ORIGINAL ARTICLE

Photomixotrophism on *in vitro* elongation of *Corymbia* hybrid clones

Fotomixotrofismo no alongamento *in vitro* de clones híbridos de *Corymbia*

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Abstract

The importance of *Corymbia* species and their interspecific hybrids has been evidenced in forestry programs, due to their wood quality and adaptation to various environmental conditions. To solve the *in vitro* culture limiting factors of a hybrid *Corymbia citriodora* × *C. torelliana* and three *C. torelliana* × *C. citriodora* hybrid clones, the present work aimed to evaluate the influence of photomixotrophism on the *in vitro* elongation phase. The tissues used to obtain the explants were nodal segments from minitumps of the four hybrid clones. The effect of photomixotrophism was assessed by three light sources (fluorescent lamp, white LEDs and red/blue LEDs); three sucrose concentrations (0, 15 and 30 g L⁻¹) and two sealing forms (rigid membrane less polypropylene caps and two membranes). Data on shoot length and number per explant, vigor, shoot oxidation and photosynthetic pigment content were collected at 35 days of *in vitro* culture. Based on the results obtained, it can be inferred that red/blue LEDs, 15 g L⁻¹ sucrose and membrane less vials are more suitable for *in vitro* elongation of hybrid clones *C. citriodora* × *C. torelliana* and *C. torelliana* × *C. citriodora*, without prejudice to the development of shoots intended for the production of micropropagated clonal plants.

Keywords: *In vitro* propagation; Light quality; Gas exchange.

Resumo

A importância das espécies do gênero *Corymbia* e de seus híbridos interespecíficos tem sido evidenciada nos programas de silvicultura, devido à sua qualidade da madeira e adaptação a diversas condições de ambiente. Para solucionar os fatores limitantes no cultivo *in vitro* de um clone híbrido de *Corymbia citriodora* × *C. torelliana* e três de *C. torelliana* × *C. citriodora*, o presente trabalho teve como objetivo avaliar a influência do fotomixotrofismo na fase de alongamento *in vitro*. Os tecidos utilizados para a obtenção dos explantes foram segmentos nodais, provenientes de minicepas dos clones híbridos. O efeito do fotomixotrofismo foi avaliado por meio de três fontes de luz (Lâmpada fluorescente, LEDs branco e LEDs vermelho/azul); três concentrações de sacarose (0, 15 e 30 g L⁻¹) e duas formas de vedação (tampas rígidas de polipropileno sem membrana e duas membranas). Dados de comprimento e número de brotos por explante, vigor, oxidação das brotações e conteúdo de pigmentos fotossintéticos, foram coletados aos 35 dias de cultivo *in vitro*. Com base nos resultados obtidos, pode-se inferir que LEDs vermelho/azul, 15 g L⁻¹ de sacarose e frascos sem membrana são mais adequadas para serem utilizadas no alongamento *in vitro* dos clones híbridos de *Corymbia citriodora* × *C. torelliana* e *C. torelliana* × *C. citriodora*, sem prejuízo ao desenvolvimento de brotações destinadas à produção de plantas clonais micropropagadas.

Palavras-chave: Propagação *in vitro*; Qualidade de luz; Troca gasosa.

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INTRODUCTION

The expansion of forest plantations to non-traditional regions and the search for better adaptation for the production of seedlings have increased the use of species and hybrid combinations. Thus, in recent years, the hybridization of *Corymbia citriodora* (Hook.) K.D. Hill & L.A.S. Johnson with *Corymbia torelliana* (F. Muell.) K.D. Hill & L.A.S. Johnson has provided excellent results, both in terms of wood quality and growth, in addition to better environmental adaptation and easier vegetative propagation (Assis, 2014; Trueman et al., 2018). In this context, alternatives aimed at improving the production process of seedlings in selected hybrid clones of the genus *Corymbia*, it has been recommended to use of the micropropagation technique via proliferation of axillary buds (Wendling et al., 2014).

Among the stages of micropropagation, the *in vitro* elongation phase is essential for obtaining shoots for the rooting of the microcuttings, which has been carried out both in the *ex vitro* (Wendling et al., 2014) and *in vitro* (Trueman et al., 2018). In recent years, research on the genus *Corymbia* has increased significantly in relation to vegetative propagation *in vitro*, aiming to obtain microstumps (Wendling et al., 2014; Brondani et al., 2018; Souza et al., 2018).

In this context, in order to solve the limiting factors of micropropagation, the need is evident for basic studies to improve the production of microstumps *in vitro*. Biotechnological techniques are being applied in order to automate the micropropagation process, including photomixotrophism, such as the quality of light in the growth rooms with LED lamps, the adjustment of the sugar content in the culture medium and the use of ventilation of the recipients (Pawłowska et al., 2018; Batista et al., 2018). These provide a more favorable environment for the development of plants *in vitro*, for the production of photoassimilates, and also as a stimulus perceived by photoreceptors that trigger photomorphogenesis in plants (Gupta & Karmakar, 2017).

Considering the importance that the genus *Corymbia* and its hybrids currently represent for the forestry sector, the present study aimed to evaluate the effect of photomixotrophism (light quality, sucrose concentration and forms of sealing) on the *in vitro* elongation of one clone's microcuttings of a hybrid of *Corymbia citriodora* × *C. torelliana* and three of *C. torelliana* × *C. citriodora*.

MATERIAL AND METHODS

Study location and experimental material

The experiments were conducted at the Tissue Culture Laboratory II of the Institute of Biotechnology Applied to Agriculture (BIOAGRO) at the Federal University of Viçosa - UFV, Viçosa / MG. The genetic material used to obtain the explants came from ministumps of a hybrid clone of *Corymbia citriodora* × *C. torelliana* (CT01) and three from *C. torelliana* × *C. citriodora* (TC01, TC02 and TC03), from the company CMPC – Celulose Riograndense, located in the municipality of Guaíba / RS.

For the elongation phase, the shoots produced in the *in vitro* multiplication phase were prepared by isolating four standardized 0.5 cm shoots and inoculated under aseptic conditions, in glass flasks (250 mL capacity), grown for 35 days, containing 50 ml of JADS medium (Correia et al., 1995), to which were added: 100 mg L⁻¹ of myoinositol, 800 mg L⁻¹ of PVP-30 (polyvinylpyrrolidone - Vetec®), 6 g L⁻¹ of agar, 0.05 mg L⁻¹ of BA (6-benzyladenine - Sigma Co.) and 0.25 mg L⁻¹ of 3-indolbutyric acid (IBA) (Sigma®).

The culture media were prepared by using deionized water and the pH adjusted to 5.8 ± 0.05 with NaOH (0.1 M) and HCl (0.1 M), before autoclaving and adding the agar. The autoclaving of the culture medium was performed at a temperature of 121 °C and a pressure of approximately 1 kgf cm⁻², for 20 minutes. The treatments were maintained in a growth room at 25 ± 2 °C for a photoperiod of 16 hours of light and irradiance of 40 µmol m⁻² s⁻¹ (quantified by radiometer, LI-COR®, LI-250A Light Meter).

Light quality

To evaluate the effects of light quality on *in vitro* elongation, the experiment was arranged in a 4 × 3 factorial arrangement, in a completely randomized design, with four hybrid *Corymbia*

clones (CT01, TC01, TC02 and TC03) and three light sources: Fluorescent lamp (F/L) (HO Sylvania T12, 110 W, São Paulo, SP, Brazil), White LED lamp (W/L) (SMD 100, 18 W, Vilux®, Vitória, ES, Brazil) and LED lamp red/blue (R/B) (LabPARLL-HR / DB-480, 11.6 W, LabLumens®, Carapicuíba, SP, Brazil), containing forty replications, composed of one explant each. At 35 days of cultivation, the length of shoots ($> 0,5$ cm), the average number of shoots per explant (> 0.5 cm); the oxidation and vigor according to a scale of notes, and the content of photosynthetic pigments were evaluated.

The light spectra were measured by a spectroradiometer (Ocean Optics Spectra-Suite, Ocean Optics, Dunedin, FL) (Figure 1).

Figure 1. Variations in absolute irradiance ($\mu\text{W cm}^{-2} \text{ nm}^{-1}$) and wavelength (nm) of light emitted by fluorescent lamps, white LEDs and red/blue LEDs used in the *in vitro* elongation experimentation of *Corymbia* hybrid clones (CT01, TC01, TC02 and TC03) obtained from the growth room of LCT II, BIOAGRO / UFV.

Sucrose concentrations

Based on the best results from the previous experiment, the red/blue LEDs light source was used for all the analyzed clones.

To test the different sucrose concentrations, the experiment was arranged in a 4×3 factorial arrangement, in a completely randomized design, with four hybrid *Corymbia* clones (CT01, TC01, TC02 and TC03) and three sucrose concentrations (0, 15 and 30 g L^{-1}), containing forty repetitions, composed of one explant each. At 35 days of cultivation, the length of shoots (> 0.5 cm), average number of shoots per explant (> 0.5 cm), oxidation and vigor according to a scale of notes was evaluated.

Sealing forms

For the treatments that provided the best results of the previous experiment, for all analyzed clones, 15 g L^{-1} of sucrose was used in the culture medium.

The experiment was arranged in a 4×2 factorial arrangement, in a completely randomized design, with four *Corymbia* hybrid clones (CT01, TC01, TC02 and TC03) and two forms of sealing, characterized by rigid polypropylene caps without membrane (W/M); and polypropylene caps with two holes (10 mm) covered with $0.45 \mu\text{m}$ membranes (MilliSeal® AVS-045 Air Vent) (2/M).

Scale of notes: vigor and oxidation of explants

Assessments of vigor and oxidation (Figure 2) were evaluated according to a scale of scores (Oliveira et al., 2016).

Figure 2. Oxidation and vigor evaluations according to a scale of notes. **(A)** 1 = Null: no oxidation; **(B)** 2 = Medium: reduced oxidation at the base of the explants (grayish-tone culture medium); **(C)** 3 = High: complete shoot oxidation (blackish-tone culture medium); **(D)** 1 = Low: no shoots development and/or senescence and death; **(E)** 2 = Good: shoots with good growth but with small leaves; **(F)** 3 = Optimal: shoots with active growth without apparent nutritional deficiency. Bar = 1,0 cm

Analysis of photosynthetic pigments content

Leaf discs (25 mg of fresh leaf material) were sampled from shoots of the *in vitro* elongation, after 35 days under the different light qualities and inoculated in 5 mL of DMSO solution (Sigma Aldrich) saturated with calcium carbonate for 48 hours in the dark (Santos et al., 2008). The absorbance of the samples was determined in triplicate in a quartz cuvette with a 10 mm optical path, using a Genesys 10UV spectrophotometer (ThermoScientific, USA). The wavelengths (665, 649 and 480 nm) and the equations for calculating the concentrations of chlorophylls a, b and total carotenoids were based on the method described by Wellburn (1994).

Data analysis

The analyses were processed using R software, version 3.0.3 (R Core Team, 2018), with the aid of the ExpDes package, version 1.1.2 (Ferreira et al., 2013). The treatment averages were used to perform the statistical analyses. The data of the variables that did not present a normal distribution according to the Shapiro-Wilk test at 5% significance were transformed into arcsen. For the significant variables, the Tukey test was performed at 5% significance level.

RESULTS

Effect of light quality on *in vitro* elongation

The different light qualities used in the *in vitro* elongation of *Corymbia* hybrid clones, influenced the average length of the shoots and vigor of the explants at 35 days after inoculation (Figure 3), but without significant interaction between the factors ($p > 0.05$). The highest average of shoot lengths and vigor was found for clone TC03 (4.54 cm and 2.83), differing statistically ($p < 0.05$) for TC01 and TC02, respectively (Figure 3A and 3E). In view of the

Figure 3. Characteristics observed at 35 days *in vitro* elongation. **(A)** Length of shoots per explant as a function of hybrid clones; **(B)** Length of shoots per explant as a function of different light qualities; **(C)** Number of shoots per explant as a function of hybrid clones; **(D)** Number of shoots per explant as a function of different light qualities; **(E)** Vigor of shoots as a function of hybrid clones; **(F)** Vigor of shoots according to different light qualities; **(G)** Oxidation of shoots as a function of hybrid clones; **(H)** Oxidation of shoots as a function of different light qualities; **(I)** Content of photosynthetic pigments as a function of hybrid clones; **(J)** Content of photosynthetic pigments as a function of different light qualities * Means followed by the same letter do not differ from each other by the Tukey test at 5% probability. Bars represent sample standard deviation.

number of shoots and oxidation, clone CT01 (on average 1.60 and 1.03) showed the best results, but did not differ between the genotypes used (Figure 3C and Figure 3G). Regarding the content of photosynthetic pigments (chlorophyll a, b and carotenoids) the results show that the TC03 clone had the highest amounts (Figure 3I).

Regarding the quality of light, the treatment with R/B stood out for all the characteristics evaluated (length of shoots, number of shoots, vigor of explants and photosynthetic pigments content), presenting the best morphological responses in *in vitro* culture (Figure 3B, 3D, 3F and 3J).

Effect of sucrose concentration on *in vitro* elongation

For the characteristics studied, a difference in response was observed between *Corymbia* clones and sucrose concentrations used in *in vitro* elongation, at 35 days of culture (Figure 4), but without significant interaction between factors ($p > 0.05$).

The length of the shoots and the vigor of the explants showed a similar behavior, with the highest averages being verified with clone TC03 (5.02 cm and 2.77), differing statistically ($p < 0.05$) for clones TC01 and TC02 (Figures 4A, 4E). As for the number of shoots and oxidation of explants, the CT01 clone stood out (on average 1.55 and 1.03) (Figure 4C and 4G).

According to the sucrose concentrations used in the shoots length, number of shoots and explant vigor characteristics, it was observed that treatments 15 and 30 g L⁻¹ were considered essential for *in vitro* elongation, with significant difference ($p < 0.05$) for treatment with 0 g L⁻¹ of sucrose (Figure 4B, 4D and 4F). For the oxidation of the explants, the lowest values obtained were the ones treated with 0 g L⁻¹ of sucrose, but did not differ statistically ($p > 0.05$) from the others (Figure 4H).

Effect of sealing forms on *in vitro* elongation

The treatments involving the different clones and forms of sealing were presented independently ($p > 0.05$). The TC03 clone showed the highest average for the length of shoots (4.87 cm), differing statistically for the other treatments (Figure 5A). For the vigor of the explants (on average: 2.88), a similar behavior was observed, but there was only a significant difference for clone TC01 (Figure 5E).

For the number of shoots per explant, the best results were compared to clone CT01 (on average: 1.35) and TC02 (on average: 1.34), with a significant difference ($p < 0.05$) for TC01 and TC03 (Figure 5C). As for oxidation of explants, clone CT01 yielded the lowest average (1.03); however, there was no difference between treatments ($p > 0.05$) (Figure 5G).

Regarding the different forms of sealing used, the treatment with W/M provided the best results for the length of shoots and vigor of explants, with a significant difference ($p < 0.05$) for 2/M (Figure 5B and 5F), showing tissue growth with no nutritional deficiency, according to the scale of notes. There was no statistical difference for the oxidation of explants ($p > 0.05$) between treatments (Figure 5H). In view of the number of shoots, he observed higher averages in treatment 2/M, differing from W/M (Figure 5D).

Figure 4. Characteristics observed at 35 days *in vitro* elongation. **(A)** Length of shoots per explant as a function of hybrid clones; **(B)** Length of shoots per explant as a function of different sucrose concentrations; **(C)** Number of shoots per explant as a function of hybrid clones; **(D)** Number of shoots per explant as a function of different sucrose concentrations; **(E)** Vigor of shoots as a function of hybrid clones; **(F)** Vigor of shoots related to different sucrose concentrations; **(G)** Oxidation of shoots for hybrid clones; **(H)** Oxidation of shoots per different sucrose concentrations * Means followed by the same letter do not differ from each other by the Tukey test at 5% probability. Bars represent sample standard deviation.

Figure 5. Characteristics observed at 35 days *in vitro* elongation. **(A)** Length of shoots per explant for hybrid clones; **(B)** Length of shoots per explant related to different sealing forms; **(C)** Number of shoots per explant according to hybrid clones; **(D)** Number of shoots per explant for different sealing forms; **(E)** Vigor of shoots by hybrid clones; **(F)** Vigor of shoots according to different sealing forms; **(G)** Oxidation of shoots in different hybrid clones; **(H)** Oxidation of shoots due to different sealing forms. * Means followed by the same letter do not differ from each other by Tukey's test at 5% probability. Bars represent sample standard deviation.

DISCUSSION

Effect of light quality on *in vitro* elongation

The light source used in the *in vitro* elongation of the explants influenced the growth of *Corymbia* hybrid clones, although there were variations in responses. Genotypic differences in *in vitro* development are reported in different species of the genus *Corymbia*, as observed for *C. torelliana* × *C. citriodora* (Souza et al., 2018), *C. citriodora* (Brondani et al., 2018) and *C. citriodora* × *C. torelliana* (Arya et al., 2009).

The use of red/blue LEDs influences the morphogenesis of several plant species grown *in vitro* (Batista et al., 2018). Results have shown that LED light is more suitable for plant development, however, responses may vary according to plant species. One cause for this differentiation is related to the regulation of physiological processes, such as photomorphogenesis, resulting in the highest quality, production and development of micropropagated seedlings (Gupta & Karmakar, 2017).

Data described in the literature corroborate those found in this study, since the combination of red/blue LEDs in *Populus euramericana* Moench (pro sp.) induced greater length, number, pigment content and quality of shoots (Kwon et al., 2015). The biosynthesis of chlorophyll a, b and carotenoids is influenced by the quality of light emitted, presenting greater efficiency in the photosynthetic process predominantly in the combined blue and red wavelengths (Gupta & Karmakar, 2017). Batista et al. (2018) confirmed the superiority of LED lamps in the *in vitro* development of plants when compared to fluorescent lamps.

Effect of sucrose concentration on *in vitro* elongation

The sucrose concentration had a direct implication on the elongation of *Corymbia* clones, mainly considering 15 g L⁻¹, in which there is a reduction of this component in the medium; since the usual concentration in *Corymbia* micropropagation is 30 g L⁻¹. Studies report the use of 30 g L⁻¹ of sucrose in the micropropagation process, being responsible for the multiplication or elongation (Arya et al., 2009; Brondani et al., 2018; Souza et al., 2018).

Costa et al. (2017), working with *Ochroma pyramidale* (Cav. ex Lam), found the highest averages for the length of shoots using the concentration of 15g L⁻¹ of sucrose. Silva et al. (2018) report its positive effect on different species, which benefits cultivation and reduces costs.

With the exclusion of the carbon source in the medium, Monfort et al. (2015) working with *Ocimum selloi* Benth, found a negative effect on the number, length and dry matter of shoots. In *in vitro* cultivation, plants partially lose autotrophism and, consequently, need an exogenous source of carbohydrates, with sucrose being the most used in plant tissue culture (Shahina & Anwar, 2014).

Effect of sealing forms on *in vitro* elongation

The use of the membraneless system had a direct implication in the *in vitro* elongation of *Corymbia* clones. The amount of CO₂ assimilated by the plant in *in vitro* propagation will depend on the genotype, resulting in plant development (Tisarum et al., 2018).

Results similar to the study were found in *Eucalyptus grandis* W. Will ex Maiden × *Eucalyptus urophylla* S.T. Blake, with greater length and quality of shoots in the system without air injection in temporary immersion bioreactors (Oliveira et al., 2014). In contrast, two varieties of *Capsicum annuum* (L.) Kuntze Batista et al. (2017) found greater length of the aerial part and number of leaves per explant in a membrane system, when compared to the conventional system.

In vitro conditions are stressful for plant growth and high CO₂ concentrations are a major cause of oxidation and reduced vigor during *in vitro* cultivation (Tisarum et al., 2018). Studies have reported various effects on plants grown *in vitro* without the use of a membrane, such as improved growth and increased content of photosynthetic pigments, mainly by maintaining

adequate CO₂ concentrations to stimulate photosynthesis (Hoang et al., 2017). However, the use of systems that increase the supply of CO₂ for some species *in vitro*, promotes the development of the photosynthetic apparatus, resulting in a higher rate of multiplication and acclimatization of seedlings to *ex vitro* conditions (Shin et al., 2014).

CONCLUSIONS

- 1) The red/blue LED light source proved to be the most suitable, with the best results for shoots length, number of shoots per explant, vigor, oxidation and photosynthetic pigment content of the microcuttings;
- 2) Sucrose significantly influenced the performance of *in vitro* culture, with a reduction to 15 g L⁻¹ giving the best results in view of the variables analyzed;
- 3) The use of flasks without membranes is effective on elongating microcuttings *in vitro*;
- 4) Clones CT01 and TC03 showed the best results in view of the evaluated characteristics, being promising for seedling production *in vitro*.

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