlorinated water. *Hort Bras (Suppl)*, 23, 591.
20. Teixeira, S. L., Teixeira, M. T., & Ribeiro, J. M. (2005). Chemical sterilization of culture medium: 2 addition of sodium hypochlorite to the medium. *Hort Bras (Suppl)*, 23, 591.
21. Ribeiro, J. M., Teixeira, S. L., & Bastos, D. C. (2011). Cultivo *in vitro* de *Sequoia sempervirens* L. em meio de nutritivo esterilizado com hipoclorito de sódio. *Ciência Florestal*, 21(1), 77–82. <https://doi.org/10.5902/198050982749>.
22. Teixeira, S. L., Ribeiro, J. M., & Teixeira, M. T. (2008). Utilização de hipoclorito de sódio na esterilização de meio de cultura para multiplicação *in vitro* de *Eucalyptus pellita* L. *Ciência Florestal*, 18(2), 185–191. <https://doi.org/10.5902/19805098456>.
23. Nepomuceno, C. F., Fonseca, P. T., Silva, T. S., Oliveira, L. M., & Santana, J. R. F. (2014). *In vitro* germination of *Hyptis leucocephala* Mart. ex Benth. and *Hyptis platanifolia* Mart. ex Benth. *Revista Brasileira de Plantas Medicinais*, 16(4), 886–895. https://doi.org/10.1590/1983-084X/12_093.
24. Pais, A. K., da Silva, A. P., de Souza, J. C., Teixeira, S. L., Ribeiro, J. M., Peixoto, A. R., & da Paz, C. D. (2016). Sodium hypochlorite sterilization of culture medium in micropropagation of *Gerbera hybrida* cv. Essandre. *African Journal of Biotechnology*, 15(36), 1995–1998. <https://doi.org/10.5897/AJB2016.15405>.
25. Gavilan, N. H., Furlan, F. C., Zorz, A. Z., Oliveira, L. S. D., Campos, W. F., & Brondani, G. E. (2018). Chemical sterilization of culture medium for *in vitro* multiplication of *Cochlospermum regium*. *Ciência Rural*, 48(9). <https://doi.org/10.1590/0103-8478cr20170581>.
26. Furlan, F. C., Gavilan, N. H., Zorz, A. Z., de Oliveira, L. S., Konzen, E. R., & Brondani, G. E. (2018). Active chlorine and charcoal affect the *in vitro* culture of *Bambusa vulgaris*. *Bosque*, 39(1), 61–70. <https://doi.org/10.4067/S0717-92002018000100061>.
27. dos Santos Ribeiro, A., de FIGUEIREDO, A. J. R., Tormen, G. C. R., da SILVA, A. L. L., Campos, W. F., & Brondani, G. E. (2020). Clonal bamboo production based on *in vitro* culture. *Bioscience Journal*, 36(4). <https://doi.org/10.14393/BJ-v36n4a2020-48169>.
28. Molinari, L. V., Souza, D. M. S. C., Avelar, M. L. M., Fernandes, S. B., Gonçalves, D. S., Faria, J. C. T., ... Brondani, G. E. (2020). Effects of chemical sterilization of the culture media, porous membranes and luminosity on *in vitro* culture of *Eucalyptus grandis* × *Eucalyptus urophylla*. *Journal of Forestry Research*, 1–12. <https://doi.org/10.1007/s11676-020-01240-5>.
29. Kneifel, W., & Leonhardt, W. (1992). Testing of different antibiotics against Gram-positive and Gram-negative bacteria isolated from plant tissue culture. *Plant cell, tissue and organ culture*, 29(2), 139–144. <https://doi.org/10.1007/BF00033619>.
30. Kritzing, E. M., Van Vuuren, R. J., Woodward, B., Rong, I. H., Spreeth, M. H., & Slabbert, M. M. (1997). Elimination of external and internal contaminants in rhizomes of *Zantedeschia aethiopica* with

- commercial fungicides and antibiotics. In *Pathogen and microbial contamination management in micropropagation* (pp. 161–167). Dordrecht: Springer. https://doi.org/10.1007/978-94-015-8951-2_19.
31. Pollock, K., Barfield, D. G., & Shields, R. (1983). The toxicity of antibiotics to plant cell cultures. *Plant cell reports*, 2(1), 36–39. <https://doi.org/10.1007/BF00269232>.
 32. Phillips, R., Arnott, S. M., & Kaplan, S. E. (1981). Antibiotics in plant tissue culture: Rifampicin effectively controls bacterial contaminants without affecting the growth of short-term explant cultures of *Helianthus tuberosus*. *Plant Science Letters*, 21(3), 235–240. [https://doi.org/10.1016/0304-4211\(81\)90094-8](https://doi.org/10.1016/0304-4211(81)90094-8).
 33. Biasi, L. A. (1995). Fitotoxicidade de três antibióticos na cultura *in vitro* de abacateiro. *Bragantia*, 54(2), 251–256. <https://doi.org/10.1590/S0006-87051995000200003>.
 34. Pereira, J. E. S., & Fortes, G. R. D. L. (2003). Toxicidade de antibióticos no cultivo *in vitro* da batata em meios semi-sólido e líquido. *Pesquisa Agropecuária Brasileira*, 38(11), 1273–1279. <https://doi.org/10.1590/S0100-204X2003001100004>.
 35. Bahuguna, R. N., Joshi, R., Singh, G., Shukla, A., Gupta, R., & Bains, G. (2011). Micropropagation and total alkaloid extraction of Indian snake root (*Rauwolfia serpentina*). *Indian J Agric Sci*, 81, 1124–1129.
 36. Nadha, H. K., Salwan, R., Kasana, R. C., Anand, M., & Sood, A. (2012). Identification and elimination of bacterial contamination during *in vitro* propagation of *Guadua angustifolia* Kunth. *Pharmacognosy magazine*, 8(30), 93. <https://doi.org/10.4103/0973-1296.96547>.
 37. SILVA, A. M. F., de MELO, N. F., de SOUZA, E. B., COELHO, A. D. S., & MARINHO, R. (2013). Limpeza clonal de mudas de videira infectadas por *Xanthomonas campestris* pv. *viticola*. *Embrapa Semiárido-Artigo em periódico indexado (ALICE)*.
 38. Kanjanawattanawong, S., Singbumrung, N., Rianthong, T., & Wamaedeesat, R. (2020). ITS2 Nucleotide Sequence Analysis and Cleaned Culture Production of *Butea monosperma* var. *lutea*. *Princess of Naradhiwas University Journal*, 12(1), 173–184.
 39. Lloyd, G., & McCown, B. (1980). Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. *Commercially-feasible micropropagation of mountain laurel, Kalmia latifolia, by use of shoot-tip culture.*, 30, 421–427.
 40. Ferreira, E. B., Cavalcanti, P. P., & Nogueira, D. A. (2014). ExpDes: an R package for ANOVA and experimental designs. *Applied Mathematics*, 5(19), 2952.
 41. Leifert, C., & Woodward, S. (1997). Laboratory contamination management; the requirement for microbiological quality assurance. In *Pathogen and microbial contamination management in micropropagation* (pp. 237–244). Dordrecht: Springer. https://doi.org/10.1007/978-94-015-8951-2_30.
 42. Cardoso, J. C., & Imthurn, A. C. P. (2018). Esterilização química do meio de cultura para o cultivo *in vitro* de gérbera utilizando dióxido de cloro (ClO₂). *Ornamental Horticulture*, 24(3), 218–224. <https://doi.org/10.14295/oh.v24i3.1222>.

43. Vargas, D. P., Formoso, R. S., Dutra, L. F., Meyer, N. A., dos Santos, J., & Ueno, B. (2016). Esterilização química para o cultivo *in vitro* de porta-enxerto de pessegueiro. In *Colloquium Agrariae*. ISSN: 1809–8215 (Vol. 12, No. 1, pp. 01–06).
44. Wilson, Z. A., & Power, J. B. (1989). Elimination of systemic contamination in explant and protoplast cultures of rubber (*Hevea brasiliensis* Muell. Arg.). *Plant Cell Reports*, 7(8), 622–625. <https://doi.org/10.1007/BF00272044>.
45. Leifert, C., Ritchie, J. Y., & Waites, W. M. (1991). Contaminants of plant-tissue and cell cultures. *World Journal of Microbiology and Biotechnology*, 7(4), 452–469. <https://doi.org/10.1007/BF00303371>.
46. Dodds, J. H., & Roberts, L. W. (1985). *Experiments in plant tissue culture*. International Potato Center.
47. Okkels, F. T., & Pedersen, M. G. (1987). The toxicity to plant tissue and to *Agrobacterium tumefaciens* of some antibiotics. *Bacterial and Bacteria-like Contaminants of Plant Tissue Cultures*, 225, 199–208. <https://doi.org/10.17660/ActaHortic.1988.225.23>.
48. Oliveira, H. S. D., Lemos, O. F. D., Miranda, V. S., Moura, H. C. D. P., Campelo, M. F., & Santos, L. R. R. D. (2011). Estabelecimento e multiplicação *in vitro* de brotos no processo de micropropagação de cultivares de bananeira (*Musa* spp.). *Acta Amazonica*, 41(3), 369–376. <https://doi.org/10.1590/S0044-59672011000300006>.
49. Chen, W. L., & Yeh, D. M. (2007). Elimination of *in vitro* contamination, shoot multiplication, and ex vitro rooting of *Aglaonema*. *HortScience*, 42(3), 629–632. <https://doi.org/10.21273/HORTSCI.42.3.629>.
50. Kulkarni, A. P., Mahal, H. S., Kapoor, S., & Aradhya, S. M. (2007). *In vitro* studies on the binding, antioxidant, and cytotoxic actions of punicalagin. *Journal of agricultural and food chemistry*, 55(4), 1491–1500. <https://doi.org/10.1021/jf0626720>.

Figures

Figure 1

Illustration of the experimental methodology employed to reduce microbial manifestation in *Eucalyptus microcorys* explants in vitro cultivated