In Vitro Multiplication of *Eucalyptus pilularis* and *Eucalyptus grandis* x *E. urophylla* (Urograndis Eucalypt): Effect of Light Quality in Temporary Immersion Bioreactor

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Abstract

Light quality is an important factor for in vitro development of explants in a bioreactor system. Based on the need to optimize, this study aimed to evaluate the in vitro multiplication of Eucalyptus pilularis and urograndis eucalypt using different light quality in a temporary immersion bioreactor (TIB) system. Different spectral qualities on in vitro multiplication were evaluated using three light sources, namely fluorescent lamp (white light), red and blue. Shoot length, number of buds, fresh weight per explant, vigor and hyperhydricity were evaluated according to an established scoring scale at 30 days. The results showed that fluorescent white light was the most appropriate for use in the in vitro multiplication of E. pilularis, and blue light for the urograndis eucalypt clone resulting in a greater shoot length (1.46 cm; 2.41 cm), number of buds (2.25; 10.20), vigor (1.8; 1.3) and fresh weight per explant (86.9 mg; 449 mg). The results can be applied to optimize clonal microplant production on a commercial scale.

Keywords*:* bud multiplication, eucalypts, in vitro culture, micropropagation technique

1. Introduction

Clonal propagation by micropropagation technique of *Eucalyptus pilularis* Sm., *Eucalyptus grandis* W. Hill ex Maiden, *Eucalyptus urophylla* S.T. Blake and their hybrids (i.e., *E. grandis* x *E. urophylla*, also known as urograndis eucalypt) is becoming increasingly important for forest improvement. Reports are available regarding the early phases of in vitro introduction (Avelar *et al*.,

2020; Molinari *et al*., 2021), bud multiplication (Máximo *et al*., 2018; Carvalho *et al*., 2019; Souza *et al*., 2020a), shoot elongation (Souza *et al*., 2020b; Zorz *et al*., 2020), rooting and acclimatization of microplants (Souza *et al*., 2019; Miranda *et al*., 2020). Tissue rejuvenation/reinvigoration can be optimized through asexual propagation, which provides higher levels of clonal productivity and adventitious rooting (Wendling *et al*., 2014; Brondani *et al*., 2018; Abiri *et al*., 2020; Souza *et al*., 2022).

Research and new technologies that can be used to optimize the in vitro multiplication phase are of paramount importance in micropropagation and consequently, in clonal production and productivity. These innovations involve new sources of illumination (e.g., spectral quality), automation of in vitro cultivation and the use of a temporary immersion bioreactor (TIB) (Ribeiro *et al*., 2016; Oliveira *et al*., 2021).

TIB is a suitable technology for in vitro cultivation as it allows greater shoots and plant production in controlled environments (Le *et al*., 2021). This type of technology can optimize the in vitro culture (Loyola-González *et al*., 2019), which increases biomass, reduces time required for propagation (Máximo *et al*., 2018) and boosts plant production per area. In this context, TIB can improve nutrient availability, gas exchange and reduction of physiological disorders leading to greater growth and development of cultures (Batista *et al*., 2018; Le *et al*., 2021).

Recent studies have reported the influence of wavelengths in 450-495 nm (blue), 620-750 nm (red), 750-850 nm (far red) and 495-570 nm (green) (Carvalho *et al*., 2019) on plant morphophysiological processes (Miler *et al*., 2019; Abiri *et al*., 2020) considering that it is necessary to establish the best luminosity source for each genetic material of interest (Souza *et al*., 2020a, 2020b).

Given the above context, research with species of the genus *Eucalyptus* is still limited, mainly on the optimization of in vitro cultivation through the effect of different wavelengths and TIB on the morphology, anatomy and physiology of plants. Therefore, this study aimed to evaluate the in vitro multiplication of *E. pilularis* and urograndis eucalypt using different light qualities in the TIB system.

2. Methodology

2.1 Tissue Source

The tissues used to obtain explants were in vitro established from ministump of the A211 hybrid clone of *E. grandis* Hill ex Maiden x *E. urophylla* S.T. Blake (i.e., urograndis eucalypt) (Souza *et al*., 2020a). Ministump of *E. pilularis* Sm. was produced from seed following the same methodology by Avelar *et al*. (2020).

Ministumps were established in a clonal minigarden under automatized semi-hydroponic system in sand-bed (Higashi *et al*., 2002; Brondani *et al*., 2012) (Figure 1). Ministumps received nutrient solution by dripping, which was distributed in four daily applications totaling 4 L m^{-2} (Table 1).

2.2 Shoot Collection and Explant Standardization

Shoots were collected 20 days after pruning the apex of the ministump. Shoots were collected 20 days after pruning the apex of the ministump. The standardization (Figure 1) and transport of explants to the laboratory for in vitro cultivation of forest species were similar to the methodology of Souza *et al*. (2020a).

2.3 In Vitro Establishment

Asepsis procedure, culture Murashige and Skoog (MS) medium preparation (Murashige and Skoog, 1962) and inoculation of the explants were carried out according to Souza *et al*. (2020b). At 30 days of cultivation, the in vitro establishment of the explants was verified (Figure 1).

2.4 In Vitro Multiplication in TIB

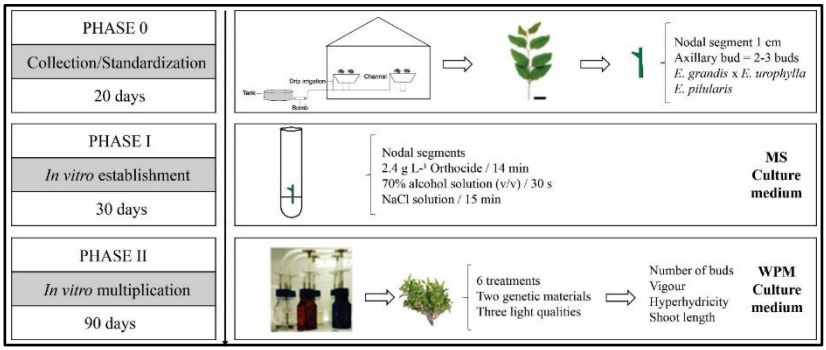
Explants were multiplied according to Souza *et al*. (2020a). During 30 days in the TIB system, tissue immersion in liquid culture medium – woody plant medium (WPM) (Lloyd and McCown, 1980) occurred for 30 s with an interval of 3 h for each immersion cycle (Figure 1).

Table 1. Nutrient solution composition for fertigation of *E. pilularis* and *E. grandis* x *E. urophylla* clonal minigarden

Element	CNS (mg L^{-1})	MW
N (form-NO3-)	60.00	14.00
N (form-NH4+)	30.00	14.00
P	12.00	30.97
Ca	30.00	40.08
K	80.00	39.10
S	18.92	32.06
Mg	12.00	24.31
Cu	0.10	63.54
Fe	2.00	55.85
Mo	0.02	95.94
Mn	1.60	54.94
Zn	1.96	65.37
B	1.08	10.81

Macro and micronutrient source	QF/MW	CNS (mg L^{-1})[(1)]
Potassium nitrate (Nuclear®, United States)	KNO^3/101.10	206.8500
Monoammonium phosphate (Mallinckrodt®, Ireland)	$NH_4H_2PO_4$/115.03	44.5700
Ammonium nitrate (Reagex®, United States)	NH_4NO_3/80.4	140.5000
Calcium sulfate (Vetec®, United States)	$CaSO_4.2H_2O$/172.17	87.1817
Calcium nitrate (Labsynth®, Brazil)	$Ca(NO_3)2.4H_2O$/236.15	57.1800
Magnesium sulfate (Mallinckrodt®, Ireland)	$MgSO_4.7H_2O$/246.48	121.6680
Manganese sulfate (Ecibra®, Brazil)	$MnSO_4.H_2O$/169.01	4.9223
Copper sulfate (Mallinckrodt®, Ireland)	$CuSO_4.5H_2O$/249.68	0.3929
Iron sulfate (Synth®, Brazil)	$FeSO_4.7H_2O$/278.02	9.9520
Sodium - EDTA (Nuclear®, United States)	Na_2-EDTA.$2H_2O$/372.24	13.3110
Sodium molybdate (Merck®, Germany)	$Na_2MoO_4.2H_2O$/241.95	0.0504
Zinc sulfate (Mallinckrodt®, Ireland)	$ZnSO_4.7H_2O$/287.54	8.6000
Boric acid (Ecibra®, Brazil)	H_3BO_3/61.83	6.2000

[(1)] The pH value of the nutrient solution was adjusted to 6.0 (±0.1) at 25 °C with HCl and/or NaOH, both at 1 M; CNS = concentration in the nutrient solution; QF = chemical formula; MW = molecular weight.



Phase 0 – collection and standardization of explants; phase I – inoculation and subsequent in vitro establishment at 30 days of cultivation; phase II – proliferation of adventitious buds on in vitro multiplication at 90 days in the TIB

Figure 1. In vitro cultivation phases of *E. pilularis* and *E. grandis* x *E. urophylla* in the TIB system

2.5 Light Source

Two cold white fluorescent lamps (Philips T10, India) (i.e., length 0.60 m, 20 W, and color 6,400-6,500 K) were used per shelf. The irradiance of the two lamps (i.e., 40 μmol m^{-2} s^{-1}) was measured by a photoradiometer (QSO-S Procheck + Sensor–PAR Photon Flux, Decagon Devices, United States).

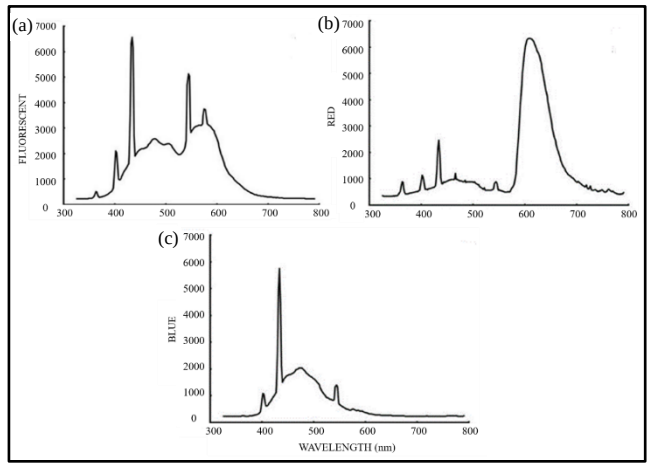
The analysis of irradiance (μW cm^{-2} nm^{-1}) and wavelength (nm) was performed using a portable spectroradiometer (SpectraPen Z850, Qubit Systems-Kingston, Canada). The different qualities of light are seen in Figure 2. Red and blue lights were provided by filtering the light output of the fluorescent lamps through double sheets of cellophane paper that were used to surround the TIB flasks.

The experiment was conducted in a growroom at 24 °C (±1 °C) under a 16-h photoperiod. At 30 days, shoots larger than 0.5 cm were evaluated for shoot length, number of buds, fresh weight per explant (mg), hyperhydricity and vigor according to the scoring scale proposed by Souza *et al*. (2020b) (Figure 3).

2.6 Data Analysis

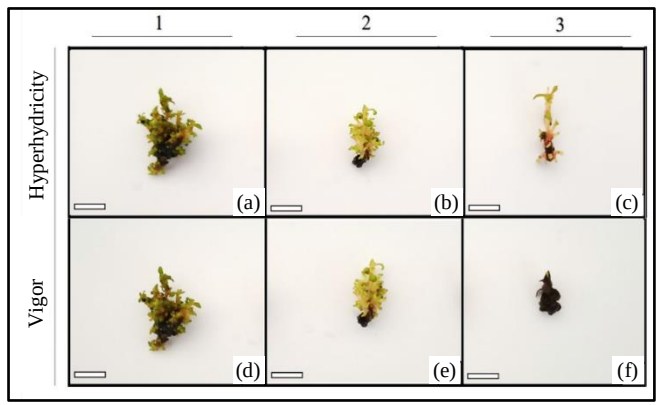
The experiment was conducted in a completely randomized design with a factorial arrangement (2 x 3) (two genetic materials: *E. pilularis* and urograndis eucalypt) and three light qualities (fluorescent white, red and blue) with 12 replicates composed of one explant each.

The data that did not have a normal distribution according to the Shapiro-Wilk ($p > 0.05$) were transformed by arcsin. The data were subjected to analysis of variance (ANOVA) ($p < 0.05$) and the averages were compared by the Tukey's test ($p < 0.05$). Analyses were processed in R software version 3.0.3 (R Core Team, 2014), ExpDes package version 1.1.2 (Ferreira *et al*., 2013). The bars represented in the graphs denote the standard deviation in relation to the mean value.



Fluorescent white light (a); red (b) and blue (c) cellophane paper

Figure 2. Wavelength (nm) and variations in absolute irradiance ($\mu W\ cm^{-2}\ nm^{-1}$) in the in vitro culture of *E. pilularis* and *E. grandis* x *E. urophylla*



Hyperhydricity: (a) 1 – no hyperhydricity; (b) 2 – reduced leaf tissue hypertrophy; (c) 3 – hypertrophy in leaf tissues and explant internodes. Vigor: (d) 1 – shoot induction and active growth without apparent nutritional deficiency; (e) 2 – shoot induction with reduced leaves; (f) 3 – low shoot induction, senescence and death; bar = 0.5 cm

Figure 3. Scale of notes used to assess hyperhydricity and vigor in *E. pilularis* and *E. grandis* x *E. urophylla* explant

3. Results and Discussion

The morphological characteristics of *E. pilularis* and urograndis eucalypt indicated the optimization of the in vitro multiplication phase in the TIB system through determining the best spectral qualities.

Results showed that shoot length (1.46 cm) (Figure 4a) and number of bud (2.25 buds per explant) (Figure 4b) of *E. pilularis* under the fluorescent white light had higher means than red (1.10 cm; 1.83 buds per explant) and blue (1.30 cm; 1.3 buds per explant) treatments. However, there was no significant difference.

An undesirable aspect of the in vitro multiplication is tissue hyperhydricity and vigor, and the results exhibited different responses according to the light quality for *E. pilularis*. According to the scoring scale (Figure 3a to 3c), the best results for hyperhydricity were obtained by blue and red lights and were significantly different from fluorescent white light (Figure 4c). No significant difference was observed in vigor (Figure 4d) with variation from 1.83 to 2.16 of the scoring scale (Figure 3d to 3f).

Fresh weight of *E. pilularis* explant varied according to treatments. The highest mean was noticed in fluorescent white light (86.9 mg per explant) and blue light (71.9 mg per explant). There was a significant difference for red light (49.2 mg per explant) (Figure 4e). Explants of *E. pilularis* showed the need for a broad light spectrum for their in vitro growth and development.

Different spectral qualities used in the in vitro culture of urograndis eucalypt affected the evaluated characteristics at 30 days. The highest mean shoot length was observed in fluorescent white (2.34 cm) and blue light (2.41 cm) treatments, and there was significant difference in red light (1.69 cm) (Figure 4a). The treatments differed significantly in terms of the number of buds per explant; the best result was obtained by blue light (7 buds per explant) (Figure 4b). High multiplication of buds denotes the potential of monochromatic light sources, especially those with peaks at blue wavelengths.

According to the scoring scale for hyperhydricity (Figure 3a to Fc), different responses were observed in relation to the different light qualities for urograndis eucalypt. Fluorescent white light source provided the highest mean (2.0), and there was a significant difference with red (1.5) and blue (1.8) lights (Figure 4c). In contrast, there was no significant difference between the

fluorescent white (1.3), red (1.4) and blue (1.3) lights for the vigor (Figure 4d); furthermore, the results were closer to a score of 1 (Figure 3d to 3f).

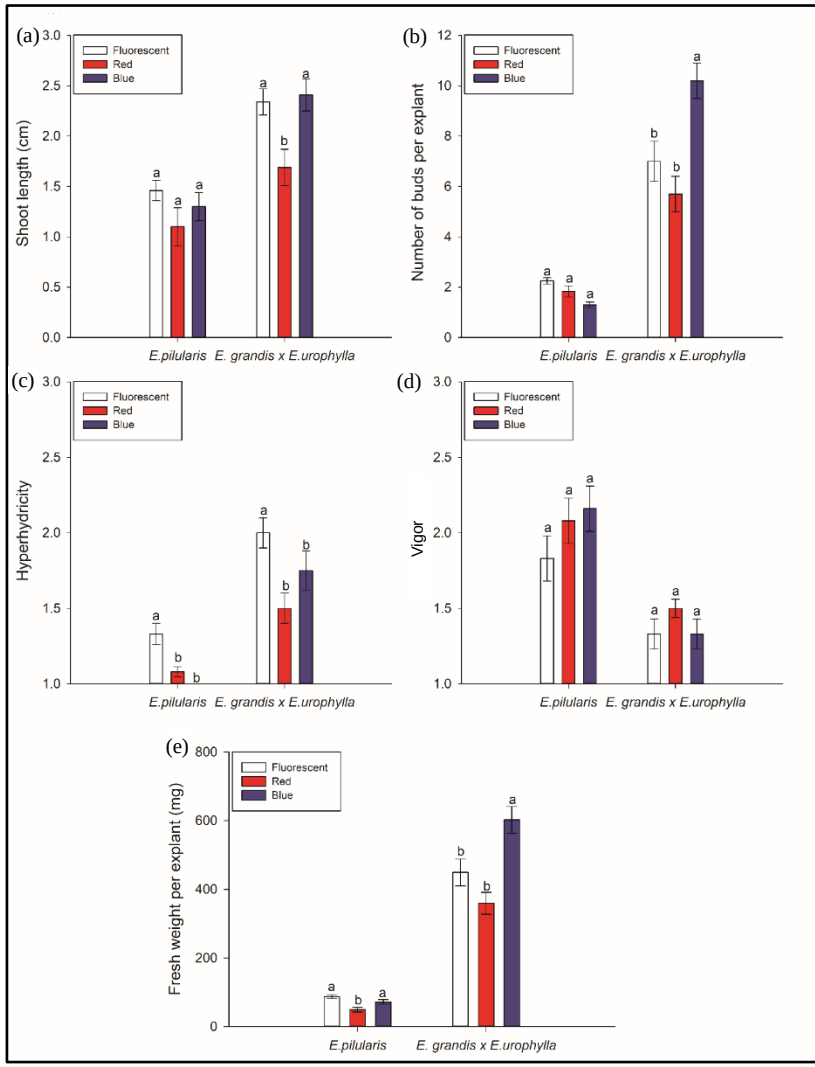


Figure 4. Morphological characteristics evaluated during the in vitro multiplication phase of *E. pilularis* and *E. grandis* x *E. urophylla* in the TIB system with different spectral qualities (fluorescent-white, red and blue lights) at 30 days

The best results for the fresh weight of urograndis eucalypt explant were observed in the blue light (602.0 mg per explant) with a significant difference

from the values obtained in fluorescent white light (449.0 mg per explant) and red light (359.0 mg per explant) (Figure 4e).

In this study, the urograndis eucalypt clone provided better result for shoot length (2.14 cm) (Figure 5a), number of buds per explant (7.63 buds per explant) (Figure 5b), vigor (1.3) (Figure 5d) and fresh weight (470 mg per explant) (Figure 5e), showing rapid growth and development. In contrast, there were different results in hyperhydricity. According to the scoring scale (Figure 3a to 3c), the explants of *E. pilularis* showed lower hyperhydricity (1.1), which statistically differed from that of urograndis eucalypt (Figure 5c). The characteristics of the explants of *E. pilularis* and urograndis eucalypt are shown in Figure 6.

Fluorescent white obtained the highest shoot length, number of buds, vigor and fresh weight for *E. pilularis*. These responses were similar to the findings for *Urtica dioica* (Coelho *et al.*, 2021). Data described in the literature corroborate the present observations since the use of a broad light spectrum for the *Corymbia torelliana* x *C. citriodora* (Souza *et al.*, 2018) and urograndis eucalypt (Souza *et al*., 2020a) induced a higher number of buds and greater shoot length. This result highlighted the importance of the wide variation in wavelength (450-650 nm) for *E. pilularis* with the best photomorphological responses when the propagules are grown in controlled environments – a useful technology for optimizing development and productivity.

Blue light attained the best shoot length, number of buds, vigor and fresh weight per explant of urograndis eucalypt. Blue wavelengths (i.e., 400-500 nm) result in different morphophysiological responses in plants, which can be expressed as changes in growth and development to favor the formation of specialized tissues in environmental conditions (Miranda *et al*., 2020). Investigations on the effect of exposure to a blue light spectrum on in vitro culture have confirmed improvements in plant morphogenesis (Miler *et al*., 2019; Abiri *et al*., 2020). Nevertheless, the growth and development can also depend on the genotype and culture conditions (Molinari *et al*., 2021; Souza *et al*., 2020a).

The results obtained here in the urograndis eucalypt clone are similar to those found by Gupta and Sahoo (2015) in *Curculigo orchioides* where they observed adequate vigor and development of the explants in the wavelengths of 450 and 600 nm. In contrast, for some species, such as *Rehmania glutinosa* (Manivannan *et al*., 2015), *Solanum lycopersicum*, *Cucumis sativus* and

Capsicum annum (Snowden *et al*., 2016), the reduction in growth was observed when the plants were grown under blue light (400-500 nm). Studies with eucalyptus urograndis provided evidence that explants need specificity in the light spectrum (400 nm) to optimize photosynthetic processes.

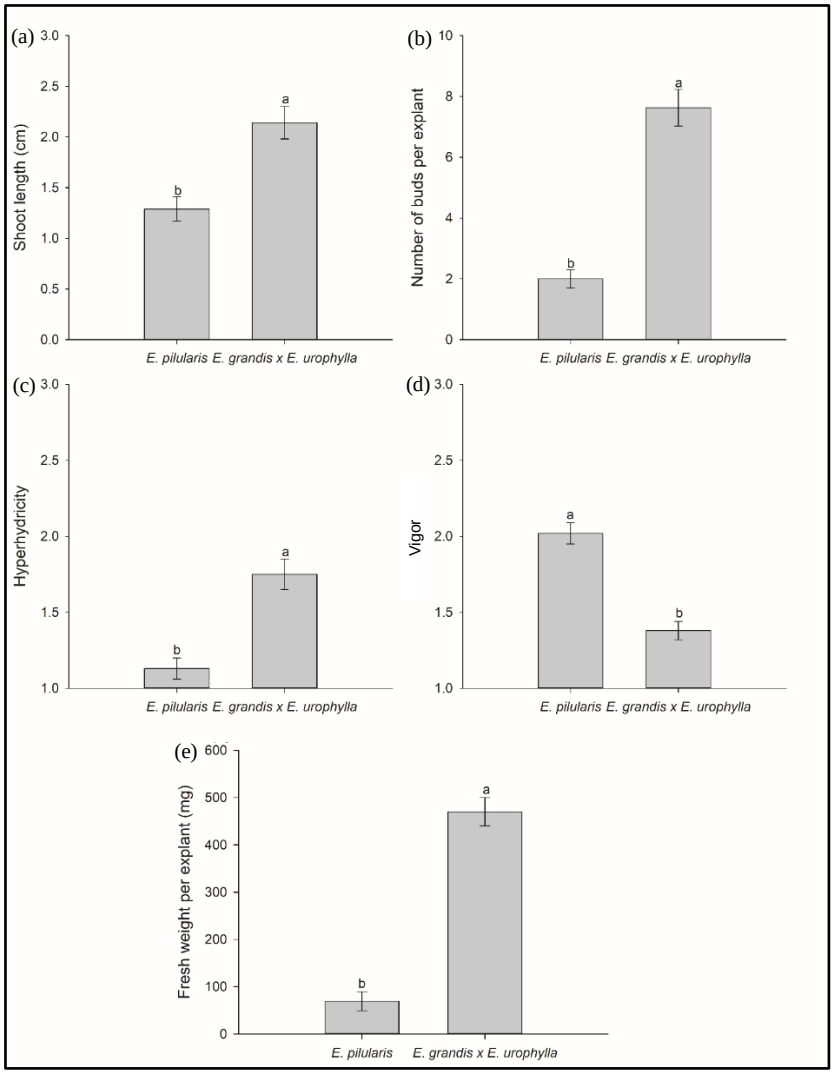
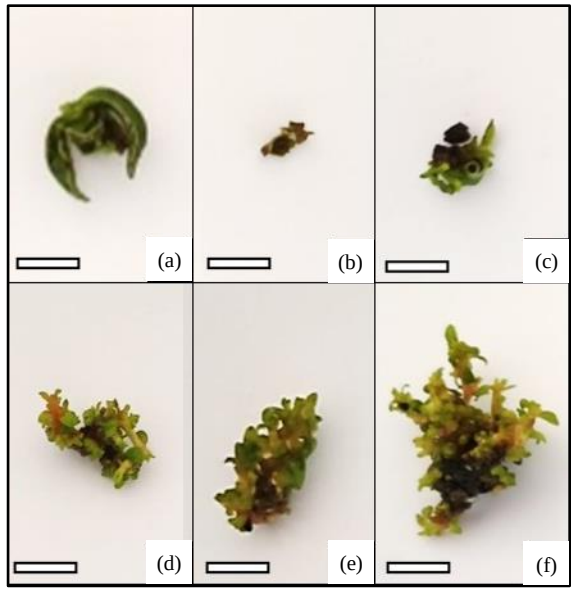


Figure 5. Morphological characteristics of *E. pilularis* and *E. grandis* × *E. urophylla* in relation to different spectral qualities in the TIB system at 30 days



Shoots of *E. pilularis* (a-c); shoots of *E. grandis* × *E. urophylla* (d-f); fluorescent white light (a and d); red light (b and e); blue light (c and f); bar = 0.5 cm

Figure 6. In vitro multiplication phase in relation to different spectral qualities in the TIB system at 30 days

The use of fluorescent white light resulted in the accumulation of water in the tissues and translucent leaves. However, it did not prevent the in vitro cultivation of eucalyptus genotypes in the TIB. These traits are among the main anomalies observed in semisolid or liquid medium cultures (Loyola-González *et al*., 2019); physiological disorders may occur under different cultivation conditions and genotype (Abiri *et al*., 2020; Le *et al*., 2021). In addition, explants can have low levels of lignin and cellulose, stomatal changes, and low chlorophyll content and percentage of dry mass (Li *et al*., 2018; Molinari *et al*., 2021). According to the analyses, the urograndis eucalypt clone presented the best results in all tested treatments.

These responses may be linked to the use of liquid culture medium in the TIB, which allowed for greater absorption of water and mineral salts by plants. Therefore, abiotic factors can directly and indirectly influence plants (Souza *et al*., 2020a). Concomitantly, research and studies that seek to minimize the effects of hyperhydricity are of paramount importance for clonal propagation.

Morphological growth and development have important implications for optimization in the TIB systems when it comes to plant productivity in

eucalyptus clones. The use of fluorescent white light for *E. pilularis* and blue light for *E. urograndis* in the TIB gave the best results for the proliferation of adventitious buds demonstrating the feasibility of propagating on a commercial scale. Thus, understanding the light quality and the TIB is essential for optimizing in vitro cultivation protocols as observed in the present study.

4. Conclusion

In vitro morphological characteristics of *E. pilularis* and *E. grandis* x *E. urophylla* cultivated in the TIB were influenced by the wavelength. On one hand, fluorescent white light was the most suitable for in vitro multiplication of *E. pilularis* resulting in a greater shoot length, number of buds, vigor and fresh weight. On the other hand, blue light resulted in more shoot length, number of buds, vigor and fresh weight for the in vitro multiplication of the *E. grandis* x *E. urophylla*. This new method can maximize the production of clonal plants on a commercial scale.

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