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Effect of light intensity and seal type on the *in vitro* elongation and adventitious rooting of *Eucalyptus grandis* × *E. urophylla*

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Abstract

Background: Rejuvenation/reinvigoration of tissues through micropropagation has become an important tool for clonal propagation in eucalypts species. This study evaluated the effect of photomixotrophism (i.e., light intensity and seal type) on *in vitro* elongation and adventitious rooting to identify the limiting factors on *in vitro* culture of the *Eucalyptus grandis* × *E. urophylla* hybrid.

Methods: Nodal segments (i.e., explants) from ministumps grown in a semi-hydroponic system were collected. The effects of light intensity and seal type on *in vitro* elongation and adventitious rooting stages were evaluated from a 40 µmol m^{-2} s^{-1} fluorescent lamp and 20, 40, and 80 µmol m^{-2} s^{-1} red/blue LEDs, with (through porous membranes) and without gas exchange.

Results: Based on the results at 35 d, 40 µmol m^{-2} s^{-1} fluorescent lamp and gas exchange combination was the most suitable for *in vitro* elongation and adventitious rooting of *Eucalyptus grandis* × *E. urophylla*. Both factors increased the vigour, shoot length, photosynthetic pigment content, xylem, phloem, stomatal number and density, root length, diameter, number of roots per explant, and adventitious rooting fraction.

Conclusions: Light intensity and seal type influences the *in vitro* elongation and adventitious rooting of *Eucalyptus grandis* × *E. urophylla*. The results contribute to optimising the cloning of commercial eucalypts species by the micropropagation technique.

Keywords: clonal propagation, micropropagation, luminosity; LED, porous membranes

Introduction

The expansion of forest plantations and the improved capacity for plant production have increased the use of *Eucalyptus* and *Corymbia* species and their hybrids. The hybridation of *Eucalyptus grandis* × *Eucalyptus urophylla* (i.e., the hybrid is known as urograndis eucalypt) has provided excellent results in terms of wood quality and growth, in addition to better soil and climate adaptation and easier vegetative propagation (Carrillo et al. 2018). In this context, different ways to improve the production of eucalypts plants using the micropropagation technique via proliferation of axillary buds have been recommended (Abiri et al. 2020; Souza et al. 2020a).

Tissue rejuvenation through *in vitro* cultivation has become an important tool for the clonal micropropagation of *Eucalyptus* and *Corymbia* species, since they usually have problematic adventitious rooting when the cutting and minicutting techniques are applied (Lopes et al. 2019; Kuppusamy et al. 2019). Among the micropropagation stages, *in vitro* elongation is essential

for obtaining shoots for rooting and for acclimatisation of microcuttings (Souza et al. 2020b; Zorz et al. 2020), performed both in the *ex vitro* (Brondani et al. 2018; Li et al. 2019) and *in vitro* conditions (Miranda et al. 2020; Molinari et al. 2020).

To optimise micropropagation protocols for *in vitro* elongation and adventitious rooting of different eucalypts genotypes, there is a need for basic research to improve the production and quality of microstumps. Biotechnology is commonly applied to enhance the *in vitro* elongation and rooting, including the control of spectral quality (Batista et al. 2018; Souza et al. 2020a;), light intensity (Miranda et al. 2020; Coelho et al. 2021), and gas exchange (Souza et al. 2019; Martins et al. 2020; Molinari et al. 2020) in plant culture. Thus, the use of systems that increase the supply of light and CO_2 to some *in vitro* species may promote more significant development of their photosynthetic apparatus, resulting in larger multiplication, growth, rooting, and the subsequent acclimatisation of plants to *ex vitro* conditions (Saldanha et al. 2012; Louback et al. 2021).

Plants require a broad light spectrum and efficient gas exchange to optimise the photosynthetic processes, reduce relative humidity, increase aeration, produce hardier plants, and, consequently, favour survival and rooting (Lazzarini et al. 2017). In order to improve ventilation of *in vitro* culture containers, lids with porous membranes allowing gas exchange are used, reducing the relative humidity inside the vases and increasing plant transpiration and water and nutrient absorption (Saldanha et al. 2012; Batista et al. 2018).

Both factors (light source and gas exchange) contribute to the reduction of stress to the explant, providing positive effects in photosynthesis, as well as photoinduction, increasing the chlorophyll and carotenoids contents (Batista et al. 2018; Abiri et al. 2020). Conversely, photosynthesis can be inefficient at low light intensity and hermetic lids that prevents gas exchange resulting in slow growth and development. Also, excessive light and high CO_2 concentrations can alter physiological processes that are important to plant survival, inducing photorespiration and damaging photosynthetic structures (Silva et al. 2017; Oliveira et al. 2021).

Despite the economic importance of eucalypts species and their hybrids to the forestry sector, studies on the effect of different light intensities and gas exchange scenarios on morphogenesis and growth for plant production are still scarce. Consequently, we hypothesised that different light intensities and seal types would trigger differences in morphophysiological and anatomical features of *Eucalyptus grandis* × *E. urophylla*.

Methods

The genetic material used to obtain the explants (i.e., 1-cm-long nodal segment and an axillary bud without leaves), previously *in vitro* established and multiplied, was acquired from ministumps of the A211 hybrid clone of *Eucalyptus grandis* Hill ex Maiden × *Eucalyptus urophylla* S. T. Blake, from Esteio Seedling Production Nursery, located in São João Del-Rei, Minas Gerais, Brazil (Souza et al. 2022).

Ministumps were established in a clonal mini hedge under a semi-hydroponic system of the large channel type in a bed of medium-coarseness sand. The plants received nutrient solution (Table 1) by dripping, four

TABLE 1: Composition of the nutritive solution for fertigation of *Eucalyptus grandis* × *E. urophylla* plants.

Nutrient	Nutritive solution[1] (mg L^{-1})	MW
$N\text{-}NO_3^-$	60.00	14.00
$N\text{-}NH_4^+$	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	CF / MW	(mg L^{-1})
Potassium nitrate (Nuclear®)	KNO_3 / 101.10	206.85
Monoammonium phosphate (Mallinckrodt®)	$NH_4H_2PO_4$ / 115.03	44.57
Ammonium nitrate (Reagex®)	NH_4NO_3 / 80.4	140.50
Calcium sulfate (Vetec®)	$CaSO_4.2H_2O$ / 172.17	87.18
Calcium nitrate (Labsynth®)	$Ca(NO_3)_2.4H_2O$ / 236.15	57.18
Magnesium sulfate (Mallinckrodt®)	$MgSO_4.7H_2O$ / 246.48	121.66
Manganese sulfate (Ecibra®)	$MnSO_4.H_2O$ / 169.01	4.92
Copper sulfate (Mallinckrodt®)	$CuSO_4.5H_2O$ / 249.68	0.39
Iron sulfate (Synth®)	$FeSO_4.7H_2O$ / 278.02	9.95
Sodium - EDTA (Nuclear®)	Na_2-EDTA.$2H_2O$ / 372.24	13.31
Sodium molybdate (Merck®)	$Na_2MoO_4.2H_2O$ / 241.95	0.050
Zinc sulfate (Mallinckrodt®)	$ZnSO_4.7H_2O$ / 287.54	8.60
Boric acid (Ecibra®)	H_3BO_3 / 61.83	6.20

[1] pH was adjusted to 6.0 (±0.2) at 25 °C with HCl and/or NaOH, both at 1 M. CF = chemical formula, MW = molecular weight.

times a day, at a total daily flow rate of 4 L m^{-2}. The electrical conductivity of the nutrient solution was kept at approximately 2.0 mS cm^{-1} (Souza et al. 2022).

***In vitro* introduction and multiplication**

Nodal segments (i.e., explants) were washed five times in running water and immersed in a fungicide solution containing 2.4 g L^{-1} of Orthocide 500® (Captan 50% as the active ingredient) for 15 min. Subsequently, they were washed five times in autoclaved deionised water and immersed in a 70% (v v^{-1}) hydroalcoholic solution for 30 s with constant agitation inside a horizontal laminar flow chamber. Then, explants were immersed in NaOCl solution Clarix® (2.0 - 2.5% of active chlorine) for 15 min. Finally, the explants were washed in autoclaved deionised water five times, and were inoculated vertically, under aseptic conditions in test tubes (25 × 150 mm), containing 10 mL of MS culture medium (Murashige and Skoog 1962) added with 30 g L^{-1} of sucrose (Synth Ltda) and 6 g L^{-1} of agar (Merck®).

Shoots produced in the *in vitro* introduction stage were isolated and standardised to 0.5 cm and inoculated under aseptic conditions in test tubes (25 mm × 150 mm), containing 10 mL of MS basic culture medium, supplemented with 30 g L^{-1} of sucrose (Vetec®), 0.5 mg L^{-1} BAP (Sigma®), 0.01 mg L^{-1} NAA (Sigma®) and 6 g L^{-1} of agar (Merck®) for 30 d. Culture medium for the experiment was prepared with deionized water, and the pH was adjusted to 5.8 (± 0.05) at 25 °C. Autoclaving was performed at 121 °C and 1.0 kgf cm^{-2} for 20 min.

***In vitro* elongation and adventitious rooting**

Shoots produced in the *in vitro* multiplication stage were prepared by isolating four standardised 0.5 cm shoots with adequate vegetative vigour. Inoculation of the explants was performed under aseptic conditions in 250 mL glass flasks.

In vitro elongation and adventitious rooting occurred simultaneously. Culture lasted for 35 d in 50 mL of MS culture medium and supplemented with 30 g L^{-1} of sucrose, 6 g L^{-1} of agar, 0.05 mg L^{-1} BAP, and 0.5 mg L^{-1} of IBA (Sigma®).

Light intensity and seal types

The experiments were conducted in a 4 × 2 factorial arrangement, comprising four light intensities: fluorescent lamp (F/L 40 µmol m^{-2} s^{-1}) and 1:1 red/blue LEDs (R/B) at 20, 40, and 80 µmol m^{-2} s^{-1}; and, two seal types: rigid polypropylene caps without a membrane (0/M) and polypropylene caps with a hole (1.0 cm in diameter) covered with a 1.0-cm^2 membrane (1/M). The experiments were arranged in a randomised design, with 40 replicates, consisting of one explant per replicate. Natural ventilation systems were obtained by the presence of porous membranes manufactured in the lids of the culture recipients, according to Saldanha et al. (2012). The membranes were made with two 10 mm diameter holes covered by a membrane composed of two layers of microporous tape C (Cremer) and a polytetrafluoroethylene (PTFE) film (Amanco) 0.05 ± 0.01 mm thick.

For the description of variations in the absolute irradiance (µW cm^{-2} nm^{-1}) and wavelength (nm) of light emitted by the different spectral qualities analyses, a SPECTRA PEN Z850 portable spectroradiometer (Qubit Systems, Kingston, Ontario, Canada) was used (Figure 1).

The experiment was conducted in a growth room at 24 °C (± 1 °C) under a 16-h photoperiod. The irradiance of each fluorescent lamp or red/blue LEDs corresponded to 20 µmol m^{-2} s^{-1} and was measured with a QSO-S Procheck + Sensor-PAR Photon Flux photoradiometer (Decagon Devices, Pullman, Washington, USA). Therefore, the light intensity of 40 µmol m^{-2} s^{-1} was achieved with two lamps; and the 80 µmol m^{-2} s^{-1}, with four lamps.

In the *in vitro* elongation stage, tissue oxidation and vigour according to a scoring scale (Oliveira et al. 2016; Souza et al. 2020a) (Figure 2A-F), number of shoots per explant (> 0.5 cm), length of shoots (> 0.5 cm), and photosynthetic pigment content (µg mg^{-1}) were evaluated. For *in vitro* adventitious rooting, the length of the largest root (cm), the diameter of the largest root (mm), number of roots per explant, and rooting fraction were evaluated.

Analysis of photosynthetic pigments

Leaf discs (i.e., 25 mg of leaf fresh matter) were sampled after 35 d under different light intensities and seal types, being inoculated in 5 mL DMSO (Sigma®) and stored for

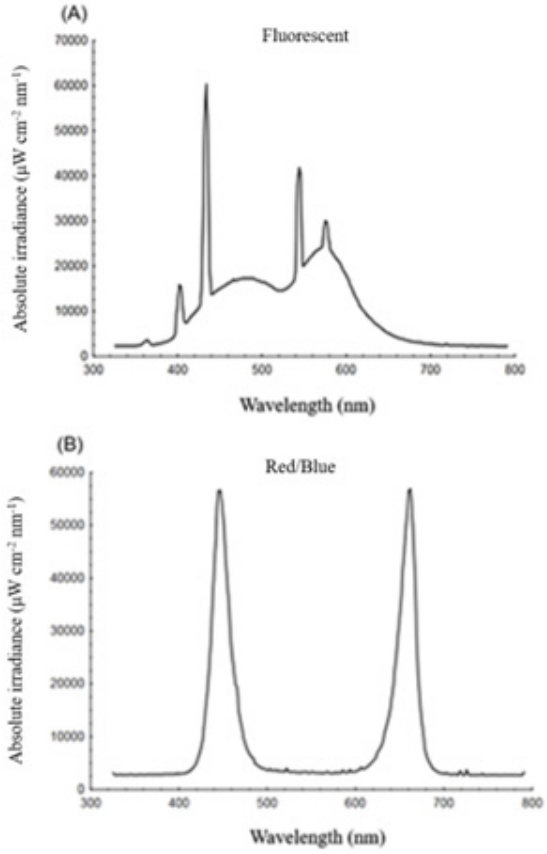

FIGURE 1: Variations in absolute irradiance (µW cm^{-2} nm^{-1}) and wavelength (nm) of light emitted on *in vitro* culture of *Eucalyptus grandis* × *E. urophylla*. (A) Fluorescent lamp (F/L); (B) Red/Blue LEDs (R/B).

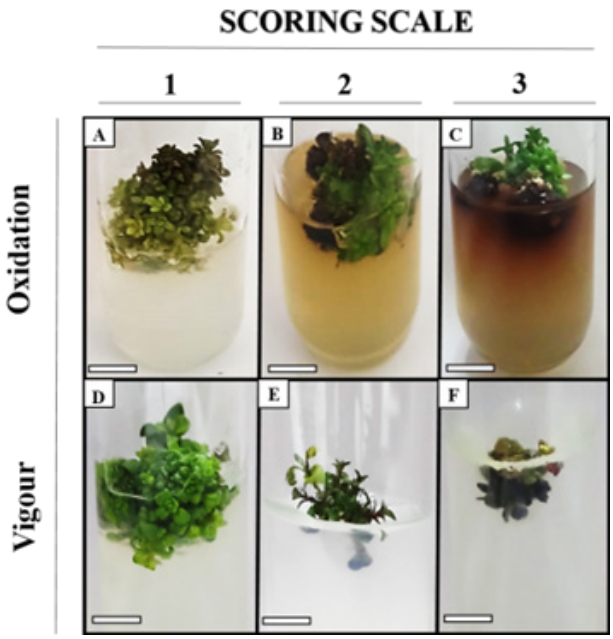

FIGURE 2: Scoring scale for the assessment of tissue oxidation and vigour of *Eucalyptus grandis* × *E. urophylla*. (A) Score 1: no oxidation; (B) Score 2: reduced oxidation at the base of the explants (grayish culture medium); (C) Score 3: complete bud oxidation (blackened culture medium); (D) Score 1: induction of shoots with active growth, with no apparent nutritional deficiency; (E) Score 2: shoot induction, but with reduced sized leaves; (F) Score 3: reduced shoot induction, senescence or death. Bar = 1.0 cm.

48 h in the dark, according to the method developed by Lichtenthaler (1987). The absorbance of the samples was determined in triplicate in a quartz cuvette with a 10-mm optical path in a Genesys 10UV spectrophotometer (Thermo Scientific, USA). The wavelengths (665, 649, and 480 nm) and the equations for calculating the concentrations of chlorophyll *a*, *b*, and *a* + *b* and total carotenoids were based on the method described by Wellburn (1994).

Leaf anatomy

Samples of the leaves of each treatment after 35 d of *in vitro* culture were collected. They were kept for 48 h in FAA solution (70% ethanol, 1:1:18), then transferred to 70% ethanol, dehydrated in an increasing ethyl alcohol series (80, 90, and 100%) for 30 min in each solution (Johansen 1940), and embedded overnight in historesin (Biosystems, Nussloch, Germany) at a 1:1 ratio in a hot oven. The blockage was processed with pure hydroxyethyl methacrylate resin, and the cross-sections (7 µm) were obtained with a manual rotating microtome. The tissues were contrasted with toluidine blue (Vetec Química Fina Ltda, Rio de Janeiro, Brazil), mounted on Entellan histological slides (Merck KGaA, Darmstadt, Germany), and photomicrographed with a coupled digital camera (AxioCam ERc5s) on a micrometric scale with 20× and 40× objective lenses.

A stomatal density assay was performed by digesting the epidermis. Leaves were clarified in a commercial solution of 50% sodium hypochlorite, neutralised in water and coloured with safranin. The blades were then mounted with 50% aqueous glycerin (Strasburger 1924), and the edges of the coverslip sealed with enamel. Images were obtained by means of a digital camera (Canon A-630) coupled to a light microscope (Olympus CBB) with a 40× objective lens. Stomatal density was determined by the number of stomata in the sampled area with the aid of Image J software.

Adaxial epidermis thickness (µm), abaxial epidermis thickness (µm), mesophyll thickness (µm), palisade parenchyma thickness (µm), spongy parenchyma thickness (µm), vascular tissue thickness (xylem and phloem) (µm), stomatal density (number per μm^2), polar diameter (µm), and equatorial diameter (µm) were measured.

To determine the anatomical features in cross-section and paradermal sections, three fields of the organ view were randomly photographed - the leaf area corresponded to 0.04 mm^2. For the features analysed, fifteen replications were used (5 anatomical sections × 3 fields of view of the organ), with one leaf each.

Data analysis

Data were processed using R Software, version 3.0.3 (R Core Team 2018), using the ExpDes package, version 1.1.2 (Ferreira et al. 2013). The main and interactive effects of light intensity and seal type on variables measured were assessed using analysis of variance (ANOVA) and then, when significant, the Tukey test was used to test specific differences between factor levels. When variables did not meet the normality and homogeneity of variance assumptions, tested using the Shapiro-Wilk and Hartley's test respectively, variables were arcsine transformed.

Results

***In vitro* elongation**

Different light intensities and seal types used in the *in vitro* elongation of the *Eucalyptus grandis* × *E. urophylla* influenced the variables evaluated 35 d after the experiment started (Figure 3A-F). There was a significant interaction between the factors only for the number of shoots per explant (Figure 3E) and shoot length (Figure 3F).

An undesirable aspect of *in vitro* elongation is the phenolic oxidation of tissues. The results showed different oxidation responses at different light intensities. According to the scoring scale, all the R/B treatments resulted in lower tissue oxidation than the F/L 40 µmol m^{-2} s^{-1} (score 1.2) (Figure 3A). Evaluating the different types of sealing, oxidation did not differ statistically between the 0/M (score 1.12) and 1/M (score 1.07) (Figure 3B). In contrast, explant vigour was poorer in the R/B 20 (2.23) compared to all other light levels (Figure 3C), while no differences in explant vigour was observed with seal type (Figure 3D).

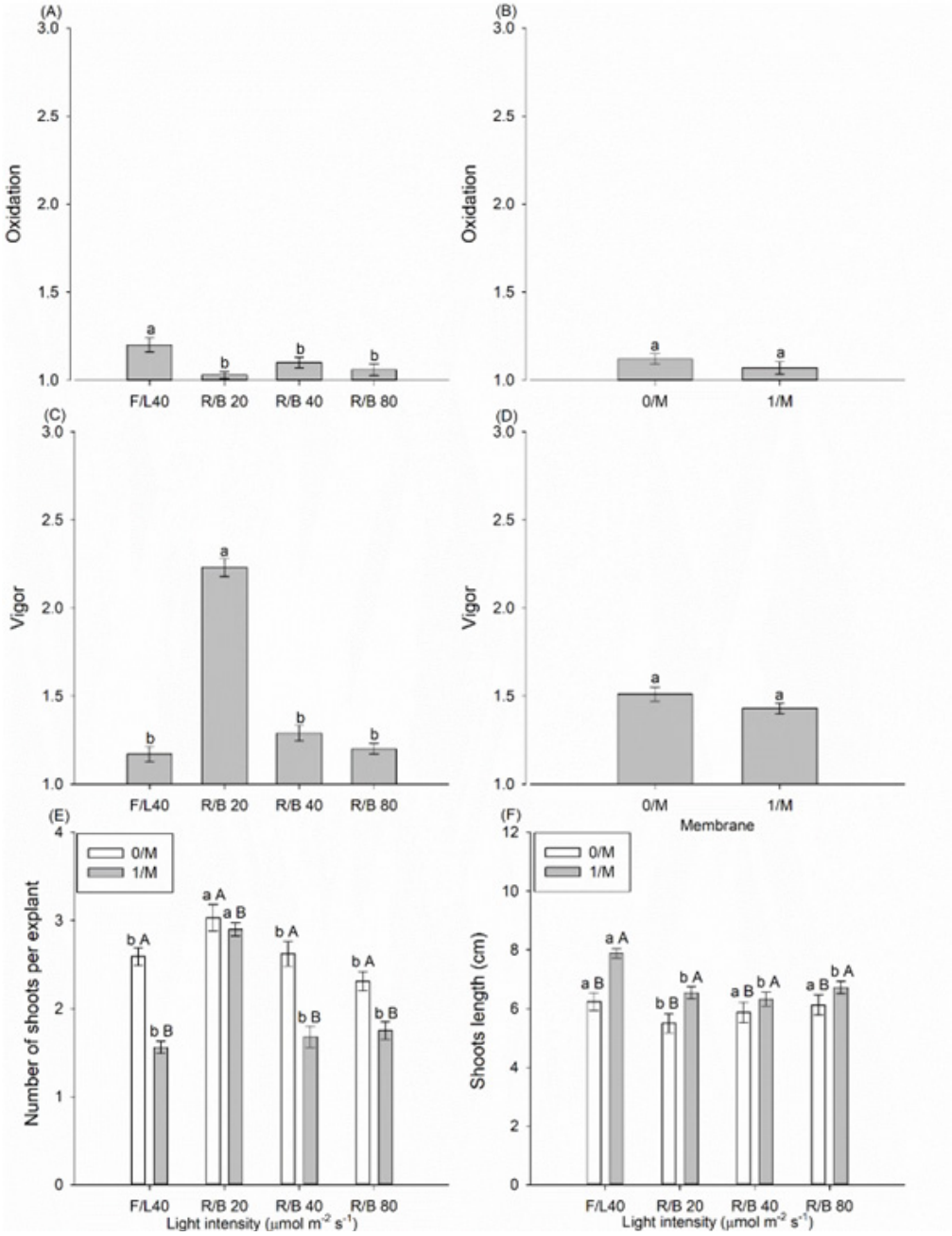

FIGURE 3: Features observed on *in vitro* elongation of *Eucalyptus grandis* × *E. urophylla* under different spectral intensities (F/L 40, R/B 20, R/B 40, and R/B 80 µmol m^{-2} s^{-1}) and seal types (0/M and 1/M). (A) Oxidation under different light intensities (scoring scale according to Figures 2A-C). (B) Oxidation under different seal types (scoring scale according to Figures 2A-C). (C) Vigour under different light intensities (scoring scale according to the Figures 2D-F). (D) Vigour under different seal types (scoring scale according to Figures 2D-F). (E) Number of shoots per explant. (F) Shoot length (cm). For (E) and (F) lowercase letters compare spectral intensities within the same seal type while capital letters compare seal types within the same light intensity. Same letters indicate insignificant differences. Bars represent means and whiskers the standard error.

The greatest number of shoots per explant was observed in the R/B 20 and 0/M treatment (3.03 shoots) (Figure 3E). Nevertheless, for shoot length, the same pattern of explant response was not observed, with highest values for the F/L 40 µmol m^{-2} s^{-1} (6.24 cm) and the 1/M seal type (Figure 3F).

Photosynthetic pigment contents differed by light intensity and seal type, but there was no interaction between the factors. The highest values for chlorophyll *a* (36.30 µg mg^{-1}), chlorophyll *b* (18.70 µg mg^{-1}), chlorophyll *a* + *b* (55.00 µg mg^{-1}), and carotenoids (6.30 µg mg^{-1}) were observed in the F/L 40 µmol m^{-2} s^{-1} (Figure 4A). Comparing the seal types, the highest values for chlorophyll *a* (30.07 µg mg^{-1}), chlorophyll *b* (16.50 µg mg^{-1}), chlorophyll *a* + *b* (46.57 µg mg^{-1}), and carotenoids (5.72 µg mg^{-1}) were found when using 1/M,

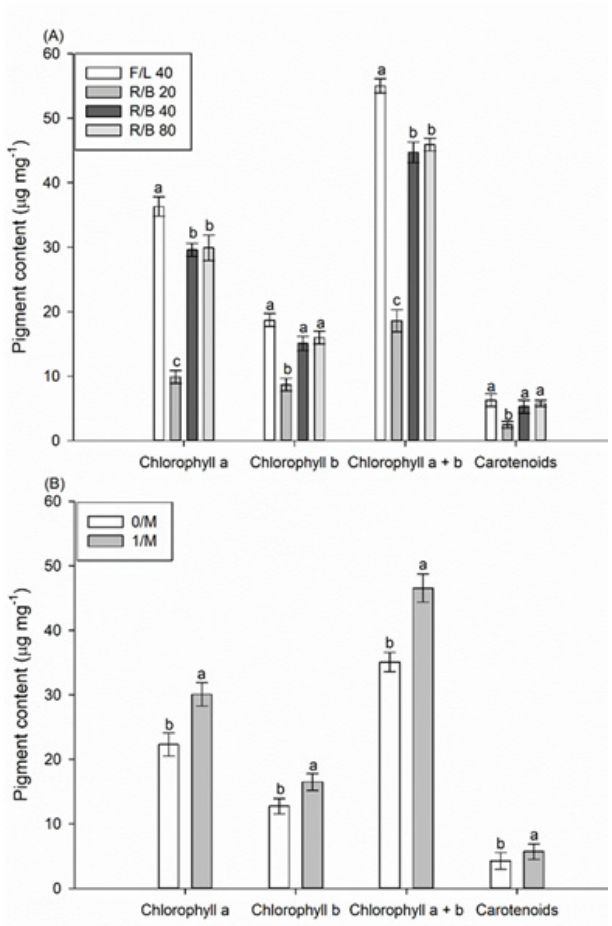

FIGURE 4: Contents of photosynthetic pigments (chlorophyll *a*, *b*, and *a* + *b*, and carotenoids) observed on *in vitro* elongation of *Eucalyptus grandis* × *E. urophylla*. (A) Spectral intensity (FL 40, R/B 20, R/B 40, and R/B 80 µmol m^{-2} s^{-1}). (B) Seal type (0/M and 1/M). Bars represent mean values and whiskers the standard error. Different letters indicate differences at $P<0.05$.

with a significant difference from the 0/M (Figure 4B).

According to these results, the efficiency of the F/L 40 µmol m^{-2} s^{-1} and the use of a membrane for *Eucalyptus grandis* × *E. urophylla* was adequate for the *in vitro* elongation stage in large-scale production systems.

Leaf anatomy

Light intensity and seal type changed the leaf anatomy observed in cross-sections. There was a significant interaction between the factors for the thickness of the adaxial epidermis, abaxial epidermis, palisade parenchyma, spongy parenchyma, and mesophyll (Figure 5).

The greatest thickness of the adaxial epidermis (15.62 and 14.11 µm) (Figure 5A), abaxial epidermis (11.12 and 10.95 µm) (Figure 5B), spongy parenchyma (75.13 and 54.58 µm) (Figure 5C), and mesophyll (110.64 and 131.56 µm) (Figure 5D) of *Eucalyptus grandis* × *E. urophylla* were observed with the 1/M seal type under R/B 80 µmol m^{-2} s^{-1} and R/B 40 µmol m^{-2} s^{-1}, respectively. In contrast, the 20 R/B level combined with the hermetic lid (0/M) exhibited the smaller values for leaf tissue variables measured (Figures 5A-E).

In addition, differences were observed in the structural organisation of the leaf cells at the anatomical level when the plants were exposed to different spectral qualities or seal types (Figures 6A-H). There was no interaction between factors for xylem, phloem, or total vascular tissues (xylem + phloem) thicknesses (Figure 7).

The largest dimensions observed in relation to the thickness of the xylem (16128 µm) (Figure 7A), phloem (15574 µm) (Figure 7C), and total vascular tissues (31703 µm) (Figure 7E) were found for the FL 40 µmol m^{-2} s^{-1}, which differed significantly from the other treatments. These results corroborate the previous results (Figures 3 and 4) on the effect of light intensity on morphological features on *in vitro* elongation stage.

Between the different seal types used (0/M and 1/M), the means of xylem thickness (9040 and 8925 µm) (Figure 7B), phloem thickness (9631 and 8613 µm) (Figure 7D), and total vascular tissue thickness (18672 and 17539 µm) (Figure 7F) showed insignificant differences.

As for the leaf anatomy features studied in paradermal sections, a significant interaction between the factors (light intensity and seal type) for stomatal number and density was observed. The polar and equatorial diameters did not differ significantly.

The highest mean stomatal number and density were found in the FL 40 µmol m^{-2} s^{-1} and 1/M treatment (357 stomata and 861.56 per μm^2) (Figure 8A-B, and 9B), which differed significantly from the other treatments. This same pattern was observed for the vascular system (xylem + phloem) and morphological features evaluated (Figures 3, 4, and 7).

Regarding the polar and equatorial diameter, the largest stomata were observed in the R/B 20 µmol m^{-2} s^{-1} (20.21 and 12.96 µm) (Figures 8C-D) and 0/M (19.07 and 12.72 µm) (Figures 8E-F), but with no significant difference from the other treatments. The appearance of the stomata in paradermal sections of the *Eucalyptus grandis* × *E. urophylla* are shown in Figures 9A-H.

***In vitro* adventitious rooting**

Features of *in vitro* rooting differed by light intensity and seal type at 35 d. There was no interaction between the factors (light intensity and seal type) for root length, root diameter, or rooting percentage, while for the number of roots per explant, the factors were dependent.

Root length and diameter were maximum under FL 40 µmol m^{-2} s^{-1} (6.45 cm and 0.87 mm, respectively), which differed significantly from the R/B 20 µmol m^{-2} s^{-1}, R/B 40 µmol m^{-2} s^{-1}, and R/B 80 µmol m^{-2} s^{-1} (Figures 10A, and 10C). The use of a membrane (1/M) also led to a higher mean root length (5.73 cm) and diameter (0.71 mm) than 0/M (4.91 cm in length and 0.61 mm in diameter) (Figures 10B, and 10D).

Similarly, the number of roots per explant was maximum under FL 40 µmol m^{-2} s^{-1} and 1/M (4.09 cm) (Figure 10E). Considering all features evaluated under

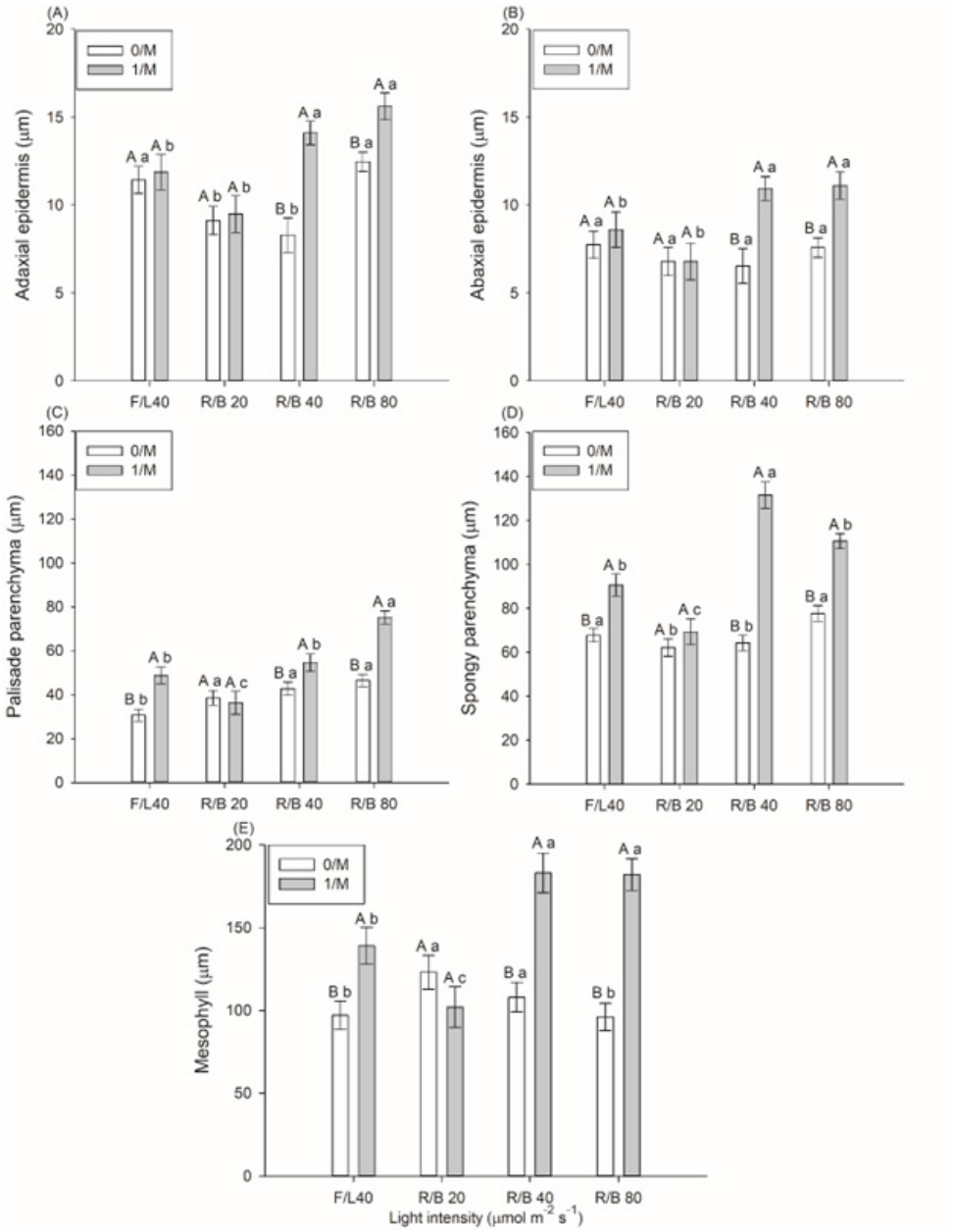

FIGURE 5: Anatomical features observed on *in vitro* elongation according to spectral intensity (FL 40, R/B 20, R/B 40, and R/B 80 µmol m^{-2} s^{-1}) and seal type (0/M and 1/M). (A) Adaxial epidermis. (B) Abaxial epidermis. (C) Palisade parenchyma. (D) Spongy parenchyma. (E) Mesophyll. Lowercase letters compare spectral intensities within the same seal type while capital letters compare seal types within the same light intensity. Bars represent mean values and whiskers the standard error. Different letters indicate differences at $P<0.05$.

different spectral intensities and seal types, the lowest performance was observed in the R/B 20 and 0/M treatment (Figures 10A-E).

Rooting of the microstumps was influenced by the light intensity and seal type, resulting in different responses of the *Eucalyptus grandis* × *E. urophylla*. The FL 40 µmol m^{-2} s^{-1} provided the highest rooting percentages (96.8%), but it differed significantly only from the R/B 20 µmol m^{-2} s^{-1} (82.8%) (Figure 11A). Comparing the seal types, 1/M yielded a significantly higher rooting percentage (95.3%) than 0/M (87.5%) (Figure 11B).

The results suggest that it is possible to produce plants with high root proliferation and rooting percentage, as observed in the FL 40 µmol m^{-2} s^{-1}, R/B 40 µmol m^{-2} s^{-1}, and R/B 80 µmol m^{-2} s^{-1}. The high rooting percentages indicate the potential to use specific light intensities and seal types. The appearance of the *Eucalyptus grandis* × *E. urophylla* microstumps in terms of the features studied in the *in vitro* elongation and rooting are shown in Figures 12A-E.

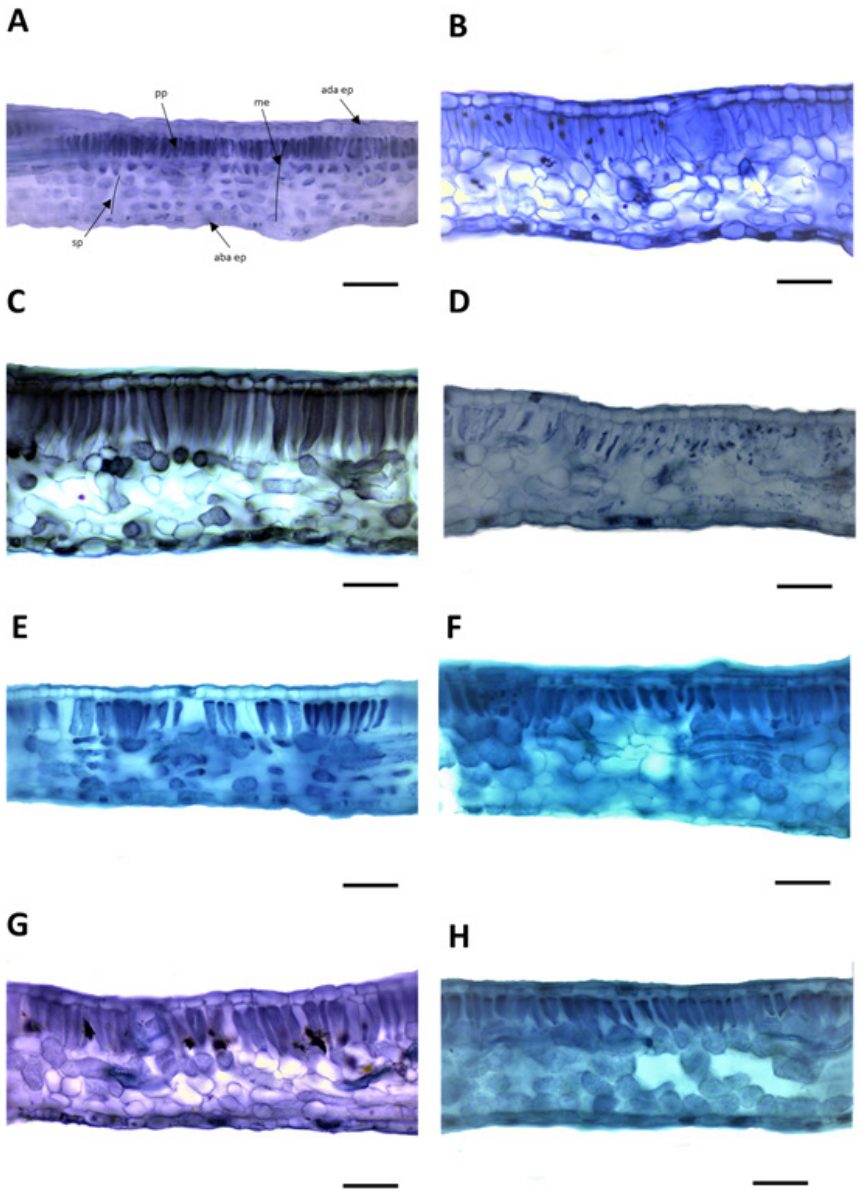

FIGURE 6: Cross-sections of the leaf blade observed on *in vitro* elongation of *Eucalyptus grandis* × *E. urophylla*. (A) FL 40 µmol m^{-2} s^{-1} and 0/M. (B) FL 40 µmol m^{-2} s^{-1} and 1/M. (C) R/B 20 µmol m^{-2} s^{-1} and 0/M. (D) R/B 20 µmol m^{-2} s^{-1} and 1/M. (E) R/B 40 µmol m^{-2} s^{-1} and 0/M. (F) R/B 40 µmol m^{-2} s^{-1} and 1/M. (G) R/B 80 µmol m^{-2} s^{-1} and 0/M. (H) R/B 80 µmol m^{-2} s^{-1} 1/M. ada ep = adaxial epidermis; aba ep = abaxial epidermis; pp = palisade parenchyma; sp = spongy parenchyma; me = mesophyll. Bar = 50 µm.

Discussion

In vitro elongation

Different light intensities and seal types influenced tissue oxidation, vigour, number of buds per explant, and shoot development of microstumps of the *E. grandis* × *E. urophylla* hybrid.

The lowest phenolic oxidation in the tissues was observed at the lowest R/B 20 and 1/M combination. Low light or darkness during *in vitro* culture of woody species reduces the phenolic oxidation in tissues of *Eucalyptus cloeziana* (Oliveira et al. 2015), *Eucalyptus benthamii* (Baccarin et al. 2015), *Corymbia citriodora* × *C. torelliana*, and *Corymbia torelliana* × *C. citriodora* (Souza et al. 2018).

In vitro conditions that are stressful for plant growth include low CO_2 concentrations and high ethylene concentrations which cause tissue oxidation, senescence, and reduced vigour (Tisarum et al. 2017), as we observed under the condition with lower gas exchange in the treatment with a hermetic lid without membrane (0/M). Using membranes increased CO_2, O_2 and H_2O exchange producing more vigorous plants (Tisarum et al. 2017).

The R/B 20 light resulted the least vigour, with apparent etiolation, reduced leaf size, senescence, and/or death (Figures 12C-D). Similarly, previous research showed that the use of light intensities above 40 µmol m^{-2} s^{-1} in the orchid *Microlaelia lundii* (Favetta et al. 2017), C*orymbia torelliana* × *C. citriodora* (Souza et al. 2018), *Urtica dioica* (Coelho et al. 2021), and

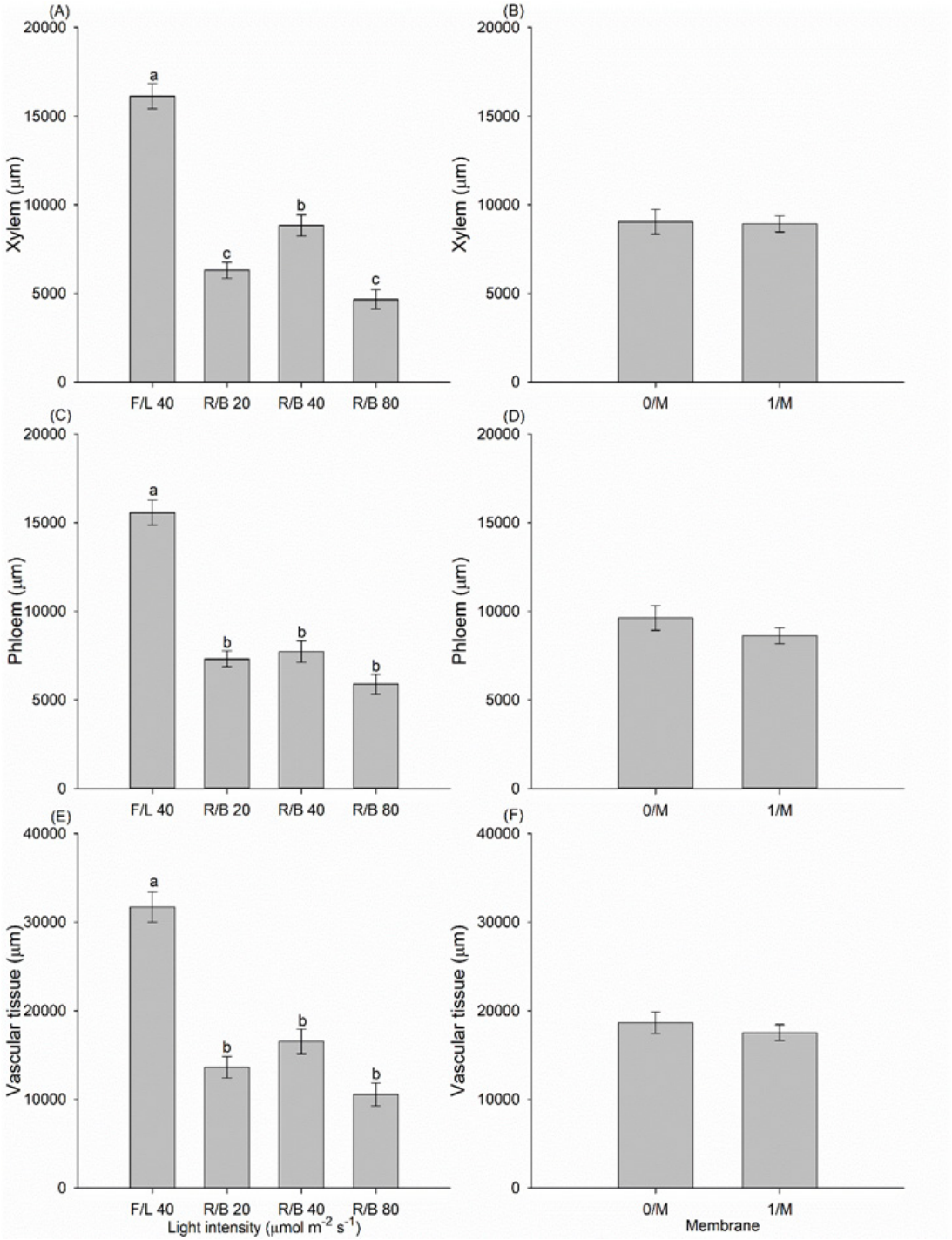

FIGURE 7: Anatomical features observed on *in vitro* elongation according to spectral intensity (FL 40, R/B 20, R/B 40, and R/B 80 µmol m^{-2} s^{-1}) and seal type (0/M and 1/M). (A) Xylem. (B) Xylem. (C) Phloem. (D) Phloem. (E) Vascular tissues. (F) Vascular tissues. Bars represent mean values and whiskers the standard error. Different letters indicate differences at $P<0.05$.

Mentha arvensis (Oliveira et al. 2021) induced larger explant development and vigour. Using membranes in flasks have also shown positive effects on vigour of *Plectranthus amboinicus* (Silva et al. 2017), which may be likely associated to increased photosynthetic rates brought about by greater CO_2 availability and lower relative humidity.

Number of shoots per explant was highest under the R/B 20 and 0/M combination, which contrast with variables related to microstump growth and development which were highest under the FL 40 and 1/M combination. Some studies have shown that a light source with a broad light spectrum induces greater *in vitro* plant development (Batista et al. 2018; Santos et al. 2020; Miranda et al. 2020).

The control of spectral quality, light intensity, and gas exchange during plant culture is essential to increase photosynthetic rates and shoot development

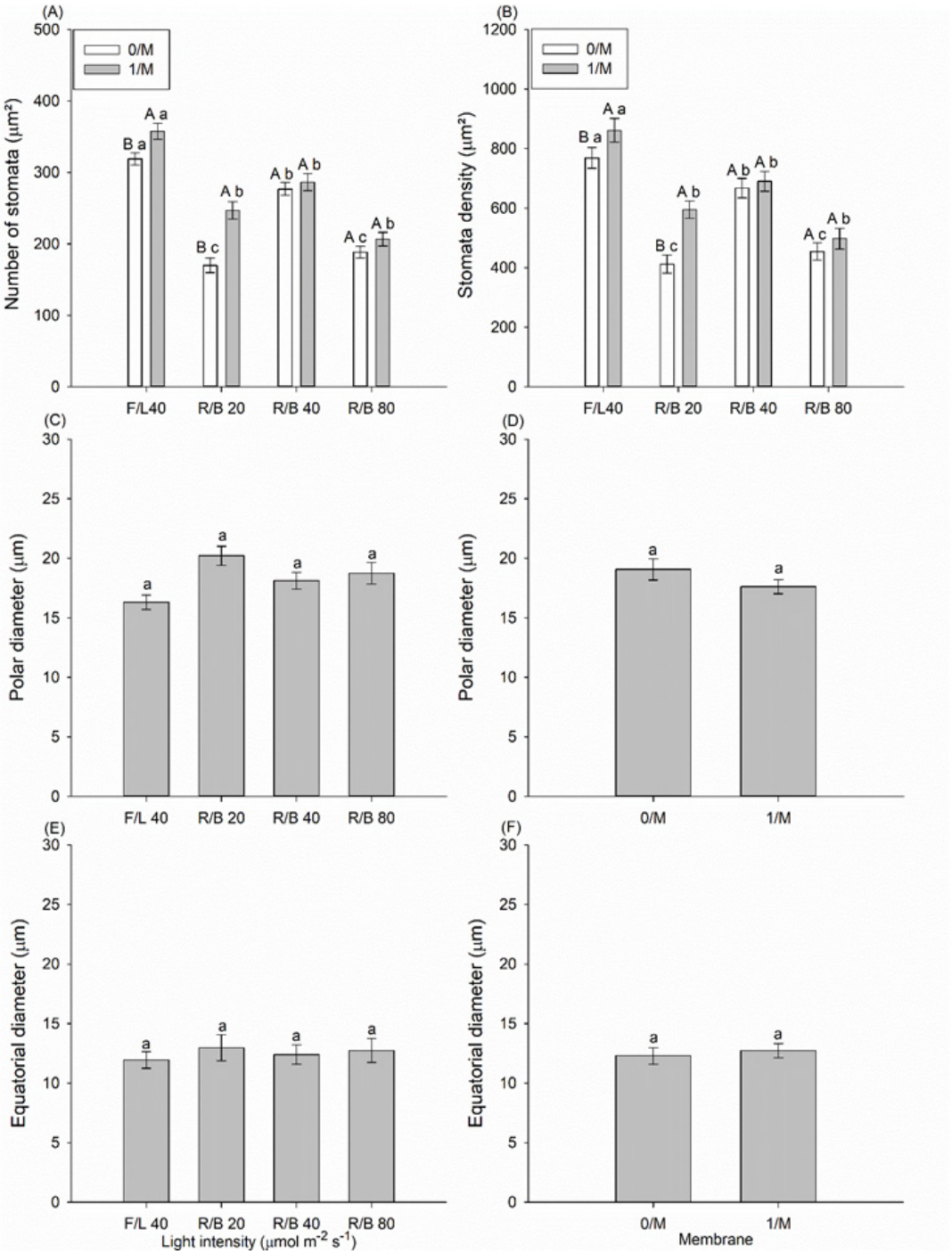

FIGURE 8: Anatomical features observed on *in vitro* elongation according to spectral intensity (FL 40, R/B 20, R/B 40, and R/B 80 µmol m^{-2} s^{-1}) and seal type (0/M and 1/M). (A) Number of stomata. (B) Stomatal density. (C) Polar diameter. (D) Polar diameter. (E) Equatorial diameter. (F) Equatorial diameter. For (A and B) lowercase letters compare spectral intensities within the same seal type while capital letters compare seal types within the same light intensity. Same letters indicate insignificant differences. Bars represent means and whiskers the standard error.

(Santos et al. 2020; Silveira et al. 2020). Therefore, *in vitro* propagation with porous membranes that allow greater gas exchange between the external and internal atmospheres of the flasks results in a higher plant growth rate (Núñez-Ramos et al. 2021).

Greatest photosynthetic pigments contents (chlorophyll *a*, *b*, *a* + *b*, and carotenoids) were observed under the FL 40 and 1/M combination. Similarly, greater photosynthetic rates and explant development have been observed under a broad light spectrum and flasks that allow greater gas exchange (Gupta & Karmakar 2017; Tian et al. 2019; Lei et al. 2021, Coelho et al. 2021). However, these results are not always consistent in the literature. For instance, Souza et al. (2020b) found greater microstump development and pigment content of *Corymbia torelliana* × *C. citriodora* under

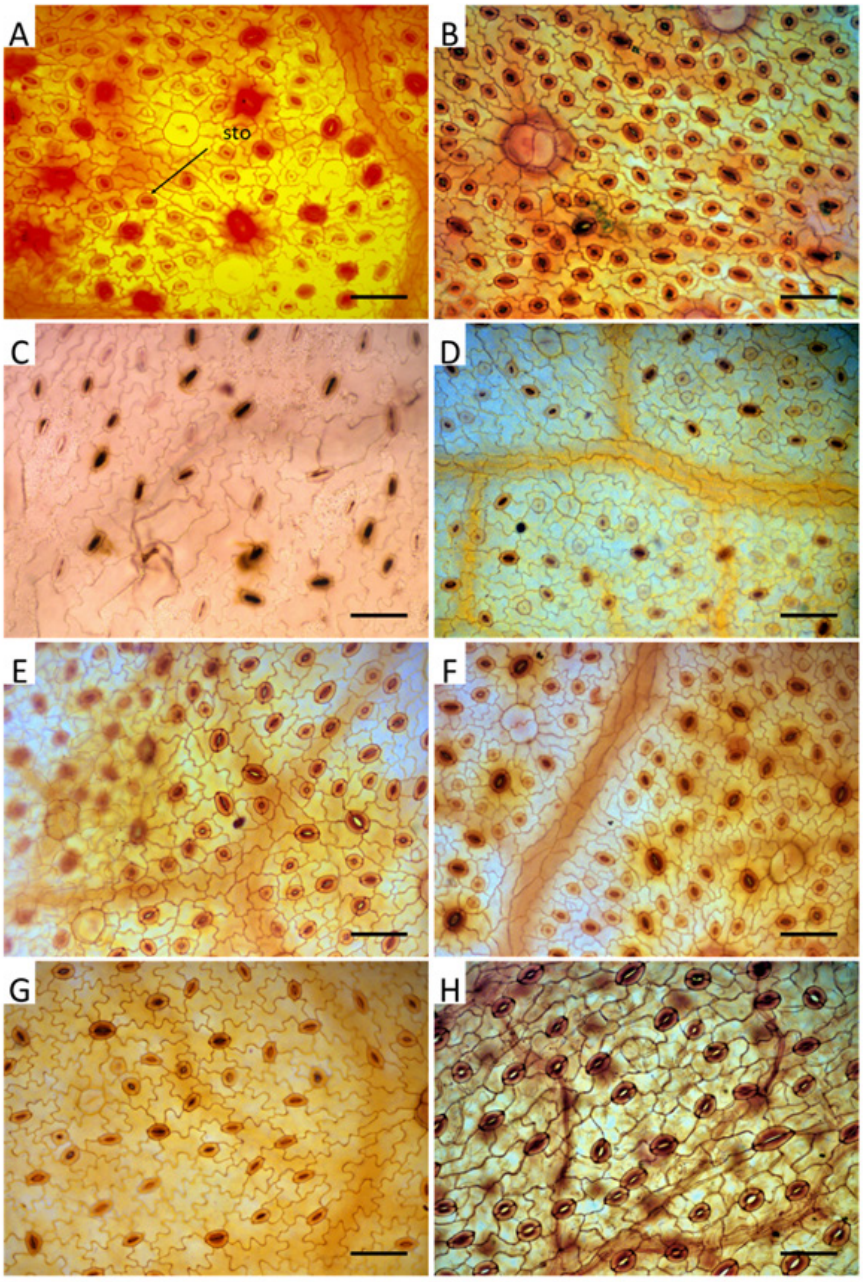

FIGURE 9: Paradermal sections of the leaf blade from the *in vitro* elongation of *Eucalyptus grandis* × *E. urophylla*. (A) FL 40 µmol m^{-2} s^{-1} and 0/M. (B) FL 40 µmol m^{-2} s^{-1} and 1/M. (C) R/B 20 µmol m^{-2} s^{-1} and 0/M. (D) R/B 20 µmol m^{-2} s^{-1} and 1/M. (E) R/B 40 µmol m^{-2} s^{-1} and 0/M. (F) R/B 40 µmol m^{-2} s^{-1} and 1/M. (G) R/B 80 µmol m^{-2} s^{-1} and 0/M. (H) R/B 80 µmol m^{-2} s^{-1} 1/M. *sto = stomata. Bar = 50 µm.

R/B 40 µmol m^{-2} s^{-1} without a porous membrane. However, as a general rule it seems that membranes allowing gas exchange have a positive effect on *in vitro* propagation, likely to be associated to regular supply of CO_2 and O_2, lower relative humidity and lower ethylene concentration inside the culture vessel (Hung et al. 2016, Souza et al. 2019; Santos et al. 2020).

Leaf anatomy

Anatomical analyses showed differences between the treatments used in *Eucalyptus grandis* × *E. urophylla* microstumps, with the largest adaxial epidermis, abaxial epidermis, palisade parenchyma, spongy parenchyma, and mesophyll thicknesses found in the R/B 40 and R/B 80 combined with the 1/M treatment. These results were similar to those from Souza et al. (2020a) in *Eucalyptus urophylla* × *E. grandis* microstumps under red/blue LEDs with a light intensity of 40 µmol m^{-2} s^{-1}. Similarly, the use of a porous membrane increased the thickness of the leaf epidermis, parenchyma, and mesophyll in *Cattleya walkeriana* (Silva et al. 2014), *Populus euramericana* (Kwon et al. 2015), and *Eucalyptus dunnii* (Souza et al. 2019).

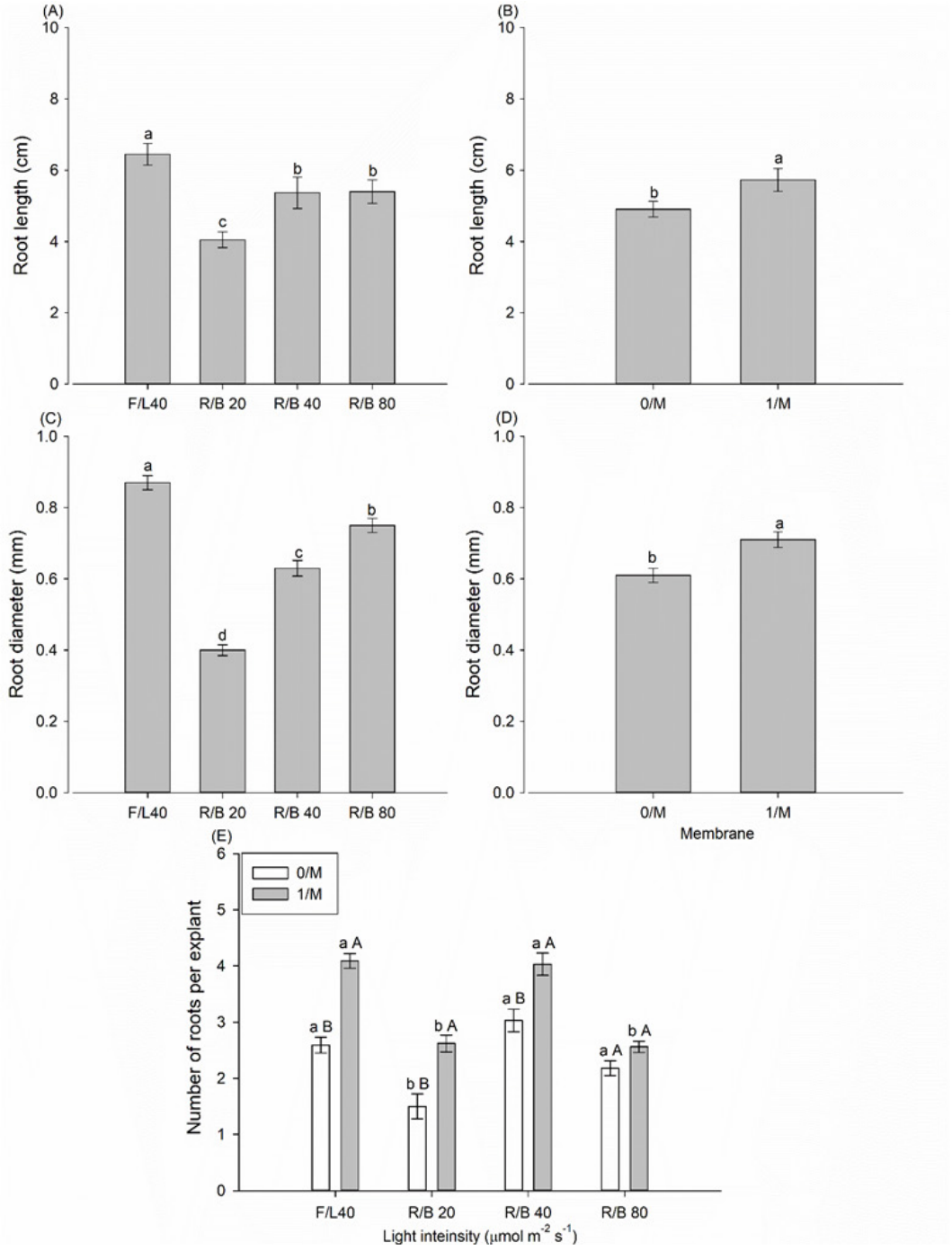

FIGURE 10: Morphological features observed on *in vitro* rooting of *Eucalyptus grandis* × *E. urophylla* according to different spectral intensities (FL 40, R/B 20, R/B 40, and R/B 80 μmol m^{-2} s^{-1}) and seal types (0/M and 1/M). (A) Root length (cm). (B) Root length (cm). (C) Root diameter (mm). (D) Root diameter (mm) (E) Number of roots per explant. For (E) lowercase letters compare spectral intensities within the same seal type while capital letters compare seal types within the same light intensity. Same letters indicate insignificant differences. Bars represent means and whiskers the standard error.

Light intensity and seal type influence the growth of plant cells, tissues, and organs, triggering different morphological and anatomical responses (Martins et al. 2020). Many studies show better *in vitro* plant development with LED lamps compared to fluorescent lamps (Batista et al. 2018), although the response to photomixotrophism also seems to depends on the genotype (Zienkiewicz et al. 2015). In addition, the thicker leaf epidermis and greater epicuticular wax deposition observed under higher light intensities and porous membranes might be considered as an adaptation to decrease water loss as suggested by Martins et al. (2020).

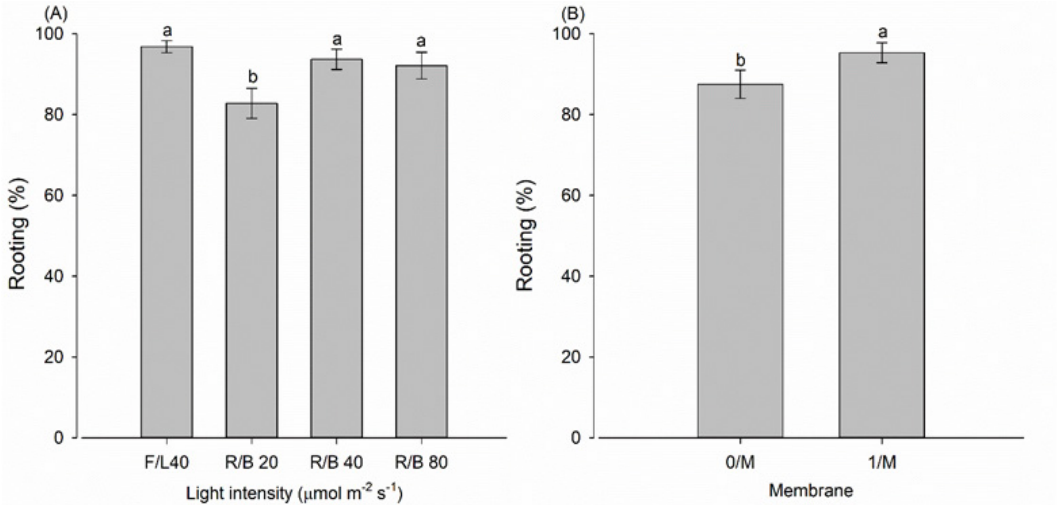

FIGURE 11: *In vitro* adventitious rooting percentage of *Eucalyptus grandis* × *E. urophylla*. (A) Spectral intensity (FL 40, R/B 20, R/B 40, and R/B 80 µmol m^{-2} s^{-1}). (B) Seal type (0/M and 1/M). Same letters indicate insignificant differences. Bars represent means and whiskers the standard error.

Vascular tissues, stomatal number, and density were higher under the FL 40 light treatment. Using low light and hermetic lids there is little supply of CO_2, and hence tissues tend to be less developed due to the lower production of photoassimilates (George et al. 2008), as was observed in the R/B 20 µmol m^{-2} s^{-1} with 0/M combination. Plants *in vitro* grown with low light intensity, low CO_2 concentration, and exogenous carbohydrates as the only energy source usually show anomalies, such as low photosynthetic capacity, stomatal malfunction, absence of leaf cuticle, and abnormal leaf parenchyma (Núñez-Ramos et al. 2021).

Castro et al. (2007) found best vascular tissue development in *Mikania glomerata* when explants were subjected to a broad light spectrum (450-700 nm) and higher light intensities (40 µmol m^{-2} s^{-1} and 80 µmol m^{-2} s^{-1}). Mohamed and Alsadon (2010) found that the use of the membranes resulted in thicker leaves and more developed vascular tissues (xylem and phloem) in *Solanum tuberosum* than the conventional system with hermetic lids. The control of light and CO_2 in plant cultivation is an important factor, and at low intensity, photosynthesis can be limited reducing development, while excessive photosynthesis can damage photosynthetic pigments and structures (Silva et al. 2017).

Another relevant effect caused by increased CO_2 availability and adequate light intensity is the increased stomatal number and density, which we observed under the FL 40 and 1/M combination. Similarly, larger

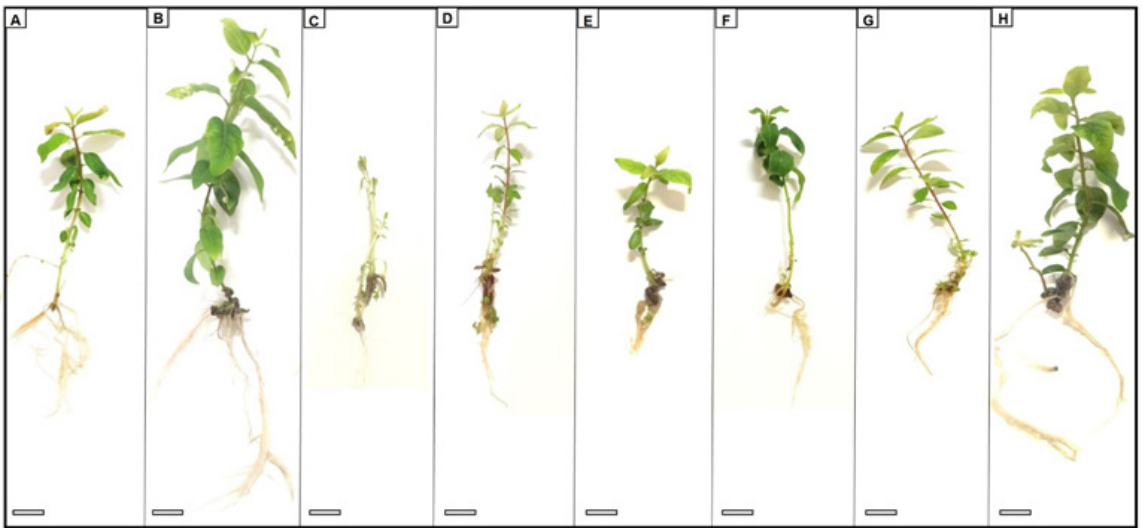

FIGURE 12: Microplants of the *Eucalyptus grandis* × *E. urophylla in vitro* elongated and rooted. (A, C, E, and G) with seal type 0/M. (B, D, F, and H) 1/M. (A and B) FL 40 µmol m^{-2} s^{-1}. (C and D) R/B 20 µmol m^{-2} s^{-1}. (E and F) R/B 40 µmol m^{-2} s^{-1}. (G and H) R/B 80 µmol m^{-2} s^{-1}. Bar = 1.0 cm.

stomatal density and better plant development were observed when using membranes in the flasks with the *in vitro* culture of *Plectranthus amboinicus*, (Silva et al. 2017). Regarding *Withania somnifera* explants, higher photosynthetic pigment production and efficiency, stomatal number, and stomatal conductance were observed at a light intensity of 60 μmol m^{-2} s^{-1} (Lee et al. 2007). In contrast, different light intensities (60, 85, 100, and 140 μmol m^{-2} s^{-1}) had no influence on the leaf anatomy of *Eucalyptus urophylla* microstumps (Miranda et al. 2020).

In vitro adventitious rooting

In the adventitious rooting stage, several factors underlie root formation in microstumps. Among these are plant hormones and the cultivation environment (e.g., light, temperature, and gas exchange), which play an important role in rhizogenesis (Almeida et al. 2017; Díaz-Sala 2020). The difficulty of propagation through *in vitro* adventitious rooting is one of the main problems in producing clonal plants of many eucalypts species (Abiri et al. 2020). In the present study, root length and diameter, number of roots per explant, and rooting percentage showed the best results in the FL 40 with 1/M combination.

The use of white or fluorescent spectral quality in the *in vitro* cultures may lead to greater root and shoot development by allowing greater light interception maximising the photosynthetic rate, when compared to blue and red monochromatic lights, as observed for *Lactuca sativa* var. *capitata* (Lin et al. 2013). Different studies also indicate better development of plants under light intensities higher than 20 μmol m^{-2} s^{-1} (Batista et al. 2016; Ferreira et al. 2017). These studies show that plants require a broad light spectrum and efficient gas exchange to optimise photosynthetic processes, reduce relative humidity, increase their aeration, become hardier and favour greater survival and rooting (Saldanha et al. 2012; Louback et al. 2021). In *Hevea brasiliensis*, *Cattleya walkeriana*, and *Eucalyptus dunnii*, the best results for length and number of roots, survival, and rooting were obtained with flasks that allowed improved natural ventilation (Tisarum et al. 2017; Souza et al. 2019).

Systems that increase the supply of CO_2 and light to *in vitro* explants would usually promote greater development of the photosynthetic apparatus, resulting in high rates of multiplication, growth, rooting, and subsequent acclimation of the plants to *ex vitro* conditions (Saldanha et al. 2012; Louback et al. 2021). Sometimes, a gradual adaptation to *ex vitro* conditions may be required for plants grown in the *in vitro* photomixotrophic systems to undergo autotrophic growth in a greenhouse (Pérez et al. 2015; Silveira et al. 2020).

Thus, the microstumps cultured with the 40 and 80 μmol m^{-2} s^{-1} light intensities and 1/M responded uniformly, with high rooting (92.1 to 96.8%) and a high probability of acclimatisation, compared to those obtained with the use of 20 μmol m^{-2} s^{-1} and 0/M, being indicative of optimisation of the *in vitro* rooting protocol. The treatments with light intensity equal to or greater than 40 μmol m^{-2} s^{-1} and 1/M were more suitable for *in vitro* adventitious rooting of the microstumps.

Conclusions

Quality and intensity of the FL 40 μmol m^{-2} s^{-1} light combined with a porous membrane (1/M) provided the most suitable conditions for *in vitro* elongation and adventitious rooting of *Eucalyptus grandis* × *E. urophylla* grown under a controlled environment. Explants under this treatment exhibited the greatest vigour, shoot length, photosynthetic pigment content, xylem phloem development, stomatal number and density, root length and diameter, number of roots per explant, and rooting percentage.

These results may contribute to optimise large-scale production of clonal plants.

List of abbreviations

LED light-emitting diode
MS Murashige and Skoog culture medium (Murashige & Skoog 1962)
BAP 6-benzylaminopurine
NAA α-naphthaleneacetic acid
IBA indole-3-butyric acid
DMSO dimethyl sulfoxide solution
FAA formalin-acetic acid-alcohol
ANOVA analysis of variance

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DMSCS conducted the experiment, perform the statistical analysis, and wrote results and discussion. SBF, VPD, and LVM conducted the experiment and contributed with the writing of the manuscript. GLT contributed revising the manuscript and interpreting results. GEB supervised the experimental work, and contributed revising the manuscript and writing the discussion. All authors revised the manuscript and approved the final version.

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