LIGHT QUALITY IN THE *IN VITRO* INTRODUCTION OF *Corymbia* HYBRID CLONES[1]

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ABSTRACT – Micropropagation via axillary bud proliferation is recommended for rejuvenation or reinvigoration of selected clones, as well as for improving clonal seedlings rooting. The success of a micropropagation protocol depends on the *in vitro* introduction, since following phases, multiplication, elongation, and rooting can only take place once the aseptic crop with vegetative vigor has been established. This study aims to assess the effect of light on the *in vitro* introduction of hybrid clones of *Corymbia torelliana* x *C. citriodora* e *Corymbia citriodora* x *C. torelliana* by the micropropagation technique through proliferation by axillary buds. The mini-stumps, suppliers of explants for *in vitro* introduction, were conducted in semi-hydroclonal mini-clonal hedge. Nodal segments from three *Corymbia torelliana* x *C. citriodora* (TC01, TC02 e TC03) clones and one *Corymbia citriodora* x *C. torelliana* (CT01) clone were collected, disinfested and inoculated in JADS culture medium, in order to compare the effects of light quality from a dark/fluorescent lamp, a fluorescent lamp, and white and red/blue LEDs. At 30 days after inoculation, the following characteristics were evaluated: average contamination percentage, oxidation, non-reactive explants, shoot length and average number of shoots per explant greater than 0.5 cm. Gathered data showed that the use of red/blue LED light source obtained the best results in all assessed characteristics in the *in vitro* introduction.

Keywords: *In vitro* propagation;Vegetative propagation; LEDs.

QUALIDADE DE LUZ NA INTRODUÇÃO IN VITRO DE CLONES HÍBRIDOS DE Corymbia

RESUMO – A micropropagação via proliferação de gemas axilares tem sido recomendado para rejuvenescimento/ revigoramento de clones selecionados, e consequentemente melhoria no enraizamento de mudas clonais. O sucesso de um protocolo de micropropagação depende da fase de introdução ***in vitro****, visto que as etapas seguintes de multiplicação, alongamento e posterior enraizamento, só podem ser executadas após o estabelecimento de culturas assépticas e com bom vigor vegetativo. Diante disso, o presente estudo teve como objetivo avaliar o efeito da qualidade de luz na introdução* ***in vitro*** *de clones híbridos de* ***Corymbia torelliana*** *x* ***C. citriodora*** *e* ***Corymbia citriodora*** *x* ***C. torelliana*** *pela técnica de micropropagação via proliferação por gemas axilares. As* mini-stumps*, fornecedoras dos explantes para introdução* in vitro*, foram conduzidas em minijardim clonal semi hidropônico. Segmentos nodais de três clones de* ***Corymbia torelliana*** *x* ***C. citriodora*** *(TC01, TC02 e TC03) e de um clone de* ***Corymbia citriodora*** *x* ***C. torelliana*** *(CT01) foram coletados, desinfestados e inoculados em meio de cultura JADS, à fim de comparar os efeitos da qualidade da luz de Escuro/lâmpada fluorescente, Lâmpada fluorescente, LEDs branco e LEDs vermelho/azul, e. Aos 30 dias após a inoculação, foram avaliadas as-características: porcentagem média de contaminação, oxidação, explantes não reativos, comprimento*

de brotos e o número médio de brotações por explante maiores que 0,5 cm. Com base nos resultados obtidos, o uso da fonte de luz LEDs vermelho/azul obteve os melhores resultados, para todas as características avaliadas na introdução ***in vitro.***

Palavras-Chave: Propagação ***in vitro****; Propagação vegetativa; LEDs.*

1.INTRODUCTION

Micropropagation through proliferation of axillary buds has been recommended for rejuvenation of selected clones in a forest area, mainly aiming at improving the production process of cloning. Success of a micropropagation protocol depends on the phase of *in vitro* introduction, given that the subsequent stages of multiplication, lengthening, and rooting can only be carried out after the establishment of aseptic cultures and with vegetative vigor (George et al., 2008; Trueman et al., 2018).

In recent years, research on vegetative propagation of the genus *Corymbia* has increased significantly. As a result, micropropagation methods have improved, maximizing clonal production, especially by vegetative rejuvenation (Wendling et al., 2014a,b). Several technologies have been proposed to automate the process, among them: innovations in environment culture, such as alternative containers allowing gas exchanges; new light sources based on LEDs; and automation of cropping systems and routine procedures operations, such as medium preparation, transplanting and acclimatization (Bianchetti et al., 2017).

Recent studies have shown how different qualities of light influence plant metabolism (Batista et al., 2018). Here, the use of a source with a blue (450 - 495 nm) and red (620 - 750 nm) lighting that acts on *in vitro* morphogenesis, plant growth, and development (Gupta and Jatothu, 2017).

The use of light-emitting diode (LED) lamps for plants seems to be advantageous in relation to fluorescents, since LEDs can provide light more efficiently, through particular points in the light spectrum, acting in a larger energy production by photosynthesis (Bugbee, 2016). LED lights have recently become the predominant light source for micropropagation use in plant growth rooms, mainly due to the variation in spectral light composition in the growing environment optimizing the growth of several plant species, becoming an alternative for replacement of cold white fluorescent lamps (Singh et al., 2015).

Considering the importance that the *Corymbia* genus and its hybrids currently represent for the forest sector, obtaining an efficient and reproducible protocol for vegetative propagation will be essential to establish rational conditions for the propagation of these plants. This knowledge will greatly contribute to the commercial production of clonal seedlings in forestry companies, producers and research institutions, to consolidate the basis of commercial forestry.

In this context, the present study aims to evaluate the effect of light quality on the *in vitro* introduction of hybrid clones of *Corymbia torelliana* x *C. citriodora* and *Corymbia citriodora* x *C. torelliana* by the micropropagation technique through proliferation of axillary buds.

2.MATERIALAND METHODS

2.1. Study location and experimental material

Experiments were conducted at the Tissue Culture Laboratory II of the Institute of Applied Biotechnology for Agriculture – BIOAGRO, Federal University of Viçosa - UFV, located in the municipality of Viçosa/MG.

The material used to obtain the explants came from ministumps of three hybrid clones of *Corymbia torelliana* x *C. citriodora* (TC01, TC02, TC03) and one of *Corymbia. citriodora* x *C. torelliana* (CT01), originating from the CMPC - Celulose Riograndense Company, located in the Guaiba/RS municipality.

The mini-stumps were established in mini-clonal hedge, under a semi hydroponic system of sand canals, in the Research Vivarium of the Department of Forestry Engineering of the Federal University of Viçosa, Viçosa/MG. The plants received nutrient solution from a dripping system, applied four times a day, in a total daily flow of 4 L m^{-2}. The nutrient solution was composed of calcium nitrate (920 mg L^{-1}), potassium chloride (240 mg L^{-1}), potassium nitrate (140 mg L^{-1}), monoammonium phosphate (96 mg L^{-1}), magnesium sulfate (364 mg L-1), hydrofiber (40 mg L^{-1}), boric acid (2,800 mg L^{-1}), zinco sulfate (0.480 mg L^{-1}), manganês sulfate (1,120 mg L-1),

copper sulfate (0,100 mg L^{-1}) and sodium-molybdate (0,040 mg L^{-1}). The electrical conductivity of the nutrient solution was maintained around 2.0 mS m^{-2}.

2.2. Explant collection and preparation

Buds were gathered (Figure 1A) from the mini-stumps second (first introduction) and fourth collection (second introduction), 60 and 120 days after the apex pruning, respectively. Nodal segments measuring between 3 and 4 cm were prepared by removing the leaves from the third and fourth nodes, from the apex of the shoots (Figure 1B). Subsequently, the explants were immersed in autoclaved deionized water and transported to the tissue culture laboratory. During the whole process, the equipment used was disinfected with 70% (v/v) alcohol solution.

2.3. In vitro introduction

The nodal segments were washed five times in running water and immersed in fungicidal solution containing 2.4 g L^{-1} of Orthocide 500® (50% as active principle) for 15 minutes. They were washed five times in autoclaved deionized water and immersed in 70% (v/v) alcohol solution for 30 seconds with constant shaking, within the horizontal laminar flow chamber. They were then immersed in 1% (v/v) NaOCl solution and Tween 20 (3 drops/100 ml solution) for 15 minutes. Finally, the nodal segments were washed in 5-fold autoclaved deionized water and inoculated vertically, under aseptic conditions, into 15 cm x 2.5 cm test tubes containing 10 ml of culture medium.

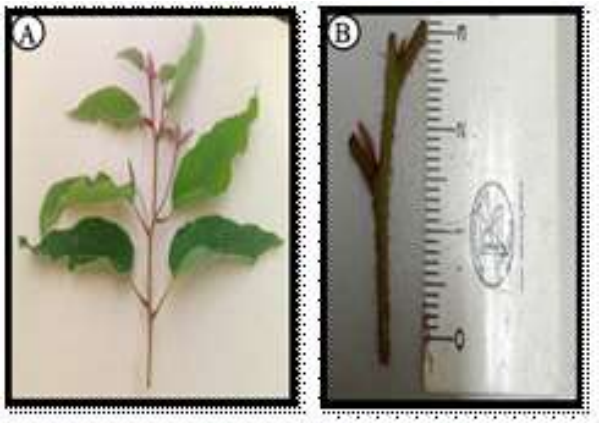

Figure 1 – Preparation of the explants of Corymbia hybrid clones for in vitro: (A) fresh budding of the mini-stumps, highlighting the portion used to obtain the explants; (B) nodal segment.

Figura 1 – *Preparo dos explantes de clones híbridos de Corymbia para introdução* ***in vitro****: **(A)** brotação recém coletada das mini-stumps, destacando a porção utilizada como explantes; **(B)** segmento nodal.*

The time from the collection of explants under field conditions until the inoculation in culture medium was less than three hours. The explants were kept immersed in autoclaved deionized water to avoid dehydration, from collection to inoculation.

The culture medium used was JADS (Correia, 1995) supplemented with 0.5 mg L^{-1} BA (6-benzyladenine – Sigma Co), 0.1 mg L^{-1} ANA (100 mg L^{-1} naphthaleneacetic – Sigma Co) acid myo-inositol (Sigma Co), 800 mg L^{-1} of PVP30 (Polyvinylpyrrolidone – Synth Ltda), 30 g L^{-1} of sucrose (Synth Ltda) and 6 g L^{-1} of agar (Merck S.A.). The pH of the solution 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 121°C and a pressure of approximately 1 kgf cm^{-2} for 20 minutes.

Three hybrid clones of *Corymbia torelliana* x *C. citriodora* (TC01, TC02, TC03) and one *Corymbia. citriodora* x *C. torelliana* (CT01) were used to make two *in vitro* introductions.

2.4. Light sources

After inoculation, the explants were kept in growth room at 25 ± 2°C for a photoperiod of 16 hours, and irradiance of 80 ìmol m^{-2} s^{-1} (quantified by radiometer, LI - COR®, LI-250A Light Meter). Three different light sources were tested: fluorescent lamp (HO Sylvania T12, 110 W, São Paulo, Brasil), white LED lamp (SMD 100, 18 W, Vilux®, Vitória, ES, Brasil) and red/blue LED lamp (LabPARLL-HR / DB-480, 11,6 W, LabLumens®, Carapicuíba, SP, Brasil). As a control, the explants were kept for seven days in the dark and afterwards transferred to the fluorescent lamp (HO Sylvania T12, 110 W, São Paulo, Brasil). Light spectra were obtained by spectroradiometer (Ocean Optics Spectra-Suite, Ocean Optics, Dunedin, FL) (Figure 2).

2.5. Design and experimental evaluations

The experiment was conducted in a 4 x 4 factorial arrangement, in a completely randomized design, with four clusters of *Corymbia* hybrids (three hybrid clones of *Corymbia torelliana* x *C. citriodora* (TC01, TC02,

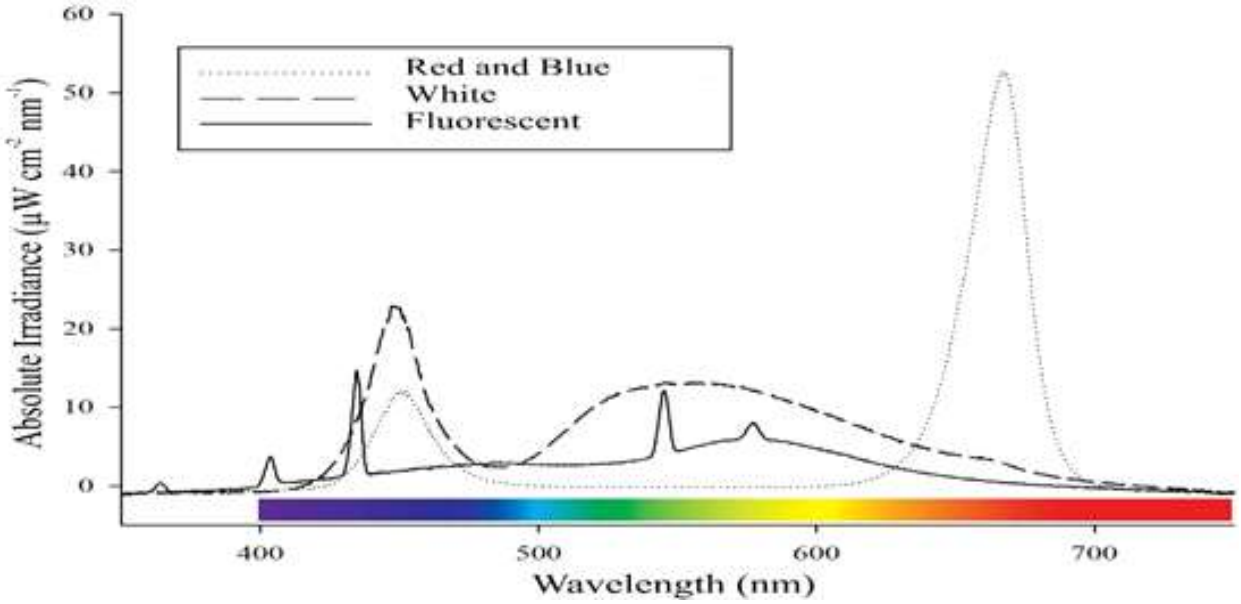

Figure 2 – Variations of absolute irradiance (iW cm-2 nm-1) and wavelength (nm) of light emitted by fluorescent lamps (HO Sylvania T12, 110 W), white LEDs (Vilux® SMD 100, 18 W) and red / blue LEDs (LabPAR LL-HR / DB-480, 11.6 W) used in induction experiments on explants in the in vitro condition of *Corymbia* hybrid clones obtained in the LCT-II growth room, BIOAGRO / UFV.

***Figura 2** – Variações da irradiância absoluta (iW cm^{-2} nm^{-1}) e do comprimento de onda (nm) da luz emitida pelas Lâmpadas fluorescentes (HO Sylvania T12, 110 W), LEDs brancos (Vilux® SMD 100, 18 W) e LEDs vermelho/ azul (LabPAR LL-HR / DB-480, 11,6 W) utilizadas na experimentação de indução em explantes na condição in vitro de clones híbridos de **Corymbia**, obtidas na sala de crescimento do LCT – II, BIOAGRO/UFV.*

TC03) and one of *Corymbia citriodora* x *C. torelliana* (CT01)) and four sources of light: Dark/Fluorescent Lamp (E/LF), Fluorescent Lamp (L/F), LED Lamp white (L/B and red/blue (V/A) with four replicates, composed of plots with eight explants.

At 30 days after inoculation, the following characteristics were evaluated: average percentage of contamination, oxidation, non-reactive explants, shoot length and average number of shoots per explant greater than 0.5 cm.

2.6. Data analysis

The analysis was processed in software R, version 3.0.3 (R Core Team, 2014), using the ExpDes package, version 1.1.2 (Ferreira et al., 2013). Data from the two subcultures at the *in vitro* introduction phase were averaged. The variables contamination, oxidation, non-reactive explant, length and number of shoots were not normally distributed with a test of Shapiro-Wilkem at 5% of significance. For significant variables, the Tukey test was done at 5% significance.

3.RESULTS

The appearance of the *Corymbia* hybrid clone explants in the *in vitro* introduction, regarding studied characteristics, is shown in Figure 3.

The characteristics of contamination, oxidation and shoot length of the observed explants presented their factors (clone and light source) acting independently. Regarding the number of nonresponsive shoots and explants, the factors had a significant interaction.

The hybrid clones of *Corymbia torelliana* x *C. citriodora* (TC01, TC02 e TC03) and *Corymbia citriodora* x *C. torelliana* (CT01) had the lowest average contamination percentage (5.46%) with the red/blue LED (Figure 4A). No significant difference ($p > 0.05$) was observed when clones were compared (Figure 4B).

The same tendency was observed regarding the phenolic oxidation of the explants, in which the red/blue LED lights promoted the lowest average of 2.34% (Figure 4C). The values in each clone were very similar (Figure 4D), and there was no significant difference ($p > 0.05$).

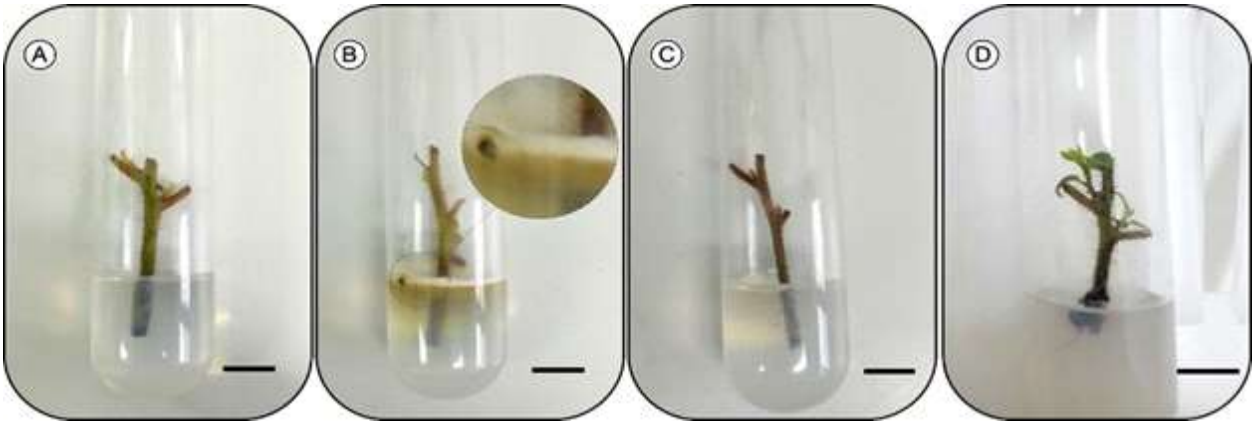

Figure 3 – Explants of *Corymbia* hybrid clones, at 30 days after inoculation in vitro condition: **(A)** nonresponsive explant; **(B)** contaminated explant; **(C)** oxidized explant; **(D)** reactive explant. Bar = 1 cm.
Figura 3 – *Explantes de clones híbridos de* ***Corymbia****, aos 30 dias após a inoculação na condição in vitro:* ***(A)*** *explante não responsivo;* ***(B)*** *explante contaminado;* ***(C)*** *explante oxidado;* ***(D)*** *explante reativo. Barra = 1 cm.*

The combination of red/blue LEDs was determinant for the lower percentage of contamination and oxidation, independent of analyzed clones. On the other hand, the highest contamination percentages and phenolic oxidation were obtained with the dark/fluorescent lamp.

Varied behavior was observed regarding shoots longer than 0.5 cm., with significant differences ($p < 0.05$) found both in clones and light sources.

The shoots (mean> 0.5 cm) from explants exposed to red/blue LED treatment showed longer lengths (average 1.02 cm), than those exposed to fluorescent lamp and dark/fluorescent lamp. No significant difference was observed between this group and that of white LED (Figure 4E).

Among the clones analyzed, clone (TC03) was statistically different regarding sprout length (mean 0.98 cm) than clone (TC02) and clone (TC01) (Figura 4F), whereas, when compared with clone (CT01), it showed no significant difference.

As for the number of shoots larger than 0.5 cm per explant, the data was significantly different ($p < 0.05$) under different treatments of light sources, as well as for clones.

Overall, the red / blue and white LEDs showed results that provided the highest numbers of shoots, resulting in a higher number of shoots per explant (2.03 and 2.11) (Figure 5A).

Data on the percentage of non-responsive explants showed a significant effect ($p < 0.05$) under different light sources, as well as clones. Regarding the response process in the induction of shoots in the explants, at 30 days after inoculation, low percentages of explants without buds were obtained, where clones (CT01) and (TC03) were the most responsive, reaching 100% in red/blue LEDs. Other treatments had similar results and no significant difference was found.

For the best light source among the clones, the clone (CT01) with red/blue LED light obtained smaller percentages of nonresponsive explants, differing statistically only for fluorescent lamp. However, clone (TC03) also had the lowest mean of non-responsive explants in detriment of the use of red/blue LEDs, with statistical difference from the dark/fluorescent lamp. The values were very close to the other clones, with no difference between the light sources.

The light source had direct influence on the development of *Corymbia* hybrid clone explants, where red/blue LED lights provided the best results based on lower oxidation, contamination, length, number of shoots and reactive explants.

4.DISCUSSION

The light source used in the *in vitro* explant cultivation had a direct influence on contamination of hybrid clones of *Corymbia torelliana* x *C. Citriodora e Corymbia citriodora* x *C. torelliana*. The red/blue

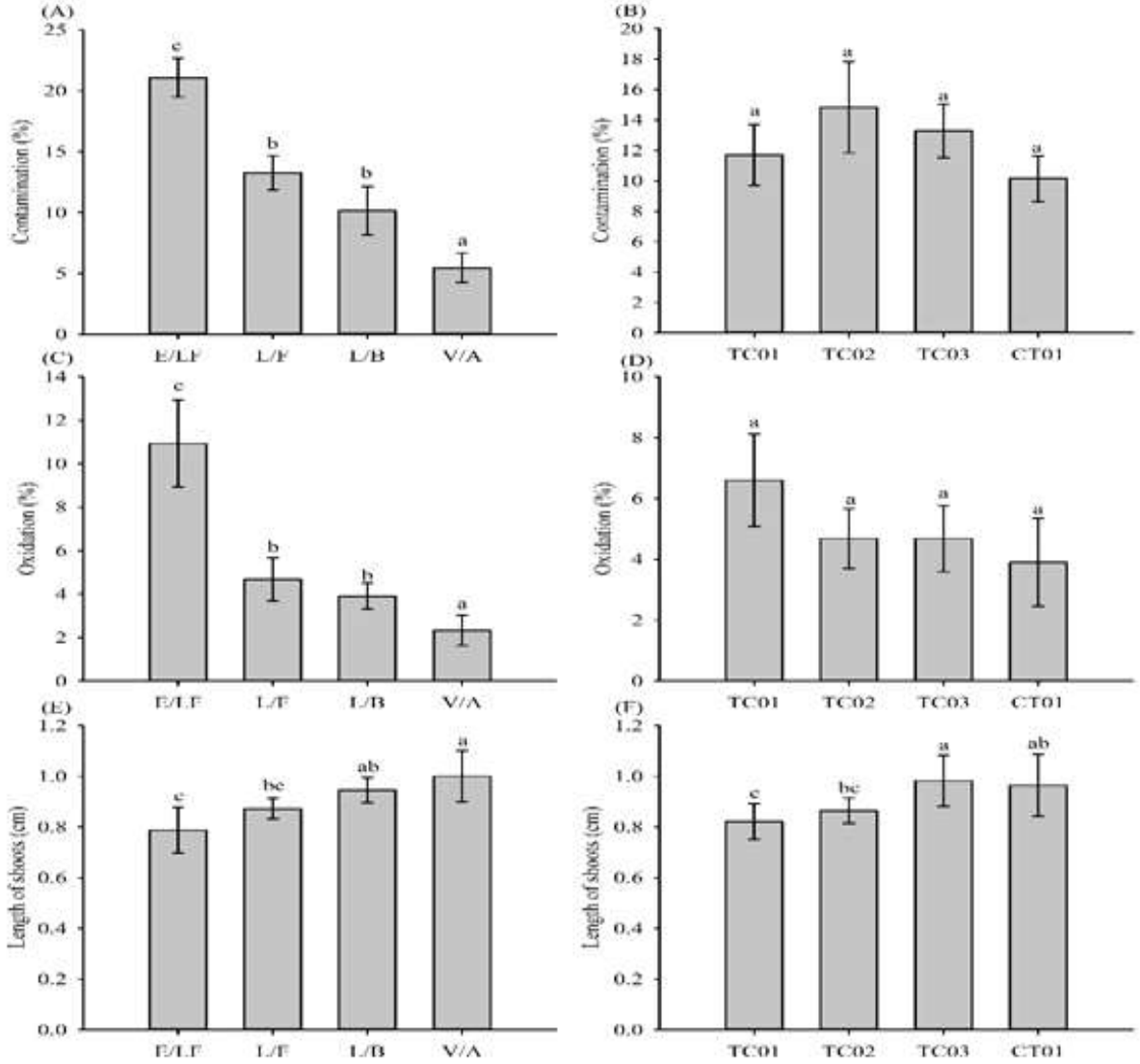

Figure 4 – Characteristics observed in the in vitro introduction according to the different sources of light Dark/fluorescent lamp (E / LF), Fluorescent lamp (L/F), white LEDs (L/B) and red / blue (V/A) LEDs and clones hybrids of *Corymbia torelliana* x *C. citriodora* (TC01, TC02 and TC03) and *Corymbia citriodora* x *C. torelliana* (CT01). (A) Percentage of contamination due to different light sources; (B) Percentage of contamination as a function of the hybrid clones; (C) Percentage of oxidation as a function of different light sources; (D) Oxidation percentage as a function of hybrid clones (E) Length of shoots according to different light sources; (F) Length of shoots according to the hybrid clones. *Averages followed by the same letter do not differ from each other, by the Tukey test at 5% probability.

Figura 4 – *Características observadas na introdução in vitro em função das diferentes fontes de luz Escuro/lâmpada fluorescente (E/LF), Lâmpada fluorescente (L/F), LEDs branco (L/B) e LEDs vermelho/azul (V/A) e clones híbridos de* ***Corymbia torelliana*** *x* ***C. citriodora*** *(TC01, TC02 e TC03) e* ***Corymbia citriodora*** *x* ***C. torelliana*** *(CT01).* ***(A)*** *Porcentagem de contaminação em função das diferentes fontes de luz;* ***(B)*** *Porcentagem de contaminação em função dos clones híbridos;* ***(C)*** *Porcentagem de oxidação em função das diferentes fontes de luz;* ***(D)*** *Porcentagem de oxidação em função dos clones híbridos* ***(E)*** *Comprimento das brotações em função das diferentes fontes de luz;* ***(F)*** *Comprimento das brotações em função dos clones híbridos. *Médias seguidas de uma mesma letra não diferem entre si, pelo teste de Tukey a 5 % de probabilidade.*

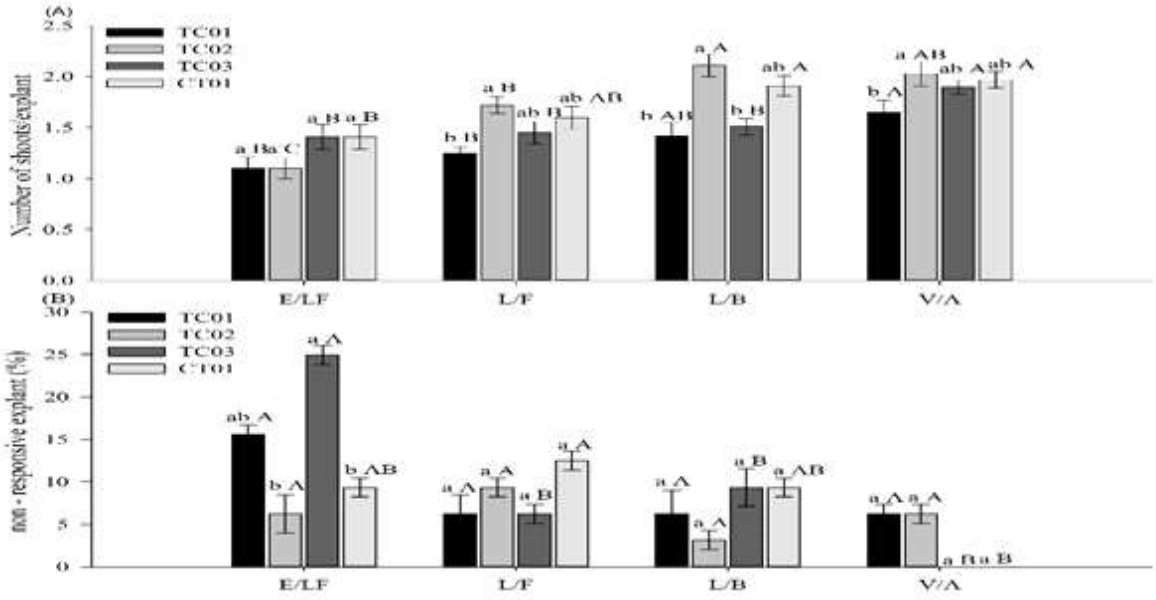

Figure 5 – Characteristics observed in the in vitro introduction according to the different sources of light Dark / fluorescent lamp (E/LF), Fluorescent lamp (L/F), white LEDs (L/B) and red / blue (V/A) LEDs and clones hybrids of *Corymbia torelliana* x *C. citriodora* (TC01, TC02 and TC03) and *Corymbia citriodora* x *C. torelliana* (CT01). (A) Number of shoots per explant; (B) Percentage of nonresponsive explant. Lower case letters establish the comparison between clones and upper case letters represent the comparison between light sources. *Averages followed by the same letter do not differ from each other, by the Tukey test at 5% probability.

Figura 5 *– Características observadas na introdução in vitro em função das diferentes fontes de luz Escuro/lâmpada fluorescente (E/LF), Lâmpada fluorescente (L/F), LEDs branco (L/B) e LEDs vermelho/azul (V/A) e clones híbridos de Corymbia torelliana x C. citriodora (TC01, TC02 e TC03) e* ***Corymbia citriodora*** *x* ***C. torelliana*** *(CT01).* ***(A)*** *Número de brotos por explante;* ***(B)*** *Porcentagem de explante não responsivo. Letras minúsculas estabelecem a comparação entre os clones e letras maiúsculas representam a comparação entre as fontes de luz. *Médias seguidas de uma mesma letra não diferem entre si, pelo teste de Tukey a 5 % de probabilidade.*

LED light provided the best responses, due to the lower percentage of contamination in relation to the other evaluated treatments.

According to Kurtzman and Martínez-Carrera (2013), several organisms with influence of blue and red lights absorb photons and transduce the energy into the cells regulating the fungal photoresponses through differential genetic expression, in the carotenoid biosynthesis. Thus, the metabolic pathway of microorganisms such as fungi can be regulated by light, mainly in the production of secondary metabolites with specific wavelength requirements, showing maximum activity at 440-470 nm (Postemsky and Curvetto, 2016).

Low intensity regarding clone response to phenolic oxidation was observed, especially those under red/blue LEDs. These results are similar to those found by Oliveira et al. (2015), who obtained less than 6% phenolic oxidation in the *in vitro* establishment of *Eucalyptus cloeziana* explants.

Phenolic oxidation has been a problem associated with the micropropagation of woody species and has been reported in several studies (Brondani, 2011; Oliveira et al., 2015; Oliveira et al., 2017). These results may be related to internal environmental factors that affect explant development, where smaller flasks tend to exhibit reduced concentrations of carbon dioxide and high concentrations of ethylene, but may also be affected by irradiation, air temperature and relative humidity (Xiao et al., 2011).

In shoots longer than 0.5 cm, light quality has been found to affect the development of *Corymbia* hybrid clones, with significant changes regarding sprouting. Wavelengths with light spectra in the red and blue range acted with greater effectiveness in

morphogenesis. The use of LED bulbs in *in vitro* cultures has been shown to be advantageous for the regulation of physiological development processes such as photomorphogenesis, leading to higher quality, production and development of micropropagated plantlets (Batista et al., 2018).

The flexibility of combining the wavelengths of the LEDs for photoreceptors can provide a higher production of metabolites, influencing morphogenesis and *in vitro* growth (Li et al., 2013; Postemsky and Curvetto, 2016). However, responses may vary according to species.

In this study, a variation between clones and light sources was observed in the *in vitro* introduction. Data found in the literature were similar to these results, showing that the combination of red/blue LEDs induced a higher number of shoots per explant in *Dendrobium officinale* and *Ajuga multiflora* (Li et al., 2013; Jeong and Sivanesan, 2018). These authors reported that LEDs are an effective alternative, especially during the *in vitro* regeneration of shoots. According to Hung et al. (2015), the red/blue ratio of LEDs significantly influenced the *in vitro* response of *Brassica chinensis* seedlings, where red LEDs strongly stimulated shoot production per explant. In this context, Bugbee (2016) considered the LED light source advantageous for micropropagation as an alternative for replacement of fluorescent lamps.

Following the same pattern of the other characteristics studied, the average number of non-responsive explants varied between clones and light sources. In general, it was observed that the clones had high rates of explants with response to shoot induction (between 75% and 100% of responsive explants). This allows continuing the multiplication phase, highlighting the importance of high induction rates. Although some explants were not responsive, some remained alive, evident by their green coloration (Erig and Schuch, 2005).

In a hybrid of *Eucalyptus urophylla* x *E. globulus* and *globulus,* 95% of explants with shoots was obtained (Borges, 2011). In contrast, Oliveira et al. (2015) obtained a 51.2% average of *Eucalyptus cloeziana* explants with shoots. Thus, different results are observed for non-responsive explants in the *in vitro* introduction phase, with varying responses according to the plant material (genotype) and the culturing conditions used.

According to Trueman et al. (2018), for the success of micropropagation it is necessary that only some explants emit shoots free of contamination, because the beginning of *in vitro* propagation is the main limiting phase. However, when a large amount of micropropagated material is required, higher rates of bud explants may be required to rapidly increase the amount of material produced.

5.CONCLUSIONS

Based on the results obtained in this study, which used hybrid clones *Corymbia torelliana x C. Citriodora* (TC01, TC02, TC03) e *Corymbia citriodora x C. torelliana* (CT01), it can be concluded that, due to the methodology adopted for the proliferation of axillary buds, the red/blue LED light source is the most adequate for *in vitro* introduction, having a direct influence over the development of the explants of *Corymbia* hybrid clones, considering: 1) a lower rate of oxidation, contamination and non-responsive explant; 2) greater shoot length and number of shoots per explant.

6.ACKNOWLEDGEMENTS

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