SCIENTIA FORESTALIS

ISSN 2318-1222 (online)

ORIGINAL ARTICLE

Plant production of *Ocotea odorifera* (Vell.) Rohwer by the micro-propagation technique

Produção de mudas de *Ocotea odorifera* (Vell.) Rohwer por meio da técnica de micropropagação

Juscelina Arcanjo Santos[1], Natália Ribeiro Paula[1], Denys Matheus Santana Costa Souza[1], Dulcinéia de Carvalho[1], Gilvano Ebling Brondani[1]

[1]Universidade Federal de Lavras – UFLA, Lavras, MG, Brasil

How to cite: Santos, J. A., Paula, N. R., Souza, D. M. S. C., Carvalho, D., & Brondani, G. E. (2022). Plant production of *Ocotea odorifera* (Vell.) Rohwer by the micro-propagation technique. *Scientia Forestalis*, *50*, e3759. https://doi.org/10.18671/scifor.v50.04

Abstract

Due to the intense exploitation of its essential oil over the years, *Ocotea odorifera* was included in the endangered species in Brazil. In this context, the micro-propagation technique is an alternative to sexual propagation, and consequently, it favors the conservation of the species. Thus, the objective of this study was to produce seedlings of *O. odorifera* through the micro-propagation technique. Seed collection was carried out in mother trees distributed in a natural stand. *In vitro* cultivation was divided into three phases: (i) *in vitro* germination and establishment; (ii) elongation and rooting and (iii) acclimatization. For explant disinfestation, a 3x2 factorial was used: three sources of propagules (seed, embryo and nodal segment) combined with two immersion times in NaClO (50%) (5 and 10 min), totaling 6 treatments. In the *in vitro* establishment, two culture media (MS supplemented with ANA and BAP) combined with or without the addition of activated charcoal were tested. In the elongation and rooting phase, the presence and absence of activated charcoal in the development of shoots was evaluated. For the acclimatization of seedlings and shoots, two *ex vitro* conditions were evaluated: in a growth room environment and in a nursery. We found significant differences between the evaluated treatments. The embryo-type propagule source and disinfested with 50% NaClO for 10 minutes showed the best morphological characteristics of germination and development. The addition of charcoal to basic MS medium, and free from plant regulators, reduced tissue oxidation in the elongation and rooting phase. Shoots of *Ocotea odorifera* developed *in vitro* and adapted well to *ex vitro* acclimatization conditions. Therefore, the micro-propagation protocol used in this work was efficient, providing the production of healthy and suitable seedlings for field conditions.

Keywords: Canela-sassafrás; Propagation; Tissue culture; Seeds.

Resumo

Devido à intensa exploração de óleo essencial ao longo dos anos, *Ocotea odorifera* foi incluída em espécies ameaçadas de extinção no Brasil. Diante desse contexto, a técnica de micropropagação é uma alternativa à propagação sexuada, e consequentemente, favorece a conservação da espécie. Desta forma, o objetivo deste estudo foi produzir mudas de *O. odorifera* por meio da técnica de micropropagação. A coleta de sementes foi realizada em árvores matrizes distribuídas em povoamento natural. O cultivo *in vitro* foi dividido em três fases: (i) germinação e estabelecimento *in vitro*; (ii) alongamento e enraizamento e (iii) aclimatização. Na desinfestação dos explantes utilizou-se um fatorial 3x2: três fontes de propágulo (semente, embrião e segmento nodal) combinado com dois tempo de imersão em NaClO (50%) (5 e 10 min), totalizando 6 tratamentos. No estabelecimento *in vitro* testaram-se dois meio de cultura (MS suplementado com ANA e BAP) combinados com ou sem a adição de carvão ativado. Na fase de alongamento e enraizamento avaliou-se a presença e ausência de carvão ativado no desenvolvimento das brotações. Para

Financial support: None.
Conflict of interest: Nothing to declare.
Corresponding author: celinarcanjo@hotmail.com
Received: 23 August 2021.
Accepted: 22 December 2021.
Editor: Mauro Valdir Schumacher.

a aclimatação das plântulas e brotações avaliaram-se duas condições *ex vitro,* em ambiente de sala de crescimento e em viveiro. Houve diferenças significativas entre os tratamentos avaliados: a fonte de propágulo do tipo embrião e desinfestado com NaClO a 50% por 10 minutos apresentou as melhores características morfológicas de germinação e desenvolvimento. A adição do carvão ao meio MS básico, e isento de fitorreguladores, reduziu a oxidação de tecidos na fase de alongamento e enraizamento. As brotações de *Ocotea odorifera* desenvolvidas *in vitro*, adaptaram-se bem às condições de aclimatização *ex vitro*. Portanto, o protocolo de micropropagação utilizado no presente trabalho foi eficiente, proporcionando a produção de mudas sadias e aptas para condução a campo.

Palavras-chave: Canela-sassafrás; Propagação; Cultura de tecidos; Sementes.

1. INTRODUCTION

Ocotea odorifera (Vell.) Rohwer, an arboreal species belonging to the family Lauraceae, has excellent quality wood and is naturally distributed in plant geographic domains naturally in the Amazon, Cerrado and Atlantic Forest biomes (Jardim Botânico do Rio de Janeiro, 2012, 2020). It is popularly known as Brazilian sassafras or American cinnamon (and in Portuguese as *canela-sassafrás*, *sassafrás*, *canela-cheirosa*, *canela-funcho*, *canela-parda*, *sassafrás-brasileiro*, and *louro cheiroso*) (Lorenzi et al., 2008). This species has been intensely exploited for the production of essential oil containing saffrole, a secondary metabolite widely used by the perfume and medicine industries (Gontijo et al., 2017), and thus became vulnerable to the destruction, fragmentation, and reduction of its habitats, and therefore is currently included in the category of "Endangered" (Jardim Botânico do Rio de Janeiro, 2012; Brasil, 2014).

In addition to the aforementioned extrinsic factors, the perpetuation of the species is threatened by its irregular production of seeds, shrinkage of the populations of the agents responsible for dispersal, and the low vigor of its seeds, which are highly attacked by insects (Carvalho, 2005). These factors, combined with the characteristics of recalcitrant seed and low germination rate in nurseries, render it difficult to produce seedlings by sexual means in forest nurseries (Carvalho, 1994)

Because it is an endangered species, it is essential to adopt strategies that aim at building germplasm banks and that will increase the number of individuals in plantations for such uses as restoration projects of degraded areas, agroforestry systems, and vegetation restoration areas. In this context, knowledge about the *in vitro* germination and propagation of the species are helpful to guide management actions and conservation strategies.

Micro-propagation is one alternative to sexual propagation. It has several advantages, such as reducing the spread of diseases, circumventing difficulties in germination or conventional propagation, and minimizing the difficulty of using recalcitrant seeds (Xavier et al., 2013; Trueman et al., 2018; Abiri et al., 2020). Several factors influence the success of *in vitro* culture of a micro-propagated species, such as the type of explant, disinfestation methods, and culture medium, which make it necessary to develop specific protocols (Abiri et al., 2020; Molinari et al., 2020; Souza et al., 2020).

During the *in vitro* culture process, contamination of plant material in the culture medium is common due to the presence of microorganisms from explants or endophytic microorganisms, especially fungi and bacteria (Sousa et al., 2007; Souza et al., 2018). This phenomenon is more pronounced when working with woody species because they have a long-life cycle, where the accumulation of polyphenols and oxidation products, such as melanin, suberin, and lignin, hinder the collection and establishment of these plants *in vitro*. (Miranda et al., 2020). Therefore, it is essential to develop more efficient disinfestation protocols for each species, as well as to understand the cultivation environment of the species (Bianchetti et al., 2017; Molinari et al., 2020)

Due to the need to develop strategies for conservation and propagation of *Ocotea odorifera* and because this species has several limiting factors to the production of seedlings in a conventional manner, studies on its *in vitro* cultivation are justified. Although protocols have been reported for the micro-propagation of *O. odorifera*, none have successfully led to the acclimation of plants to nursery conditions. The objective of this study was to develop a micro-propagation protocol for *O. odorifera* for the propagation and conservation of this species.

2. MATERIAL AND METHODS

2.1 Source of propagules

The plant material (fruits and branches) was collected from six parent trees located in the municipality of Lavras on the campus of the Federal University of Lavras (21°13'38" S and 44°58'20" W). The fruits were collected at the physiologically mature stage in October 2019. After collection, they were taken to the laboratory for manual removal of seeds and embryos. The branches that would be used to induce the emission of epicormic shoots were collected from the six mother trees selected based on visual criteria such as a straight stem, no pathogen attacks, and the presence of branches in the lower portion of the canopy, aiming to minimize effects of ontogenetic aging (Avelar et al., 2020) and to facilitate the cutting and harvesting of branches.

The branches were sectioned to approximately 50 cm in length and placed in a climate-controlled greenhouse with controlled relative humidity and temperature (RH > 80%; temperature between 20 and 35 °C). The experiment was divided into three phases: (i) germination and *in vitro* establishment; (ii) elongation and rooting; and (iii) acclimatization

2.2 Phase I: Germination and *in vitro* establishment of *Ocotea odorifera*

2.2.1 Disinfestation experiment

Different sources of propagules and disinfestation time in NaClO were evaluated for *in vitro* establishment (Table 1). Epicormic shoots (4-5 cm), for treatments B5 and B10, were collected from branches (Figure 1A) after 45 days in a greenhouse. The fungicide dimethyl 4,4'-(o-phenylene)bis(3-thioallophanate) was applied at a concentration of 0.5 g L^{-1} 48 hours before collection.

In the laboratory, the excision of the nodal segments (2-3 cm) was performed, and after washing in running water, they were immersed in a 70% (v/v) alcohol solution for 30 seconds with constant agitation. Finally, the explants were immersed in Clarix® NaOCl solution (1.00-1.50% active chlorine) for 5 and 10 minutes, followed by three washes in deionized and autoclaved water. In treatments S5 and S10, peeling was performed manually (Figure 1B). Next, the seeds were washed for 10 minutes in running water. After washing, disinfestation was performed with NaClO at 5 and 10 minutes, followed by three washings in autoclaved water inside the flow hood. For treatments E5 and E10, the embryos were removed from the interior of the seed (Figure 1C).

Table 1. Treatments with different propagule sources and disinfestation time in 50% NaClO, used in the *Ocotea odorifera* disinfestation experiment.

Treatments	Propagule source	Immersion time (NaClO 50%)
S5	Seed	5 minutes
S10		10 minutes
E5	Embryo	5 minutes
E10		10 minutes
B5	Epicormic shoots	5 minutes
B10		10 minutes

The explants were inoculated vertically (Figure 1D-F), in test tubes (2.5 cm x 15 cm), containing 10 mL of MS culture medium (Murashige & Skoog, 1962), supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar. The pH was adjusted to 5.8 before autoclaving. After inoculation, the explants were kept in a growth room at 24 ± 1 °C with a photoperiod of 16 hours of light and an irradiance of 40 µmol m^{-2} s^{-1} (quantified by radiometer, LI -250A Light Metre, LI-COR®) provided by a cold white fluorescent lamp. At 30 days after inoculation, the percentages of contamination, oxidation, and *in vitro* germination were evaluated. The explants were transferred to a new culture medium every 30 days.

2.2.2 *In vitro* establishment of *Ocotea odorifera*

Ninety days after inoculation, the explants with roots and developed shoots, were transferred to a new culture medium (Table 2). Every 30 days, the materials were transferred to a new culture medium at the same concentrations. At the end of the material establishment phase, the percentage of oxidation and *in vitro* establishment was evaluated (Figure 1G).

Table 2. Treatments used in the *in vitro* establishment phase of *Ocotea odorifera*

Treatments	Culture medium
NR/WC	Basic MS, without plant regulators and with the addition 0,05 g L^{-1} of activated carbon
NR/NC	Basic MS without plant regulators and without activated carbon
WR/NC	Basic MS with 2.22 µM of BAP, 0.27 µM of ANA and without activated carbon
WR/WC	Basic MS with 2.22 µM of BAP, 0.27 µM of ANA and with 0.05g L^{-1} activated carbon

2.2.3 Statistical analysis - Disinfestation and *in vitro* establishment

For the disinfestation experiment, a completely randomized design (DIC) was used in a factorial scheme of 2 (immersion times) x 3 (propagule sources), with 15 replicates per treatment, each replicate represented by a test tube. The establishment experiment was also carried out in a completely randomized design in a factorial scheme of 2 (with and without growth regulator - 2.22 µM of BAP, 0.27 µM of ANA) x 2 (without and with activated charcoal - 0.05 g L^{-1}), totaling four treatments. Each treatment contained 11 replicates, each replicate consisting of one test tube. The data were subjected to analysis of variance and when found significant to the TUKEY test at 5%. All analyses were processed in R Core Team software (2018), with the aid of the ExpDes package, version 1.1.2 (Ferreira et al., 2013).

2.3 Elongation and rooting *in vitro* of *Ocotea odorifera* (Phase 2)

After 120 days of *in vitro* establishment, the explants were started on the *in vitro* elongation and rooting phase (Figure 1H and I). In the elongation phase, the percentage of survival and morphological characteristics related to shoot growth of *in vitro* shoots were evaluated, such as shoot length, number of shoots per explant, number of leaves per explant, oxidation, and vigour. In the rooting phase, the following morphological characteristics were evaluated: rooting percentage, number of calli per explant, root length (cm), and number of roots per explant. The experiment was conducted in a completely randomized design with two treatments and 11 replicates per treatment. Each replicate consisted of a glass containing 40 mL of MS culture medium (Murashige; Skoog, 1962) supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar. The pH was adjusted to 5.8 before autoclaving. The flasks were kept in a growth room under the same conditions as above. The treatments are described in Table 3.

Table 3. Treatments used in the elongation and rooting phase of *Ocotea odorifera*

Treatments	Culture medium
NR/WC	Basic MS, without plant regulators and with the addition 0.05 g L^{-1} of activated carbon
NR/NC	Basic MS without plant regulators and without activated carbon

2.4 Acclimatization of *Ocotea odorifera* (Phase 3)

Seedlings and shoots, vigorous and with the presence of roots, obtained after 150 days in the *in vitro* cultivation (establishment phase), were transferred to the acclimatization phase. After cleaning the roots with double-distilled water, the seedlings were transplanted into a plastic cup containing 50 g of the autoclaved vermiculite substrate (Figure 1J). To maintain relative humidity, the material was placed on a tray covered with plastic film. Every three days, irrigations of 10 ml of water were performed in each plastic cup. At 15 and 21 days after the start of acclimatization, 15 mL of nutrient solution (without carbohydrates) was added per plastic cup with a concentration of (1/4 DM) and (1/2 DM), respectively.

The trays were placed in a in a growth room, at a temperature of 24 °C ± 1 °C under 40 µmol m^{-2}·s^{-1} irradiation and a 16-hour photoperiod (quantified by the LI-250A Light Metre) provided by a cold white fluorescent lamp. Sixty days after the beginning of acclimatization, the seedlings were transferred to plastic bags measuring 10 cm x 15 cm, which were manually filled halfway with the commercial organic substrate, vermiculite, and carbonized rice husk, in a 1:1:1 ratio. The seedlings remained in the nursery, shade house with 50% shading (Figure 1K).

The evaluations were carried out in two stages of acclimatization: acclimatization in a growth room and acclimatization in a nursery. The morphological characteristics evaluated were: percentage of survival, shoot length, number of leaves per explant, oxidation, vigor, percentage of callus production and number of roots per explant. The evaluation of *ex vitro* acclimatization was performed after 30 days in the nursery.

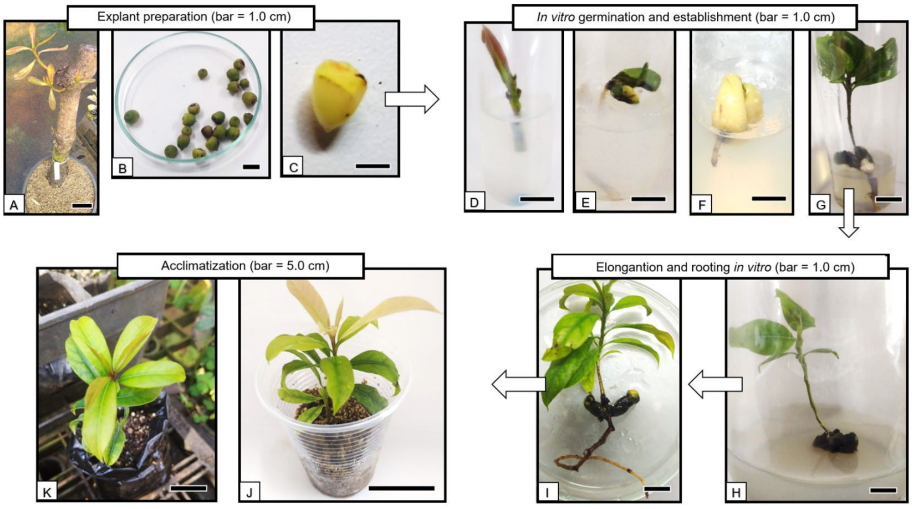

Figure 1. Micro-propagation of *Ocotea odorifera*. Explant preparation: (A) epicormic shoots; (B) seeds and (C) embryo. *In vitro* germination and establishment: (D) Epicormic shoots; (E) seed; (F) embryo and (G) explant *in vitro* established. *In vitro* elongation and rooting: (H) without activated carbon and (I) with activated carbon. *Ex vitro* acclimatization: (J) in a growth room and (K) in a nursery.

3. RESULTS AND DISCUSSION

3.1 Disinfestation and *in vitro* establishment of *Ocotea odorifera*

Thirty days after the introduction *in vitro* a significant interaction was observed between the factors tested (time of immersion in NaClO and sources of propagule) for all the characteristics evaluated (percentage of contamination, oxidation and *in vitro* germination) (Figure 2). In the *in vitro* establishment, there was no interaction between the factors (growth regulators and activated charcoal), however, a significant difference was observed for the evaluated characteristics (percentage of oxidation and *in vitro* establishment) (Figure 3).

For the percentage of contamination, treatment E10 presented the lowest values (on average: 20% contamination), with a significant difference from the other treatments (Figure 2A). In contrast, treatments with epicormic shoots, B5 (on average: 86.6% contamination) and B10 (on average: 80% of contamination) had the highest percentages of contamination (Figure 2A). These results indicate that the asepsis protocol used in epicormic shoots was not efficient. The fact that the epicormic shoots were obtained in greenhouses with conditions of high temperature and humidity may have provided a better condition for the proliferation of contaminating agents, which contributed to the high rate of contamination (Avelar et al., 2020).

Molinari et al. (2020), evaluating the use of active chlorine, reported that the concentration of active chlorine in the asepsis process influenced the number of shoots per explant of *E. grandis* × *E. urophylla* clones, resulting in a higher *in vitro* establishment rate and less contamination of the culture medium. Santa-Catarina et al. (2001) evaluated the disinfection of *O. odorifera* with

NaClO and ethanol with an immersion time of 30 minutes and reported that the asepsis was efficient, with a percentage of germination similar to ours.

For the phenolic oxidation of the explants, treatments E5 and E10 showed the lowest means, with 15% and 10%, respectively (Figure 2B), but there was no statistical difference between them. The highest percentage of oxidation was observed in treatments that used the seed as a source of propagule (S5 and S10), being 33.3% and 66.6%, respectively (Figure 2B). The higher rate of oxidation of the culture medium can be attributed to the release of oxidative substances (Souza et al., 2018; Molinari et al., 2020) from the seeds of species of the Lauraceae family.

Evaluating shoot induction, treatment E10, using the embryo as a propagule source, had the highest percentage of germination (on average: 78.6%) when compared to treatments with propagules via seed and epicormic shoots (Figure 2C). For treatments with epicormic shoots (B5 and B10), null germination was observed (Figure 2C). Although these treatments did not show the highest percentages of oxidation, there was greater contamination, which resulted in the death of these propagules throughout the days of the experiment. Thus, the results show the efficiency of asepsis using the NaClO agent in *Ocotea odorifera* embryos, but without effectiveness for the propagules via seed and epicormic shoots (Figure 2C).

The seeds of *Ocotea* species have a high oil content, and can result in oxidation and rapid deterioration, reducing the germination percentage, and consequently *in vitro* establishment (Moritz et al., 2009). We observed that by removing the embryos from the interior of the seeds, oxidation was reduced, resulting in a higher percentage of *in vitro* germination.

In vitro germination can be used for embryo rescue in conservation and genetic improvement programmers, in the recovery of incompatible hybrid crosses, and as an explant source for tissue culture (Xavier et al., 2013; Trueman et al., 2018). The species *O. odorifera* has slow germination, with irregular fruit production throughout the year, which hinders the production of seedlings in a conventional manner, indicating difficulties in the propagation of the species. *In vitro* germination is an alternative to circumvent these problems with this species. Therefore, the protocol used is a viable alternative for propagation of the species.

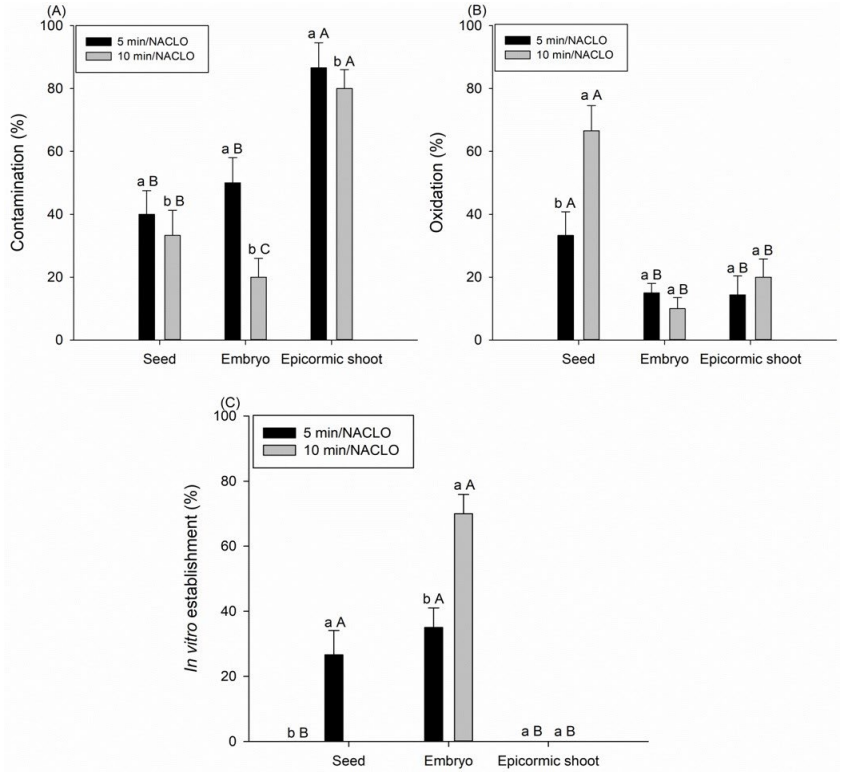

Figure 2. Morphological characteristics observed in *in vitro* introduction with immersion time in hypochlorite (5 and 10 minutes) and different sources of propagules (seed, embryo and epicormic bud). (A) Percentage of contamination; (B) Percentage of oxidation; (C) Percentage of establishment (or induction) *in vitro*.* Mean of three (or five) replicates. Means followed by different letters, uppercase

(between immersion times in NaClO) and lowercase (between propagule sources), differ by Tukey's test (5%). Bars represent the standard deviation from the mean value.

In the establishment phase, as for the phenolic oxidation of the explants, the lowest values were observed in the no-regulator/with-carbon (NR/WC) treatment (null percentage). For the no-regulator/no-carbon (NR/NC) treatment, there was 82% phenolic oxidation in the explants (Figure 3A). Moritz et al. (2009) observed that the use of activated charcoal can adsorb growth regulators and oxidative substances, contributing to the reduction of phenolic oxidation in *Ocotea porosa* explants, as also verified in the present study (Figure 3A).

Activated charcoal acts as an antioxidant, promotes the adsorption of plant exudates and toxic metabolites (Kim et al., 2019). This is due to the presence of a pore network in the activated carbon structure that can adsorb many inhibitory substances in the medium or released by the explants (Perales Aguilar et al., 2016). Therefore, the adsorption capacity of activated carbon acting as an antioxidant may explain the similarity between the no-regulator/with-carbon (NR/WC) and with regulator/with carbon (WR/WC) treatments. Although the WR/WC contains the regulator, the presence of activated carbon may have adsorbed the regulators, neutralizing its effect.

For the percentage of *in vitro* establishment, the best results were also found in the treatments with activated charcoal: NR/WC (100% *in vitro* establishment) and WR/WC (82% *in vitro* establishment), with a significant difference between treatments (Figure 3B). Similar results were observed by Santa-Catarina et al. (2001) when evaluating the establishment phase of *Ocotea odorifera* embryos. The authors tested the concentrations of 0 and 4.4 µmol N^6-benzylaminopurine in the culture medium and found no differences in the germination or predevelopment of embryos.

These results are also similar to those found in explants of *Abelmoschus esculentus* (Irshad et al., 2017), *Psidium guajava* Linnaeus (Perales Aguilar et al., 2016), and *Cattleya crispata* (Souza et al., 2021). The use of antioxidants (PVP and activated charcoal) reduced the effects of phenolic oxidation, promoting better development in the micro-propagation of cultures.

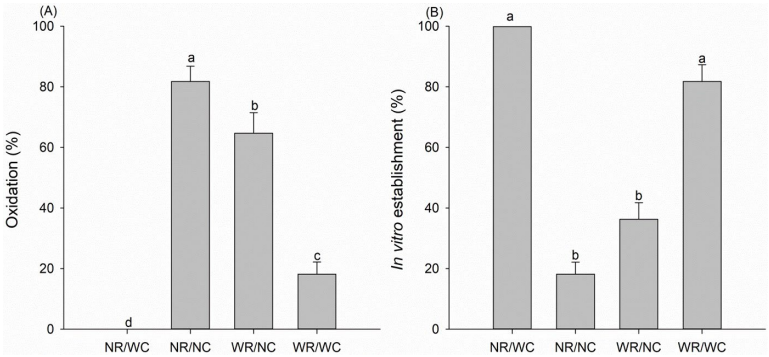

Figure 3. Morphological characteristics observed in *in vitro* establishment with the presence or absence of plant regulators ANA and BAP and activated carbon in MS culture medium: (A) Percentage of oxidation; (B) Percentage of *in vitro* establishment. *Means followed by different letters, differ from each other, by Tukey's test (5%). Bars represent the standard deviation from the mean value.

3.2 Elongation and rooting *in vitro* of *Ocotea odorifera*

There was a significant difference between treatments for all morphological characteristics evaluated (Figure 4). The culture medium (basic MS), free of plant regulators and with the addition of activated carbon (NR/WC) was superior in all evaluated characteristics, when compared to the culture medium without plant regulators and without activated carbon (NR/NC). For rooting induction, there was a significant difference for all evaluated traits, aside for the number of roots per explant (Figure 5D). The highest percentage of rooting (91%) (Figure 5A), the greatest root length (4.18 cm) (Figure 5C) and the morphological characteristics

of the shoots were observed in the NR/WC treatment (Figure 4). However, number of calli per explant was higher in NR/NC than in NR/WC (Figure 5B).

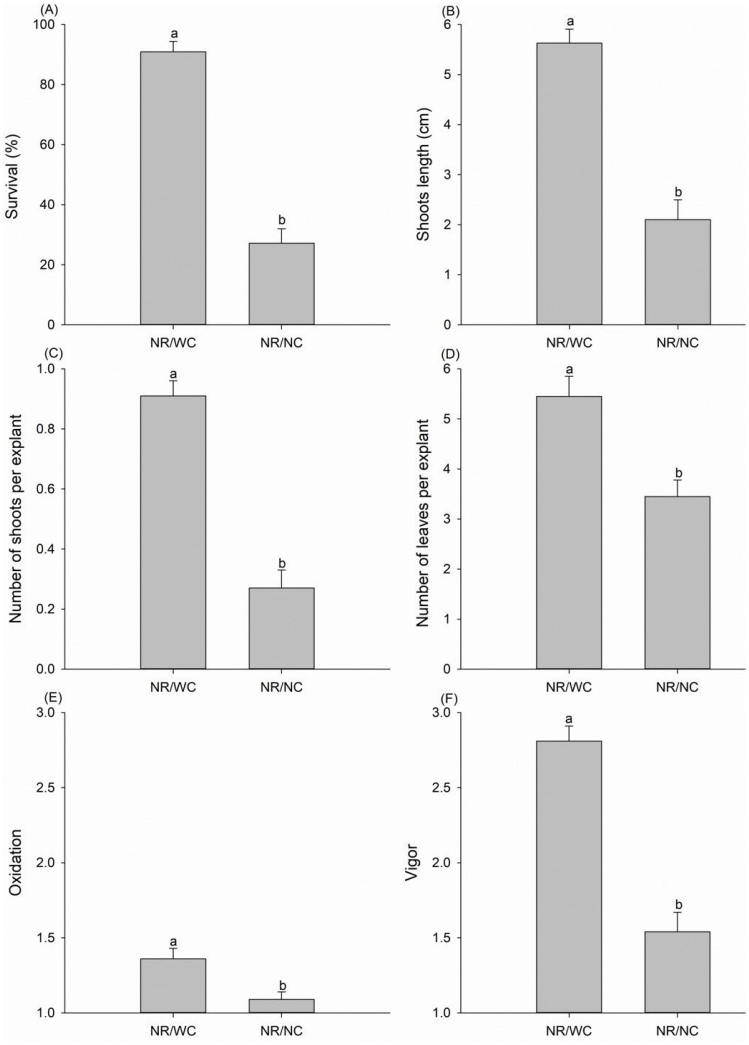

Figure 4. Morphological characteristics observed in *in vitro* elongation with the presence or absence of plant regulators ANA and BAP in MS culture medium: (A) Percentage of survival; (B) shoot length (cm); (C) Number of shoots per explant; (D) Number of leaves per explant; (E) Oxidation; (F) vigor. *Means followed by the same letters do not differ from each other, by Tukey's test at 5% error probability. Bars represent the standard deviation from the mean value.

The morphological characteristics evaluated in the species *O. odorifera* provided data to help optimize the elongation and rooting phase *in vitro* through supplementation with activated charcoal. The use of antioxidants (PVP and activated carbon) resulted in lower oxidation and greater survival and development in *Terminalia amazonia* (Gmel.) Explants during micro-propagation (Méndez Álvarez & Abdelnour-Esquivel, 2014). In the orchid hybrid *Brassocattleya* pastoral × *Laeliocattleya* Amber Glow, the use of activated charcoal can promote a greater number of shoots (Villa et al., 2014), as observed in the present study.

The addition of activated charcoal to the culture medium increases the adsorption of substances that inhibit plant development, such as phenols and ethylene, and minimizes the toxicity of growth regulators or exogenous substances with harmful effects, contributing to greater shoot proliferation and root system development of explants *in vitro* (Kim et al., 2019; Perales Aguilar et al., 2016).

However, supplementation of the culture medium with an antioxidant may have positive or negative effects, depending on the species, type of explant, or cultivation phase in which it is

being propagated (Pankaj et al., 2014). *In vitro* conditions are stressful for plant growth, and high concentrations of exogenous antioxidants can affect development during *in vitro* cultivation (Tisarum et al., 2018). In *Opuntia tuna*, the concentration of activated charcoal in the nutrient medium should not exceed 320 mg L^{-1}, as it can influence the growth, rooting, and vigour of the plant material (Ribeiro & Teixeira 2016), supporting the concentration of 50 mg L^{-1} activated charcoal used for the *in vitro* elongation and rooting of *O. odorifera*.

The absence of adventitious root induction is one of the main causes that limits cloning by mini-cutting and micro-propagation techniques (Almeida et al., 2017). Findings on the micro-propagation of *Cattleya walkeriana* (Sousa et al., 2007) and *Eugenia pyriformis* (Assis et al., 2018) confirm those found in this study, since supplementation in the culture medium with activated charcoal reduced oxidation and callus production and afforded greater development of the aerial part and root system when compared to no antioxidant. Therefore, knowledge of the relationship between the use of activated carbon, growth pattern, and rooting of explants allows us to better understand the micro-propagation protocols in *O. odorifera*, showing that these factors exert a great influence on *in vitro* culture.

3.3 Acclimatization of *Ocotea odorifera*

There was no significant difference in the percentage of survival, number of leaves per explant, oxidation, vigour, percentage of callus production or number of roots per plant between the two conditions studied (Figure 6A; C; D; E and Figure 7). Only shoot length differed by condition, with a mean of 11.09 cm for seedlings under *ex vitro* conditions (acclimatization in nursery) and 6.46 for acclimatization in growth room, which gives a 4.63-cm difference (Figure 5B).

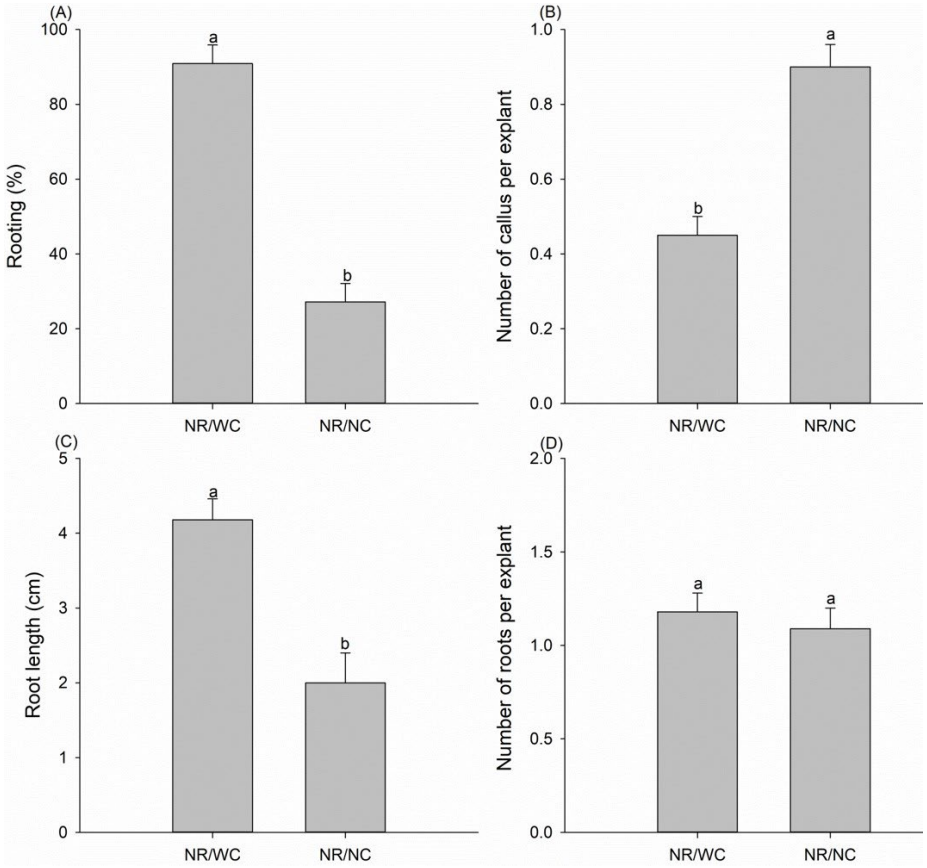

Figure 5. Morphological characteristics observed in *in vitro* rooting in relation to the presence or absence of activated carbon. (A) Percentage of rooting; (B) Number of callus per explant; (C) Root length (cm); (D) Number of roots per explant. *Averages followed by different letters differ from each other by Tukey's test (5%). Bars represent the standard deviation from the mean value.

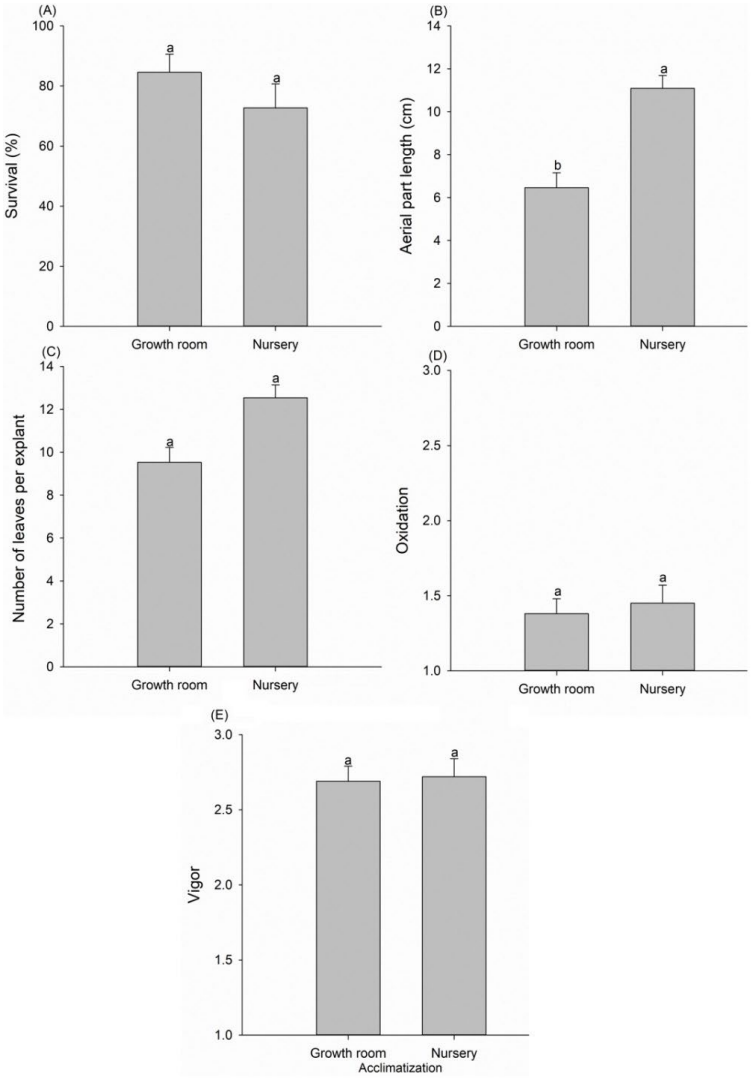

Figure 6. Morphological characteristics observed in acclimatization: growth room and nursery (A) Percentage of survival; (B) aerial part Length (cm); (C) Number of leaves per explant; (D) Oxidation; (E) Vigor. *Means followed by the same letters do not differ from each other, by Tukey's test at 5% error probability. Bars represent the standard deviation from the mean value.

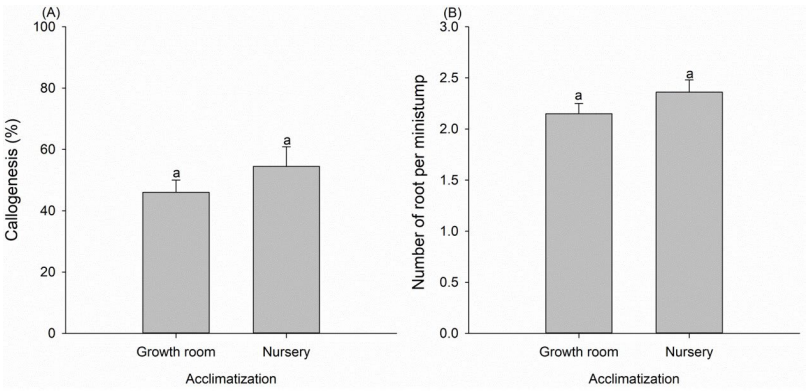

Figure 7. Morphological characteristics observed in the acclimatization: in a growth room and in nursery after 30 days. (A) Percentage of callus formation; (B) Number of root per mini-stump. *Means followed by the same letters do not differ from each other, by Tukey's test at 5% error probability. Bars represent the standard deviation from the mean value.

The seedlings of *Ocotea odorifera* were subjected to *two* acclimatization *ex vitro* conditions to understand their morphological development, especially the characteristics related to rooting (Figure 8J and 8K). The rooting phase in micro-propagation protocols can be performed either under *ex vitro* (Xavier et al., 2013) or *in vitro* conditions (Nourissier & Monteuuis, 2008). Evaluating seedlings under *in vitro* and *ex vitro* conditions is important because there is commercial interest in reducing the number of phases in micro-propagation protocols and thereby reducing the total time of seedling production.

Well-developed shoots in the elongation phase significantly influence the rooting process in the acclimation of the species (Souza et al., 2020a, 2020b), which can be conducted *ex vitro* or *in vitro*. The morphological characteristics of *Ocotea odorifera* observed were statistically similar between two *ex vitro* acclimatization conditions, including the number of roots per micro-stem, indicating that the shoots reached adequate development conditions during the elongation phase (Figure 8H and 8I), so they were capable of inducing rooting in the two conditions tested (Figure 6).

Eucalyptus benthamii shoots showed good rooting development under *in vitro* and *ex vitro* conditions in the study conducted by Brondani et al. (2018). However, the authors recommended *ex vitro* rooting for the reduction of the stress of successive transplantations and the achievement of high acclimation rates when compared to *in vitro* rooting. Gallo et al. (2017) also used rooting under *ex vitro* conditions in the micro-propagation of *Eucalyptus* spp. and reported good rooting and seedling survival.

One of the main problems with micro-propagated woody species is the low survival of micro-plants when they are transferred *ex vitro* (Miranda et al., 2020). The seedlings of *Ocotea odorifera* transferred *ex vitro* in this study showed excellent development, a high rate of survival, and great aptitude for planting and field survival. The good performance of the species showed that the *in vitro* germination protocol was efficient and that the number of phases of the protocol can be reduced. The superiority of the shoot length *ex vitro* also confirms the good adaptation of the species to the new conditions (Figure 6B).

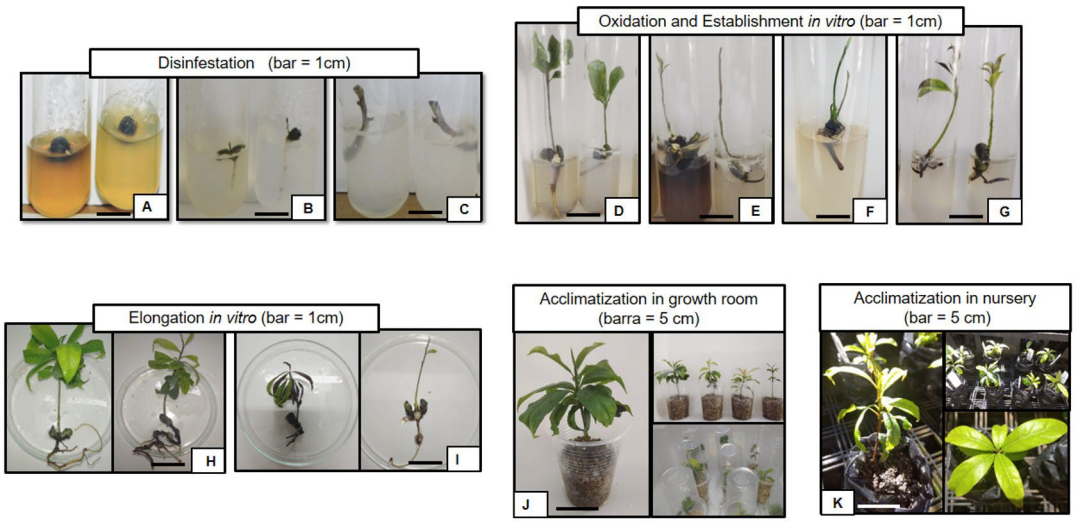

Figure 8: Treatments used in the *in vitro* micropropagation experiments of *Ocotea odorifera*. Disinfestation:(A): Seed with NaClO (5 and 10 minutes); (B): Embryo with NaClO (5 and 10 minutes); C: Epicormic shoots with NaClO (5 and 10 minutes); *In vitro* establishment: (D) NR/WC (Basic MS, without plant regulators and with activated carbon; (E): NR/NC (Basic MS without plant regulators and without activated carbon); (F): WR/NC (Basic MS with plant regulators and without activated carbon); (G): WR/WC (Basic MS with plant regulators and with activated carbon); *In vitro* elongation – (H): NR/WC (Basic MS, without plant regulators and with activated carbon); (I): NR/NC (Basic MS without plant regulators and without activated carbon); Acclimatization: (J): in a growth room; (K): in a nursery.

4. CONCLUSION

Based on our results, we concluded that: 1) In the germination and establishment phase, the embryo propagule source with 50% NaClO for 10 minutes presented a lower percentage of contamination and oxidation and a higher percentage of germination. 2) In the elongation and rooting phase, the addition of activated carbon to the basic MS medium promoted an increase in the survival, greater length and number of shoots per explant, increase in rooting percentage, and greater root length. 3) In the acclimatization phase, the *Ocotea odorifera* seedlings adapted well to the two tested conditions: acclimatization in a growth room and acclimatization in a nursery. 4) The protocol of *in vitro* germination of *Ocotea odorifera* was efficient, providing healthy seedlings and being amenable to adaptation in the field. This protocol can shorten the total production time.

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Authors contribution: JAS: conceptualization, data curation, formal analysis, methodology, writing, Investigation; NRP: methodology, writing, Investigation; DMSC: data curation, formal analysis, methodology, writing, Investigation; DC: conceptualization, data curation, formal analysis, methodology, writing, Supervision; GEB: conceptualization, data curation, formal analysis, methodology, writing, Supervision.