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# ESSENTIAL OIL INCREASES LEAF NUMBER IN EXPLANTS OF WOODY SPECIES NATIVE TO SOUTHERN BRAZIL

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**Resumo**

*Óleo essencial aumenta número de folhas em explantes de espécie lenhosa nativa do sul do Brasil*. Este estudo avaliou o efeito do óleo essencial (OE) de *Nectandra grandiflora* Nees & Mart. ex Nees sobre diferentes parâmetros no estabelecimento *in vitro* de *Eugenia involucrata* DC. Para isto, alíquotas de OE foram adicionadas ao meio ½ MS em concentrações finais de 0.05, 0.075, 0.1, 0.125, 0.15, 0.175 e 0.2 µL $mL^{-1}$, além do controle positivo (meio nutritivo) e controle negativo (diluente). Matrizes de *E. involucrata* cultivadas em casa de vegetação foram utilizadas como doadoras de explantes. Após 30 dias de cultivo *in vitro* foram avaliadas as variáveis sobrevivência, estabelecimento, contaminação bacteriana, contaminação fúngica, oxidação fenólica e número de folhas. Ademais, visando explicar os resultados observados, a composição química do OE foi analisada por cromatografia gasosa. Os resultados indicaram que, apesar de não inibir a contaminação por micro-organismos e não influenciar na oxidação fenólica, o OE a 0,075 µL $mL^{-1}$ possibilitou a emissão de um maior número de folhas, enquanto que o estabelecimento dos explantes (86,66%) não diferiu estatisticamente dos controles ($p < 0,05$). Estes resultados indicam que o OE possui potencial para ser utilizado no desenvolvimento de um bioestimulante para explantes de *E. involucrata*.
*Palavras-chave*: compostos voláteis, canela-amarela, micropropagação, cerejeira-do-mato.

**Abstract**

This study evaluated the effects of *Nectandra grandiflora* Nees & Mart. ex Nees essential oil (EO) on various parameters related to the *in vitro* establishment of *Eugenia involucrata* DC. To this end, aliquots of EO were added to the ½ MS medium at final concentrations of 0.05, 0.075, 0.1, 0.125, 0.15, 0.175, and 0.2 µL $mL^{-1}$, along with a positive control (nutritive medium) and a negative control (diluent). Individuals grown under greenhouse conditions were used as donors of explant material for *E. involucrata*. After 30 days of *in vitro* cultivation, assessments included survival, establishment, bacterial contamination, fungal contamination, phenolic oxidation, and number of leaves. Furthermore, to help interpret the observed results, the EO chemical composition was analyzed by gas chromatography. The results showed that, despite not inhibiting microbial contamination and not influencing phenolic oxidation, the EO at 0.075 µL $mL^{-1}$ promoted the emergence of a greater number of leaves. At the same time, explant establishment (86.66%) did not differ statistically from the controls ($p < 0.05$). These results indicate that the EO has the potential to be used in the development of a biostimulant for *E. involucrata* explants.
*Keywords:* volatile compounds, canela-amarela, micropropagation, cerejeira-do-mato.

## INTRODUCTION

Forest resources have increasingly been the focus of research aimed at generating products with high added value. Considerable efforts have been devoted to developing strategies that promote the conservation of native flora. Therefore, micropropagation techniques have gained importance in forest research due to their advantages in producing seedlings with superior genetic, physiological, and sanitary quality (XAVIER *et al.*, 2021).

However, to develop an efficient micropropagation protocol, it is necessary to add growth regulators to the culture medium. In general, establishment difficulties can be overcome by adding cytokinins and gibberellins, which facilitate cell division, cell elongation, and increase cuticle plasticity (TAIZ *et al.*, 2017). Conversely, the rooting of regenerated aerial parts is often difficult to achieve, and the medium should be supplemented with auxins (XAVIER *et al.*, 2021).

The *in vitro* establishment of woody species is also associated with challenges, such as phenolic oxidation, which is caused by enzymes that oxidize phenolic compounds into quinones, leading to growth inhibition and explant death in many species. To minimize oxidation, antioxidants are often added to the culture medium (XAVIER *et al*., 2021; STEFANEL *et al*., 2021a).

Additionally, microbial contamination is also a frequent obstacle in *in vitro* cultivation. While some contaminated cultures may result from inadequate surface sterilization or insufficient aseptic practices, endophytes could contaminate *in vitro* cultures by passing through surface-sterilized tissues. Although most of these microorganisms are not pathogenic, their growth is stimulated in the culture medium, resulting in competition for nutrients with the explants. Therefore, substances with germicidal or antibiotic properties have been used in *in vitro* cultivation (ORLIKOWSKA *et al*., 2016; STEFANEL *et al*., 2021b; XAVIER *et al*., 2021).

Despite the positive aspects of supplementing the nutritive medium with biostimulants and/or growth regulators, antioxidants, and/or antimicrobials, this approach also presents a significant drawback: an increase in the production costs of seedlings with high genetic homogeneity and sanitary quality. To overcome the problems in establishing native woody species *in vitro*, plant secondary metabolites such as essential oils (EOs) components may be an alternative. EOs and their constituents perform various functions in plants, such as protection against pathogens (bacteria, fungi, and viruses), mitigation of oxidative stress, and signaling between plant organs, among others. Additionally, they are less toxic to humans and less environmentally hazardous compared to synthetic products (FRIESEN *et al*., 2011; CHOUHAN *et al*., 2017).

Considering the biological activities already described for *Nectandra grandiflora* Nees & Mart. ex Nees leaves EO, such as antifungal (SILVA *et al*., 2016) and antimicrobial (FERRAZ *et al*., 2018), this extract emerges as a potential alternative for controlling *in vitro* contaminants and/or phenolic oxidation. In *in vitro* propagation studies of *Eugenia involucrata* DC. (Myrtaceae), difficulties in explant establishment are mainly due to the occurrence of phenolic oxidation and endogenous contaminations (GOLLE *et al*., 2013; STEFANEL *et al*., 2021b). This native woody species grows in areas of the South, Southeast and Midwestern Brazil, as well as the state of Bahia, and in neighboring South American countries. Due to its wood quality and durability, the species is highly valued; it also possesses medicinal properties and produces highly appreciated fruits (CARVALHO, 2008).

In view of the economic and ecological importance of E. *involucrata* and the problems detected in previous attempts for its *in vitro* micropropagation, as well as the biological activities already reported for *N. grandiflora* EO, this study aimed to evaluate the effects of the EO on various parameters related to the *in vitro* establishment of *E. involucrata*. In this context, particular attention was given to the EO's potential to control endogenous contamination and/ or phenolic oxidation in *E. involucrata* explants. In addition, to support the interpretation of the results, the chemical composition of the *N. grandiflora* EO was described.

## MATERIAL AND METHODS

### Plant material for essential oil extraction

Leaves of *N. grandiflora* were collected in the summer of 2016 from a native population in Southern Brazil, located in Jaguari, Rio Grande do Sul State (29°26'S and 54°40'W). The plant material was identified by the Forest Engineer Dr. Solon Jonas Longhi, and a voucher specimen has been deposited at the Herbarium of the Forest Sciences Department, Universidade Federal de Santa Maria, Brazil (HDCF 13.162).

### Essential oil extraction and chemical analysis

The EO was obtained from fresh leaves by hydrodistillation for 3 hours. Yield determination was performed by calculating the ratio of fresh leaf weight to EO volume (w/v). The chemical composition was analyzed using a 7890A GC (Agilent) equipped with a 5975C mass selective detector (GC–MS) and a DB5-MS capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness). Quantitative evaluation was calculated based on GC peak areas, using a GC coupled to a flame ionization detector (GC-FID). Analysis parameters followed those described by Silva *et al*. (2016).

### Explants

Nine-year-old *E. involucrata* plants grown under greenhouse conditions were used as explant donors. For the superficial disinfestation of the matrices and explants, the methodology described by Golle *et al*. (2013) was followed. Nodal segments without the apical ones were selected as explants. The nutritive medium used was ½ MS, which was autoclaved at 121 °C at 1 atm for 15 min.

### Inoculation of explants

Aliquots of the extract were added to the culture medium at final concentrations of 0.05, 0.075, 0.1, 0.125, 0.15, 0.175, and 0.2 µL $mL^{-1}$, previously diluted in ethanol (1:1) (v/v). The positive control consisted of only the nutritive medium, while the negative control contained the medium supplemented with ethanol at the highest

concentration used to dilute the EO samples. Previously, a pilot test was performed with EO at 0.1, 0.5, 1.0, and 3.0 µL $mL^{-1}$ under the same conditions. However, the three highest concentrations inhibited optimal explant establishment in the nutritive medium.

In a laminar flow chamber, foliar remnants were removed from explants. Each explant contained two dormant axillary buds. After inoculation, the vials were kept in a growth room. The experiment was conducted in a completely randomized design in a unifactorial arrangement, with five replicates each consisting of three explants. After 30 days of *in vitro* culture, the following variables were evaluated: survival (indicated by the green coloration of the explant) (%), establishment (defined as the development of leaf primordia in the explant) (%), bacterial contamination (presence of bacterial colonies near the explant) (%), fungal contamination (presence of fungal mycelia adjacent to explants) (%), phenolic oxidation (presence of dark brown coloration covering at least half the explant surface) (%), and number of leaves formed per explant.

**Statistical analysis**

After testing the normality of errors using the Kolmogorov-Smirnov test, the means were transformed using the function $[\sqrt{(x + 0.5)}]$, where x represents the observed value. The variables were subjected to analysis of variance (ANOVA) and, when the F value was significant, Tukey's test was applied for mean comparison at $p < 0.05$. For this, the statistical program Sisvar version 5.1 was employed.

## RESULTS

The EO extraction of *N. grandiflora* leaves showed a yield of 0.4302 (±0.0260)% and a density of 0.9116 (± 0.008) g $mL^{-1}$. Chemical analysis of the EO allowed the identification of 24 constituents (accounting for 71.83% of the total composition), and the major ones were (+)-dehydrofukinone (23.34%), dehydrofukinone epoxide (8.69%), kaurene (5.58%), selin-11-en-4-α-ol (4.71%), rimuene (4.53%), eremophilene (4.32%) and eremophilan-11-en-10-ol (4.08%). The complete composition of the EO is presented in the Supplementary Material.

A significant effect of EO concentrations on explant establishment ($p = 0.0015$) and the number of leaves ($p = 0.0000$) was observed. Conversely, no significant effects were detected for survival (mean value of 51.85%), oxidation (37.04%), fungal contamination (mean value of 17.78%), and bacterial contamination (mean value of 50.37%).

According to Table 1, the use of EO did not affect the *in vitro* establishment of explants, as there were no clear differences among the treatments, including controls. On the other hand, the use of 0.075 µL $mL^{-1}$ of the extract produced a significant increase in the number of leaves in *in vitro* cultures, differing statistically from both controls but not from concentration of 0.100 µL $mL^{-1}$ (Table 1).

Thus, although *N. grandiflora* EO at 0.075 µL $mL^{-1}$ did not reduce microbiological contamination or phenolic oxidation, nor did it influence *in vitro* establishment, it promoted a significant increase in the number of leaves in cultures. The observed results are important, as leaf emergence of explants, along with an increase in the photosynthetic area, enhances plant vigor and enables it to withstand acclimatization.

Table 1. Establishment (Estab.) (%) and number of leaves formed (N. Leaves) of *Eugenia involucrata* at 30 days of *in vitro* cultivation after inoculation in ½ MS medium containing different concentrations of *Nectandra grandiflora* leaf essential oil.

Tabela 1. Estabelecimento (Estab.) (%) e número de folhas formadas (N. Folhas) de *Eugenia involucrata* a 30 dias de cultivo *in vitro* após inoculação em meio ½ MS contendo diferentes concentrações de óleo essencial de *Nectandra grandiflora.*

| EO treatments (µL $mL^{-1}$) | Estab. (%) | N. Leaves |
|---|---|---|
| Positive control | 40.00 ab | 12.00 b |
| Negative control | 33.33 ab | 11.00 b |
| 0.050 | 40.00 ab | 12.00 b |
| 0.075 | 86.66 a | 47.00 a |
| 0.100 | 46.66 ab | 22.00 ab |
| 0.125 | 20.00 b | 4.00 b |
| 0.150 | 6.66 b | 4.00 b |
| 0.175 | 13.33 b | 3.00 b |
| 0.200 | 0.00 b | 0.00 a |
| MEAN | 31.85 | 12.77 |
| CV | 17.22 | 29.91 |
| SE | 0.07 | 0.14 |

Means in the same column followed by different letters indicate significant differences by Tukey´s test, $p < 0.05$. CV: coefficient of variation; SE: standard error.

**DISCUSSION**

Regarding the extraction yield of *N. grandiflora*, Amaral *et al*. (2017) found values of 2.08% and 1.71% for fresh and dried leaves, respectively. Ferraz *et al*. (2018) reported that EO yields were higher in spring (0.23%) and autumn (0.2%), while the lowest yield was found in winter (0.08%). Several factors can affect the yield of EOs, among which genetic characteristics are some of the most important, as they account for variability, especially in native species that grow spontaneously, such as *N. grandiflora*. In addition, the growth phase and phenological stage at the time of collection, the extraction method, and the geographical environment, which affects the growth of the plant and some physiological responses, also influence the yield (NI *et al*., 2021). Concerning the EO chemical composition found in this study, our results are in agreement with the data previously described by Silva *et al*. (2016), which reported oxygenated sesquiterpenoids as major components, being dehydrofukinone (26.85%) the major one. However, in the latter cited study, the authors did not evaluate the seasonal variability of chemical composition. Ferraz *et al*. (2018) also identified compounds from the oxygenated sesquiterpenoids class, but the major components were isobicyclogermacrenal, spathatulenol, and rosadiene. Although the chemical composition of the EO described in this study differed considerably from that described by Ferraz *et al*. (2018), the presence of a single diterpenoid kaurene, in similar percentages (approximately 5%) is noteworthy. This is not surprising, considering that this compound is a biosynthetic intermediate of gibberellin phytohormones (OSHIKAWA *et al*., 2024). On the other hand, Amaral *et al*. (2017) found as a major compound the ketone di-hydro-karanone (30.9%), beyond the hydrocarbon α-selinene (2.7%). Regarding the density of EO, a literature value of 0.926 g $mL^{-1}$ for EO has already been reported (SILVA *et al*., 2016), similar to the value found in our study. This density is in accordance with the chemical composition of the EO, since oils consisting mainly of terpenoids are less dense than water.

In the pilot test, the explants did not survive when exposed to EO concentrations higher than 0.5 µL $mL^{-1}$. In a previous study, the effects of the commercial biostimulant Promalin® (1.8% benzyladenine (BAP), cytokinin (CK), and 1.8% gibberellin (GA, GA4 + GA7) were evaluated in the promotion of sprouting on blackberry (*Rubus* spp.) cv. Brazos. root cuttings. The authors reported decreased values with increasing biostimulant concentrations (DIAS *et al*., 2012). Furthermore, according to these authors, cytokinins and gibberellins at low concentrations promote the growth and vegetative development. However, high concentrations of growth regulators are often toxic to explants. On the other hand, the toxic effects on the explants at higher EO concentrations could also result from allelopathy, as reported by Melo *et al*. (2017), who observed an allelopathic effect of isolated sesquiterpenoids and EOs with sesquiterpenoids as major compounds. Thus, we decided to evaluate lower concentrations of the EO on the explant culture.

When lower concentrations of extractive were used, a significant effect of EO concentrations on the establishment and the number of leaves was found. However, no significant effects were detected for fungal and bacterial contamination. This indicates that the plant extract did not control the appearance of fungi and bacteria, but allowed the explants to develop in the culture medium. The lack of microbial contamination control was unexpected, since *in vitro* antimicrobial activity of the EO has already been documented (SILVA *et al*., 2016; FERRAZ *et al*., 2018).

Several hypotheses explain the greater number of leaves observed in the explants submitted to 0.075 µL $mL^{-1}$. The structural relationship of the sesquiterpenoids, a class of major components of *N. grandiflora* EO, with gibberellins may be an explanation for the stimulation of explant development.

Furthermore, some cytokinins also have terpenoid structure, such as abscisic acid, which is derived from monocyclic sesquiterpenes and plays important roles in plant responses to environmental variations, as well as in the regulation of seed development and germination (PAN *et al*. 2020). In addition, this plant hormone contributes to the adaptation of vegetative tissues to conditions of environmental stress and is also associated with senescence. Furthermore, in situations of drought, it can regulate the stomatal activity of plants, reduce transpiration, improve the efficiency in reactive oxygen species (ROS) scavenging, and increase the activity of antioxidant systems (ALAMGIR, 2018).

Another possibility to explain the greater number of leaves produced by the explants when exposed to EO at low concentrations could be a hormesis effect. This phenomenon can be observed in all groups of organisms, such as bacteria, fungi, angiosperms, and animals. It occurs when a toxic substance at high doses shows beneficial effects when applied at low doses and, in this case, stimulates plant development (CALABRESE; BLAIN, 2009). Regarding the concentration, salicylic acid applied at low concentrations resulted in increased development and a higher number of leaves compared to other treatments. Similarly, foliar application of salicylic acid at low concentrations stimulated different morphological and growth parameters of tomato plants, whereas reverse effects were observed at high concentrations. Thus, low concentrations of *N. grandiflora* EO may have assisted in the establishment of *E. involucrata* explants. However, additional studies should be carried out to explain the underlying mechanism (CALABRESE, 2005; SADEGHIAN *et al*., 2013).

Although the contaminants are not desirable for *in vitro* cultures, when microbial growth does not prevent the development of the explant, the associated cultivation can be an alternative (FRIESEN *et al*., 2011; STEFANEL *et al*., 2021b). This statement is in line with the findings in our work, as the EO seems to promote a balance between the contaminating agents and the explant. Thus, low EO concentrations permitted the explant to establish itself effectively, even when in contact with fungal and bacterial organisms.

According to Danielli *et al*. (2017), the use of *Nectandra* sp. EO, combined with commercial fungicide, showed an additive and synergistic effect, reducing the active concentration of the antifungal agent when used in association. Thus, considering that EO showed no toxicity when tested at concentrations between 0.075 and 1.0 µL $mL^{-1}$ (Table 1), further experiments should be conducted using combinations of extractives as EO obtained from this species with growth hormones and/or with synthetic antimicrobials. Thus, a reduction of the active concentration of the commercial product can be achieved, resulting in reduced costs.

To our knowledge, this is the first study that evaluates the use of an EO to stimulate explant establishment and/or control contamination and phenolic oxidation of *E. involucrata* in *in vitro* cultures. However, additional micropropagation experiments should be conducted with other woody species, with the aim of developing an effective biostimulant or plant growth regulator. Besides reducing the costs of obtaining seedlings by micropropagation, the generated product may add value to *N. grandiflora* EO, increasing its economic importance and contributing to the conservation of this native species.

## CONCLUSION

- The EO of *N. grandiflora* stimulates the production of leaves in *E. involucrata* explants cultured *in vitro* at 0.075 µL $mL^{-1}$, without interfering with microbial contamination, phenolic oxidation and establishment.
- Despite the absence of antimicrobial or antioxidant effects *in vitro*, the EO promoted a significant increase in leaf number at an optimal concentration.
- The establishment rate of explants (86.66%) treated with EO did not differ statistically from the positive and negative controls, indicating the absence of phytotoxic effects at the tested concentrations.
- The chemical composition of the EO, analyzed by gas chromatography, provides insights into potential bioactive compounds that may act as biostimulants.
- Thus, the evaluated EO and its constituents have the potential to be used in the development of a natural biostimulant for the *in vitro* propagation of *E. involucrata*.

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