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MIXOTROPHISM EFFECT ON *IN VITRO* ELONGATION AND ADVENTITIOUS ROOTING OF *Eucalyptus dunnii*

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HIGHLIGHTS

Different types of sealing and sucrose concentrations influence *in vitro* elongation and adventitious rooting.

Higher gas exchange (CO_2) favors the *in vitro* plant growth.

The autotrophic system for the *in vitro* cultivation of *Eucalyptus dunnii* was not efficient.

Keywords:
Micropropagation
Gas exchange
Porous membrane

ABSTRACT

The relevance of *Eucalyptus dunnii* has been evidenced mainly for its wood quality and cold tolerance among cultivated subtropical eucalypts. However, rooting is a challenge for its propagation, particularly when adult material is involved. This study aimed to assess the mixotrophism on the *in vitro* elongation and adventitious rooting phases in *Eucalyptus dunnii* microcutting. The experimental material used was obtained from a ministumps of *Eucalyptus dunnii* clones. In order to evaluate gas exchange and sucrose supplementation on *in vitro* elongation and adventitious rooting, the experiment was prepared in a 3×4 factorial arrangements with three forms of sealing (rigid polypropylene caps with no membrane (0/M), with a membrane (1/M), with three membranes (3/M) and four sucrose concentrations (0, 10, 20 and 30 $g{\cdot}L^{-1}$). At 30 days in the elongation phase it was evaluated (length, number of shoots per explant, oxidation, bud vigor, pigment content, leaf area and anatomy) and rooting (length, root diameter and rooting). Results show that sucrose should be added in the culture medium for *in vitro* elongation and can be reduced to concentrations between 10 and 20 $g{\cdot}L^{-1}$. *In vitro* rooting requires the use of 30 $g{\cdot}L^{-1}$ of sucrose. The use of flasks with membranes that allow gas exchange is an effective alternative to promote the in vitro elongation and adventitious rooting of *Eucalyptus dunnii* microcutting.

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INTRODUCTION

Eucalyptus dunnii is a subtropical climate of great importance due to its wood quality and cold tolerance among cultivated subtropical eucalyptus (Smith and Henson, 2007). However, specie rooting is a challenge in clonal propagation, particularly when adult material is involved. The rejuvenation of propagules has allowed advances on *in vitro* elongation and adventitious rooting phases (Trueman et al., 2018). In the search for alternatives to rooting improvement in the cloning production process, micropropagation via axillary buds proliferation has been recommended (Xavier et al., 2013). Recently, investigation on the *Eucalyptus dunnii* species has increased significantly in relation to *in vitro* vegetative propagation (Oberschelp et al., 2015), as well as *in vitro* elongation (Navroski et al., 2015) and adventitious rooting (Oberschelp et al., 2015; Brondani et al., 2018).

Among micropropagation stages, *in vitro* elongation is primordial in obtaining sprouts for microcutting rooting, which has been performed as much under *ex vitro* (Xavier et al., 2013) or *in vitro* (Trueman et al., 2018) conditions. However, research for improvements in the *in vitro* elongation phase has been intensified, aiming to obtain microstumps. Scientific studies on this field have focused on the adequacy of protocols, on the reduction of the carbon source in the culture medium, as well as knowledge of the most efficient environment for the system (Bianchetti et al., 2017).

Controlling environmental factors such as the use of mixotrophism is considered important for *in vitro* morphogenetic response (Heringer et al., 2017). Generally, a positive response to micropropagation among the various factors is due to the reduction or exclusion of the sucrose source, as well as the use of porous membranes, in which the relative humidity of the bottles, characterized by the presence of water and nutrients in the plant, is reduced (Bacillus et al., 2005), thus improving its *ex vitro* acclimatization process (Saldanha et al., 2012; Jiménez et al., 2015; Batista et al., 2017; Tisarum et al., 2018). Silva et al. (2017) reported its positive effect in different species, besides the possibility of variations in the concentration of sucrose, which benefits the cultivation and reduces expenses.

Seeking to optimize a protocol for micropropagation of *Eucalytpus dunnii* in the clones production process, this study aimed to assess mixotrophism on *in vitro* elongation and adventitious rooting phases.

MATERIALS AND METHODS

Study and experimental material location

The experiments were conducted at the Laboratory of *In Vitro* Culture of Forest Species, Department of Forestry Sciences (DCF), Federal University of Lavras - UFLA, Lavras, MG. The genetic material used to obtain the explants came from microstumps of *Eucalyptus dunnii* species donated by the Institute of Research and Forest Studies (IPEF).

For the elongation phase, shoots produced in the *in vitro* multiplication phase were prepared by isolating four 0.5 cm standard shoots and inoculated under aseptic conditions in glass vials (250 mL capacity) grown for 30 days, containing 50 mL of MS medium (Murashige and Skoog, 1962), added with 6 $g{\cdot}L^{-1}$ agar, 0.05 $mg{\cdot}L^{-1}$ BAP (6-benzylaminopurine - Sigma Co.), and 0.5 $mg{\cdot}L^{-1}$ of indole-3-butyric acid (IBA) (Sigma®) (Trueman et al., 2018).

In vitro rooting, microcuttings with size about 2 cm, were prepared from elongated buds in the *in vitro* elongation phase. Microcuttings were inoculated under aseptic conditions, cultured for 30 days in glass vials (250 mL) containing 50 mL of MS medium, with the addition of 6 $g{\cdot}L^{-1}$ agar, 1 $mg{\cdot}L^{-1}$ IBA and 0.5 $mg{\cdot}L^{-1}$ of naphthalene acetic acid (NAA).

The culture medium was prepared using deionized water, and its pH was adjusted to 5.8 ± 0.05 with NaOH (0.1 M) and HCl (0.1 M) prior to autoclaving and agar addition. Autoclaving of the culture medium was performed at a temperature of 127°C and a pressure of approximately 1.5 $kgf{\cdot}cm^{-2}$ for 20 min. The treatments were kept in a growth room at 25 ± 1°C for a photoperiod of 16 h light and irradiance of 40 $\mu mol\ m^{-2}{\cdot}s^{-1}$ (quantified by radiometer, LI-COR®, LI-250A Light Meter).

DESIGN AND EXPERIMENTAL EVALUATIONS

To evaluate packaging and sucrose variations in *in vitro* elongation, the experiment was organized in a 3×4 factorial arrangements, with three types of sealing: rigid polypropylene lids without membrane (0/M), polypropylene lids with a hole (1 cm in diameter) covered with a membrane of 1.0 cm^2 (1/M), polypropylene lids with three holes (1.0 cm in diameter each) covered with a membrane of 1.0 cm^2 (3/M), and under four different sucrose concentrations (0, 10, 20 and 30 $g{\cdot}L^{-1}$).

As for the experiment in the *in vitro* rooting phase, 3×4 factorial arrangements were used, with three sealing forms: rigid polypropylene lids without membrane (0/M), polypropylene lids with a hole (1 cm in diameter) covered with a membrane of 1.0 cm^2 (1/M), polypropylene lids with four holes (1.0 cm in diameter each) covered with a membrane of 1.0 cm^2 (3/M), and three sucrose concentrations (0, 10, 20 and 30 $g{\cdot}L^{-1}$). The natural ventilation systems were obtained by porous membranes manufactured in the lids of the culture vessels, as those in Saldanha et al. (2012).

For all experiments, a completely randomized design with thirty replicates, composed of a split-plot with one explant, was used. After the inoculation in the *in vitro* elongation phase, the following parameters were evaluated: oxidation and vigor (Figure 1), shoot length (> 0.5 cm), mean shoot number per explant (> 0.5 cm), photosynthetic pigment, leaf area, and anatomy.

Leaf area was measured using WinFOLIATM software by the EPSON PERFECTION V700 PHOTO scanner. Five explants from each treatment were used. In each seedling, one area was evaluated and a leaf was removed from the second pair, counted from the apex of the aerial part to the root.

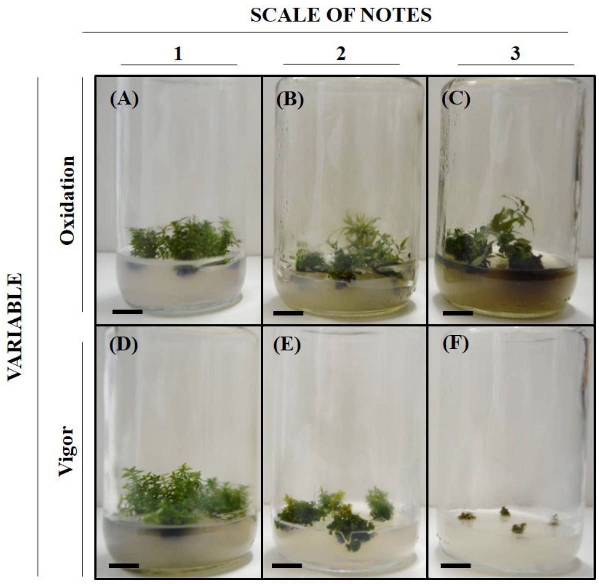

FIGURE 1 Oxidation and vigor assessments according to a scale of notes. (A) 1 = Null: no oxidation; (B) 2 = Average: reduced oxidation at the base of the explants (medium with grayish tonality; (C) 3 = High: complete oxidation of shoots); (D) 1 = Optimum: induction of shoots with active growth, without apparent nutritional deficiency; (E) 2 = Good: induction of shoots, but with leaves of reduced size; (F) 3 = Low: no induction of shoots and, senescence and death). Bar = 1 cm.

The length of adventitious root was assessed at 30 days (roots observed at the lower end of the vial), as well as the root number from the base of the stem, diameter and length of the largest root (cm).

Photosynthetic pigment analysis

Once the *in vitro* elongation culture (30 days) under different forms of sealing and sucrose concentrations was over, leaf discs (25 mg of fresh leaf matter) were withdrawn and inoculated into 5 mL of DMSO solution (Sigma aldrich) for 48 hours in the dark (Lichtenthaler, 1987). Sample absorbance was determined by triplicate in a 10 mm quartz cuvette of optical path in a Genesys 10UV spectrophotometer (ThermoScientific, USA). The wavelengths (665, 649 and 480 nm) and the equations for calculating chlorophyll concentrations a, b, and total carotenoids were based on the method described by Wellburn (1994).

Leaf anatomy

To perform the histological sections, leaves collected from each treatment were maintained in a 70% solution of formaldehyde acetic acid for 48 hours, and then the material was transferred to 70% ethanol (Johansen, 1940). The plant material was dehydrated in an alcoholic-ethylic series in increasing concentrations (80, 90 and 100%) for 30 minutes in each solution (Johansen, 1940), and it was finally stored in 100% alcohol and histresin solution (Leica®) in a 1:1 ratio in a hot oven (overnight). The embedding was with pure hydroxyethyl methacrylate resin and the cross sections obtained with the manual rotary microtome and knife with a thickness of 7 μm. They were then stained with toluidine blue, mounted on histological slides with stained glass finisher (Paiva et al., 2006) and photomicrographs with a coupled digital camera (AxionCam ERc5s) in a 20x and 40x objective micrometer scale.

Data analysis

Data were processed in R Software, version 3.0.3 (R CORE TEAM, 2018), with the help of the ExpDes package, version 1.1.2 (Ferreira et al., 2013). Treatment means were used to perform the statistical analyses and adjustments of regression equations. Non-parametric variables assessed with a 5% significance test were transformed into arsenic. For the significant variables, Tukey's test was done at 5% of significance.

RESULTS AND DISCUSSION

Mixotrophism effect on *in vitro* elongation

Variations according to studied characteristics, different types of sealing and sucrose concentrations used on *in vitro* elongation were observed at 30 days of culture with the clone of *Eucalyptus dunnii* (Figure 3). Shoot length, number of shoots per explant and leaf area observed on *in vitro* elongation showed significant interaction ($p < 0.05$) (packaging and sucrose). Oxidation, explants vigor, and pigment content were factors that acted independently. The regression curves showed polynomial behavior of the second degree.

Shoot length showed the same behavior as the number of shoots per explant and leaf area regarding sucrose concentrations, where treatment with 10 g·L^{-1} achieved the best results (Figures 2A, 2B, and 2I). The number of shoots per explant 1/M (on average 6.15) (Figure 2B) was higher with 3/M (average 3.68 cm) shoots (Figure 2A), and leaf area 3/M (on average 7.01 cm^2) (Figure 2I). Costa et al. (2017), working with *Ochroma pyramidale*, verified similar results, obtaining higher averages for length, number of shoots per explant and leaf area using the concentration of 10 g·L^{-1} of sucrose. Silva et al. (2017) report their positive effect on different species, which benefits cultivation and reduces costs, and can be applied to *Eucalyptus dunnii*.

However, *in vitro* propagation with the use of porous membranes allows the exchange of gases between the external and internal atmosphere of the flasks through natural ventilation, provided that the concentration of CO_2 is adequate, resulting in increased growth (Kozai, 2010; Martins et al., 2015). In *Capsicum annuum*, Batista et al. (2017) verified higher shoot length and number of leaves per explant in membrane system when compared to conventional system. For *Vaccinium ashei* Reade, the membrane-based system provided higher shoot length, number of shoots, leaf area and chlorophyll content (Hung et al., 2016).

Oxidation evaluations according to grade scale evidenced lowest averages without sucrose supplementation (1.21) (Figure 2D) and S/M (Figure 2C), but no difference was observed otherwise (p > 0,05). *In vitro* conditions are stressful to plant growth, and high concentrations of exogenous sugar are a major cause of oxidation during *in vitro* culture (Tisarum et al., 2018).

In contrast, the concentration of 30 g L^{-1} sucrose (mean 2.85) (Figure 2F) and 3/M (Figure 2E), although not statistically different (> 0.05), stands out. For *Alocasia amazonica*, according to Jo et al. (2009), the concentration of 30 g·L^{-1} of sucrose achieved the best results in the morphophysiological aspects and also in explants development.

However, desired results with membrane use may be associated with increased photosynthesis caused by high CO_2 availability. The *in vitro* culture of *Plectranthus amboinicus,* an improvement in explant vigor was observed with the use of three membranes in the containers (Silva et al., 2017).

Best results regarding pigment content were obtained with the use of membranes (Figure 2G) and 20 g·L^{-1} sucrose (Figure 2H), evidencing the importance of greater gas exchange (CO_2), and an external carbohydrate source in plant growth under these conditions. Sucrose levels had an effect on shoot regeneration and bioactive compounds content in *Ajuga multiflora* (Jeong and Sevanesan, 2018). According to Yuan et al. (2015), the biosynthesis of chlorophyll and carotenoids is also regulated by gas exchange and sucrose. Prolonged exposure to high concentrations of exogenously applied sucrose in most plants may result in an inhibition of photosynthesis, which is associated with inhibition of chlorophyll biosynthesis (McCarthy et al., 2016).

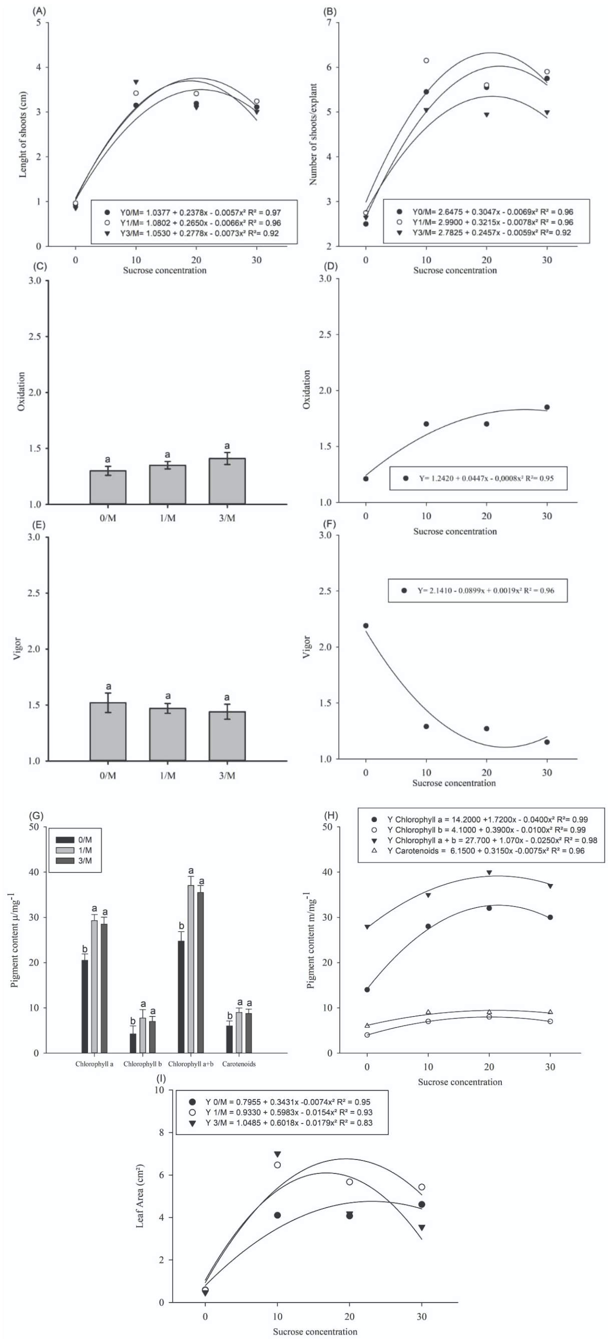

FIGURE 2 Characteristics observed on in vitro elongation of *Eucalyptus dunnii* regarding different sealing (0/M, 1/M and 3/M) and sucrose concentrations (0, 10, 20 and 30 g·L^{-1}). (A) Shoot length; (B) Number of shoots per explant; (C and D) Shoot oxidation; (E and F) Shoots vigor; (G and H) Pigment content; (I) Leaf area. *Averages followed by the same letter do not differ from each other, by Tukey's test at 5% probability.

Results report several effects of membrane use on plants grown *in vitro*, such as improved growth and increased content of photosynthetic pigments, mainly by maintaining adequate concentrations of CO_2 to stimulate photosynthesis (Saldanha et al., 2012). Bandeira et al. (2007) studies with the species *Thymus vulgaris* and Saldanha et al. (2012) with *Pfaffia glomerata*, this system improved the *in vitro* growth and increased the photosynthetic pigment content of the seedlings compared to the conventional system. Rodrigues et al. (2012) verified a lower number of shoots, photosynthetic pigment content and increased senescence in conventional system, in the *Azadirachta indica* species when comparing membrane use.

The use of the membrane system and sucrose concentration (10 $g{\cdot}L^{-1}$) was directly implicated on *in vitro* elongation of *Eucalyptus dunnii,* being determinant for shoot length, number of shoots per explant, pigment content and leaf area, where a reduction of this component in the medium is evidenced, since the most used in the micropropagation of *Eucalyptus* is 30 $g{\cdot}L^{-1}$ (Brondani et al., 2012). The lack of carbohydrate source in the culture medium showed the worst results in length, number, vigor, leaf area, and pigment content. *In vitro* culture plants partially lose autotrophism and, consequently, need an exogenous source of carbohydrates, being the most used sucrose in plant tissue culture (Parveen and Shahzad, 2014).

(A)
(B)
(C)

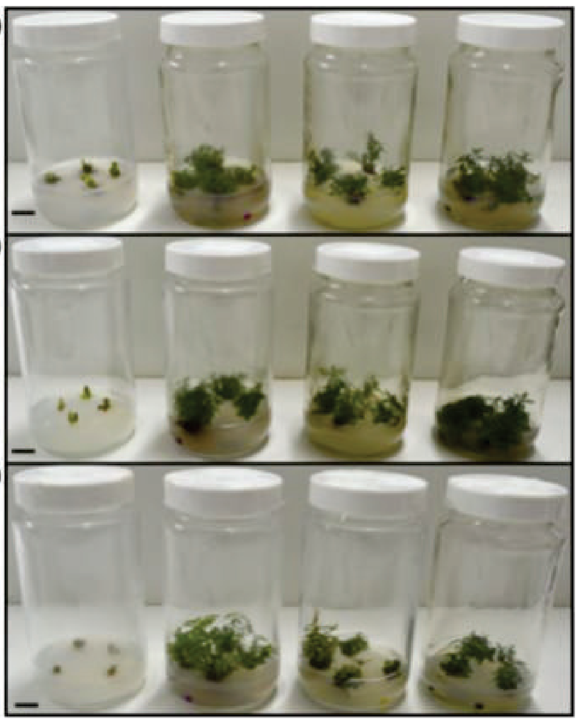

FIGURE 3 Explants of the clone of *Eucalyptus dunnii* at 30 days in the *in vitro* elongation phase. (A) Without a membrane (0/M); (B) With a membrane (1/M); (C) With three membranes (3/M). * From left to right (0, 10, 20 and 30 $g{\cdot}L^{-1}$ sucrose) Bar = 1 cm..

Anatomical structure of the foliar cells can also be influenced by the packaging system of the containers used on *in vitro* culture. The literature reports that the natural ventilation system, for instance, makes leaf cells more organized and consequently they form more rustic leaves. As stated by Silva et al. (2014), natural ventilation provided an increase in the thickness of *Cattleya walkeriana* leaf mesophyll and stomata, making them more functional. Mohamed and Alsadon (2010) evaluated the anatomy of potato seedlings cultured *in vitro*, and found that the use of natural ventilation resulted in thicker leaves with more developed xylem than the conventional system. Results from this study show that membrane-sealing systems provided more developed vascular bundles (Fig. 4A to 4C) and reduced intercellular spaces of mesophyll cells (Figure 4D to 4F).

Mixotrophic effect on *in vitro* rooting

Length, number of shoots, and root diameter acted independently regarding sealing, in face of the sucrose concentrations. These factors were significant (p<0.05) for adventitious rooting. Regression curves showed polynomial behavior of the second degree.

At 30 days on *in vitro* rooting phase, sealing and sucrose concentration showed the same tendency, with the best results obtained for the treatment with 1/M and 30 $g{\cdot}L^{-1}$ (Figure 5), due to length (Fig. 6A and 6B), shoot number per explant averaging 1.70 and 1.98 (Figure 6C and 6D), and mean root diameter of 0.17 and 0.28 cm (Figure 6E and 6F). In *Vaccinium ashei*, the ventilation system provided greater length and number of roots (Hung et al., 2016). The use of systems that increase the CO_2 supply to *in vitro* plants are conditions that can increase plant growth, improve physiological characteristics and facilitate seedlings acclimatization to *ex vitro* conditions, promoting the development of the photosynthetic apparatus (Shin et al., 2014).

Adventitious rooting is a challenge in clonal propagation, with variations from 4 to 46.5% (Brondani et al., 2011; Obserschelp et al., 2015). However, for *Eucalyptus dunnii*, it found a better response to rooting with 66.66% using 1/M and 30g L^{-1} sucrose (Figure 5G). The increase in CO_2 concentration promotes rooting and reduces growth anomalies, as it may improve the photosynthetic rate (Cha-Um et al., 2011). This condition is related to the reduction of water loss due to the deposition of epicuticular wax and to the production of functional stomata (Martins et al., 2015; Hoang et al., 2017). According to Moreira et al. (2013), the effects of the use of membranes to increase the gas exchange infer

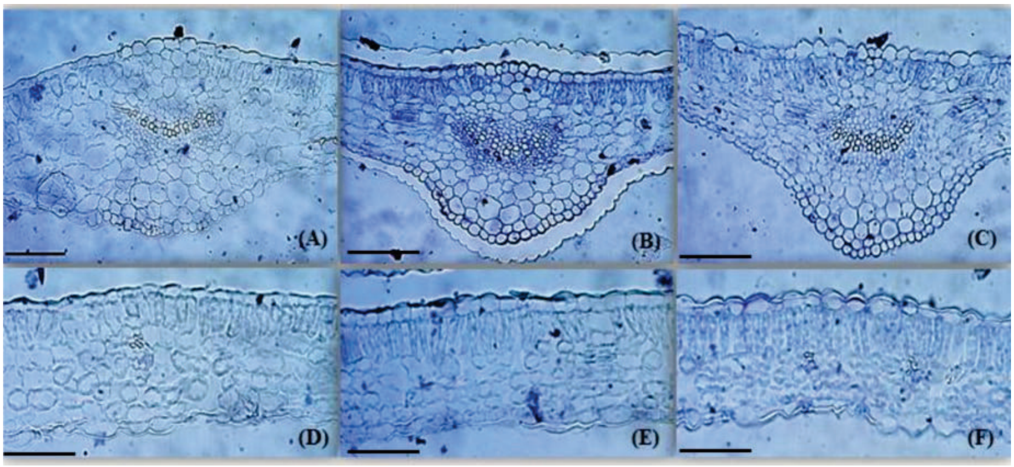

FIGURE 4 Cross sections of the central rib and leaf blade of *Eucalyptus dunnii* as a function of different sealing systems and 30 g·L⁻¹ sucrose. (A and D) Without a membrane (0/M); (B and E) With a membrane (1/M); (C and F) With three membranes (3/M). Bar = 100 μm.

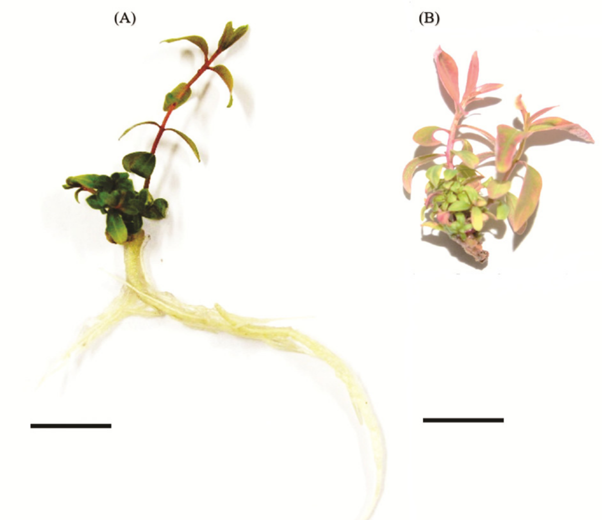

FIGURE 5 Explants of the clone of *Eucalyptus dunnii* at 30 days in the *in vitro* rooting phase in the treatment with a membrane (1/M) and 30 g·L⁻¹ of sucrose. (A) Rooted explant; (B) Explant without root. Bar = 1 cm..

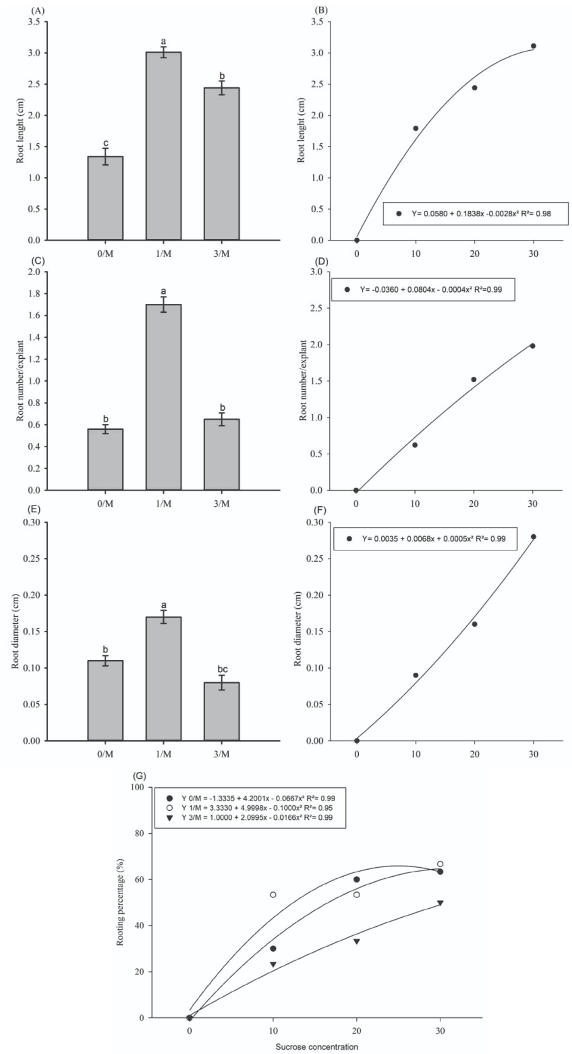

FIGURE 6 Characteristics observed in the *in vitro* rooting of *Eucalyptus dunnii* as a function of different sealing (0/M, 1/M and 3/M) and sucrose concentrations (0, 10, 20 and 30 g·L⁻¹). (A and B) Root length; (C and D) Number of roots per explant; (E and F) Root diameter; (G) Rooting percentage. *Averages followed by the same letter do not differ from each other, by Tukey's test at 5% probability.

in the reduction of relative humidity, increase of aeration, production of more rustic plants and, as a consequence, greater survival and rooting. The use of sucrose was also considered paramount. Using bioreactors in *Populus hybrids*, sucrose provided higher percentages of survival and rooting when compared without the carbon source (Arencibia et al., 2017).

In contrast to the aforementioned results, for *in vitro* cultivation and *ex vitro* acclimatization of *Hevea brasiliensis*, better results for root length and number, survival and rooting were obtained without sucrose and flasks that allowed natural ventilation (Tisarum et al., 2018). Sometimes gradual adaptation to the *ex vitro* condition may be required for plants grown in *in vitro* mixotrophic systems to undergo autotrophic growth in greenhouse (Perez et al., 2015).

CONCLUSIONS

Sucrose should be added on *in vitro* culture medium for the elongation of *Eucalyptus dunnii* clones, which may be reduced to concentrations between 10 and 20 g·L^{-1}.

The use of flasks with membranes that allow higher gas exchange is an effective alternative to improve *in vitro* elongation and adventitious rooting.

The concentration of 30 g·L^{-1} sucrose should be added in the culture medium for *in vitro* adventitious rooting in *Eucalyptus dunnii*.

Absence of carbohydrate was ineffective for the *in vitro* elongation and adventitious rooting of *Eucalyptus dunnii*.

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REFERENCES

ARENCIBIA, A.D.; GÓMEZ, A.; POBLETE, M.; VERGARA, C. High performance micropropagation of dendroenergetic poplar hybrids in photomixotrophic temporary immersion bioreactors (TIBs). **Industrial Crops and Products**, v. 96, p.102-109, 2017.

BACCARIN, F.J.B.; BRONDANI, G.E.; ALMEIDA, L.V.; VIEIRA, I.G.; OLIVEIRA, L.S.; ALMEIDA, M. Vegetative rescue and cloning of *Eucalyptus benthamii* selected adult trees. **New Forests**, v.46, p. 465-483, 2015.

BANDEIRA, J.M.; LIMA, C.S.M.; RUBIN, S.; RIBEIRO, M.V.; FALQUETO, A.R.; PETERS, J.Á. Diferentes tipos de vedações dos frascos e concentrações de sacarose na micropropagação de *Thymus vulgaris* L. **Revista Brasileira de Biociências**, v. 5, n. 2, p. 472-474, 2007.

BATISTA, D.S.; DIAS, L.L.C.; RÊGO, M.M. DO; SALDANHA, C.W.; OTONI, W.C. Flask sealing on *in vitro* seed germination and morphogenesis of two types of ornamental pepper explants. **Ciência Rural**, v. 47, n. 3, p. 1-6, 2017.

BATISTA, D.S.; FELIPE, S.H.S.; SILVA, T.D.; CASTRO, K.M.; RODRIGUES, T.C.M.; MIRANDA, N.A. Light quality in plant tissue culture: does it matter. **In Vitro Cellular and Developmental Biology - Plant**, v. 54, n. 3, p. 195-215, 2018.

BIANCHETTI, R.E.; RESENDE, C.F.de; PACHECO, V.S.; DORNELLAS, F.F.; OLIVEIRA, A.M.S. de; FREITAS, J.C.E.; PEIXOTO, P.H.P. An improved protocol for *in vitro* propagation of the medicinal plant *Mimosa pudica* L . **African Journal of Biotechnology**, v. 16, n. 9, p. 418-428, 2017.

BRONDANI, G.E.; DUTRA, L.F.; WENDLING, I.; GROSSI, F.; HANSEL, F.A.; ARAUJO, M.A. Micropropagation of an *Eucalyptus* hybrid (*Eucalyptus benthamii* × *Eucalyptus dunnii*). **Acta Scientiarum. Agronomy**, v. 33, n. 4, p. 655-63, 2011.

BRONDANI, G.E.; WIT ONDA, H.W.de; BACCARIN, F.J.B.; GONÇALVES, A.N.; ALMEIDA, M. de. Micropropagation of *Eucalyptus benthamii* to form a clonal micro-garden. **In Vitro Cellular Developmental Biology - Plant**, v. 48, n. 5, p. 478-487, 2012.

BRONDANI, G.E.; OLIVEIRA, L.S.de;, KONZEN, E.R.; SILVA, A.L.L. da; COSTA, J.L. 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**, v. 90, p. 2409-2423, 2018.

CHA-UM, S.; CHANSEETIS, C.; CHINTAKOVID, W.; PICHAKUM, A.; SUPAIBULWATANA, K. Promoting root induction and growth of *in vitro* macadamia (*Macadamia tetraphylla* L. 'Keaau') plantlets using CO_2-enriched photoautotrophic conditions. **Plant Cell, Tissue and Organ Culture**, v.106, n.3, p.435 - 444, 2011.

COSTA, M.B.T.; ARRUDA, A.S.; VIEIRA, M.C.; PAULA, M.S.P.; LUZ, J.P. Estabelecimento *in vitro* de *Ochroma pyramidale* em diferentes concentrações de meio MS e sacarose. **Revista Agrotecnologia**, v. 8, p. 1-9, 2017.

FERREIRA, E.B.; CAVALCANTI, P.P.; NOGUEIRA, D.A. ExpDes: **Experimental Designs package**. R package version 1.1.2. 2013.

HERINGER, A. S.; REIS, R. S.; PASSAMANI, L. Z.; SOUZA-FILHO, G. A.; SANTA-CATARINA, C.; SILVEIRA, V. Comparative proteomics analysis of the effect of combined red and blue lights on sugarcane somatic embryogenesis. **Acta Physiologiae Plantarum,** v.39, p. 39-52, 2017.

HOANG, N.N.; KITAYA, Y.; MORISHITA, T.; ENDO, R.; SHIBUYA, T. A comparative study on growth and morphology of wasabi plantlets under the influence of the micro-environment in shoot and root zones during photoautotrophic and photomixotrophic micropropagation. **Plant Cell, Tissue and Organ Culture**, v. 130, n. 2, p. 255–263, 2017.

HUNG, C.D.; HONG, C.H.; KIM, S.K.; LEE, K.H.; PARK, J.Y.; DUNG, C.D.; NAN, M.W.; LEE, H.I. *In vitro* proliferation and *ex vitro* rooting of microshoots of commercially important rabbiteye blueberry (*Vaccinium ashei* Reade) using spectral lights. **Scientia Horticulturae**, v. 211, p. 248-254, 2016.

JEONG, B.R.; SIVANESAN, I. Impact of light quality and sucrose on adventitious shoot regeneration and bioactive compound accumulation in *Ajuga multiflora* Bunge. **Scientia Horticulturae**, v. 236, p. 222-228, 2018.

JOHANSEN, D.A. **Plant microtechnique**. London: McGraw-Hill Book Company, 1940, 510p.

JO, E.A.; TEWARI, R.K.; HAHN, E.J.; PAEK, K.Y. *In vitro* sucrose concentration effects growth and acclimatization of *Alocasia amazonica* plantlets. **Plant Cell, Tissue and Organ Culture**, v. 96, p. 307-315, 2009.

KOZAI, T. Photoautotrophic micropropagation. Environmental control for promoting photosynthesis. **Propagation of Ornamental Plants**, v. 10, p. 188-204, 2010.

KRAUS, J.E.; ARDUIN, M. **Manual básico de métodos em morfologia vegetal**. Seropédica, RJ: EDUR, 1997, 198p.

LICHTENTHALER, H.K. ChlorolShylls and carotenoids: pigments of photosynthetic biomembranes. **Methods in Enzymology**, v. 148, p. 350-382, 1987.

MARTINS, J.P.R.; PASQUAL, M.; MARTINS, A.D.; RIBEIRA, S.F. Effects of salts and sucrose concentrations on *in vitro* propagation of *Billbergia zebrina* (Herbert) Lindley (Bromeliaceae). **Australian Journal of Crop Science**, v. 9, n. 1, p. 85-91, 2015.

MCCARTHY, A.; CHUNG, M.; IVANOV, A.G.; KROL, M.; INMAN, M.; MAXWELL, D.P. An established *Arabidopsis thaliana* var. Landsberg erecta cell suspension culture accumulates chlorophyll and exhibits a staygreen phenotype in response to high external sucrose concentrations. **Journal of Plant Physiology**, v. 199, p. 40-51, 2016.

MOHAMED, M.A.; ALSADON, A.A. Influence of ventilation and sucrose on growth and leaf anatomy of micropropagated potato plantlets. **Scientia Horticulturae**, v. 123, p. 295-300, 2010.

MOREIRA, A.L.; SILVA, A.B.; SANTOS, A.; REIS, C.O.; LANDGRAF, P.R.C. Crescimento de *Cattleya walkeriana* em diferentes sistemas de micropropagação. **Ciência Rural**, v. 43, n. 10, p. 1804-1810, 2013.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, v. 15, p. 473-497,1962.

NAVROSKI, M.C.; REINIGER, L.R.S.; PEREIRA, M.O. Alongamento *in vitro* de rebentos de *Eucalyptus dunnii* em função de diferentes genótipos e concentrações de ácido 1-naftil-acético (ANA). **Revista de Ciências Agrárias**, v. 38, n. 1, p. 79-86, 2015.

OBERSCHELP, G.P.J.; GONÇALVES, A.N.; MENEGHETTI, E.C.; GRANER, É.; ALMEIDA, M.de. *Eucalyptus dunnii* Maiden plant regeneration via shoot organogenesis on a new basal medium based on the mineral composition of young stump shoots. **In Vitro Cellular and Developmental Biology - Plant,** v. 51, p. 626-636, 2015.

PAIVA, J.G.A.de; FANK DE CARVALHO, S.M.; MAGALHÃES, M.P.;RIBEIRO, D.G. Verniz vitral incolor 500® : uma alternativa de meio de montagem economicamente viável. **Acta Botânica Brasileira**, v. 20, n. 2, p. 257-264, 2006.

PARVEEN, S.; SHAHZAD, A. Factors affecting *in vitro* plant regeneration from cotyledonary node explant of *Senna sophera* (L.) Roxb. – A highly medicinal legume. **African Journal of Biotechnology**, v. 13, p. 413-422, 2014.

PÉREZ- JIMÉNEZ, M.; LÓPEZ-PÉREZ, A.; OTÁLORA-ALCÓN, G.; MARÍN-NICOLÁS, D.; PINERO, M.C.; AMOR, F.M. A regime of high CO_2 concentration improves the acclimatization process and increases plant quality and survival. **Plant Cell, Tissue and Organ Culture,** v. 121, p. 547-557, 2015.

RODRIGUES, M.; COSTA, T.H.F.; FESTUCCI-BUSELLI, R.A.; SILVA, L.C.; OTONI, W.C. Effects of flask sealing and growth regulators on *in vitro* propagation of neen (*Azadirachta indica* A.Juss.). **In Vitro Cellular Developmental Biology - Plant**, v. 48, n. 1, p. 67-72, 2012.

SALDANHA, C.W.; OTONI, C.G.; AZEVEDO, J.L.F.de; DIAS, L.L.C.; REGO, M.M.do; OTONI, W.C. A low-cost alternative membrane system that promotes growth in nodal cultures of Brazilian ginseng [*Pfaffia glomerata* (Spreng) Pedersen]. **Plant Cell, Tissue and Organ Culture**, v. 110, p. 413-422, 2012.

SILVA, A.B.; LIMA, P.P.; OLIVEIRA, L.E.S.; MOREIRA, A.L. *In vitro* growth and leaf anatomy of *Cattleya walkeriana* (Gardner, 1839) grown in natural ventilation system. **Revista Ceres**, v.61, n. 6, p. 883-890, 2014.

SILVA, J.A.T.D.; HOSSAIN, M.M.; SHARMA, M.; DOBRÁNSZKI, J.; CARDOSO, J.C.; SONGJUN, Z.E.N.G. Acclimatization of *in vitro*-derived *Dendrobium*. **Horticultural Plant Journal**, v. 3, n. 3, p. 110-124, 2017.

SHIN, K.S.; PARK, S.Y.; PAEK, K.Y. Physiological and biochemical changes during acclimatization in a *Doritaenopsis* hybrid cultivated in different microenvironments *in vitro*. **Environmental and Experimental Botany**, v. 100, p. 26-33, 2014.

SMITH, H.J.; HENSON, M. Achievements in forest tree genetic improvement in Australia and New Zealand : Tree improvement of *Eucalyptus dunnii* Maiden Achieve. **Australian Forestry**, v. 70, n. 1, p. 17-22, 2007.

TISARUM, R.; SAMPHUMPHUNG, T.; THEERAWITAYA, C.; PROMMEE, W.; CHA, S. *In vitro* photoautotrophic acclimatization direct transplantation and *ex vitro* adaptation of rubber tree (*Hevea brasiliensis*). **Plant Cell, Tissue and Organ Culture**, v. 133, n. 2, p. 215-230, 2018.

TRUEMAN, S.J.; HUNG, C.D.; WENDLING, I. Tissue culture of *Corymbia* and *Eucalyptus*. **Forests**, v. 9, n. 2, p. 1-42, 2018.

WELLBURN, A.R. The spectral determination of chlorophylls a and b as well as total carotenoids, using various solvents with spectrophotometers of different resolution. **Journal of Plant Physiology**, v.144, n. 3, p. 307-313, 1994.

XAVIER, A.; WENDLING, I.; SILVA, R.L.DA. **Silvicultura clonal**: princípios e técnicas. 2°. ed. Viçosa, MG: Ed. UFV, 2013, 272p.

YUAN, H.; ZHANG, J.; NAGESWARAN, D.; LI, L. Carotenoid metabolism and regulation in horticultural crops. **Horticulture Research**, v. 2, n. 15036, p. 1 - 11, 2015.