Spectral quality and temporary immersion bioreactor for in vitro multiplication of *Eucalyptus grandis* × *Eucalyptus urophylla*

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Abstract

Spectral quality is an important factor for in vitro development of explants in a bioreactor system. Based on the need to optimize micropropagation for *E. grandis* × *E. urophylla* clones, the aim of the study was to assess the spectral quality of in vitro multiplication in temporary immersion bioreactor (TIB). The tissue used to generate the explants (i.e., the nodal segment with 1 cm of length and two axillary bud without leaves) was previously in vitro established and multiplied, it derived from ministumps of *E. grandis* × *E. urophylla* clone grown in a semi-hydroponic system. The spectral quality of in vitro multiplication was assessed through five light sources (i.e., fluorescent lamp, red, green, blue, and yellow cellophane). Morphological and anatomical features of tissues grown in TIB were evaluated at 90 days. Based on the results, yellow and blue spectral qualities were the most suitable to be adopted for in vitro multiplication of *E. grandis* × *E. urophylla*, since they enabled lesser hyperhydricity, favors high number of shoots per explant and shoot length, as well as thicker mesophyll and spongy parenchyma; arise as an alternative for large-scale production of eucalypts clonal plants.

Keywords Cloning · In vitro cultivation · Vegetative propagation · Wavelength

Introduction

The expansion of forest plantations to non-traditional regions and the search for better adaptation in plant propagation has increased the use of species and hybrid combinations. The hybridization of *Eucalyptus grandis* W. Hill ex Maiden with *Eucalyptus urophylla* ST Blake has provided

excellent results, both in terms of wood quality and growth, in addition to better environmental adaptation and easier vegetative propagation (Trueman et al. 2018). However, alternatives for the tissue rejuvenation/reinvigoration of selected hybrids of the *Eucalyptus* genus, aiming at greater multiplication of plants, and micropropagation technique has been recommended, allowing considerable advances in clonal propagation (Wendling et al. 2014; Zhang et al. 2020).

Studies focused on investigating the micropropagation of *Eucalyptus grandis*, *Eucalyptus urophylla*, and of their hybrids, have been important for the industrial forestry sector. These studies comprise in vitro establishment (Moura et al. 2017; Keret et al. 2018), bud multiplication (Businge et al. 2017; Máximo et al. 2018; Trueman et al. 2018; Carvalho et al. 2019), shoot elongation (Gallo et al. 2017; Miranda et al. 2020), as well as adventitious rooting and acclimatization phases (Nakhoda and Watt 2017; Brondani et al. 2018).

Basic studies focused on improving microcutting production processes to solve the limiting factors in each micropropagation phases are fundamental. Several technologies have been suggested to optimize micropropagation processes. Innovations in plant growth environments such as the use of

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new lighting sources based on their spectral quality, as well as the automation of culture system operations and routine procedures through the use of temporary immersion bioreactors (TIB) stand out among these technologies (Batista et al. 2018; Carvalho et al. 2019).

The TIB consists of an automated system used to grow plant tissues. It can be applied to improve plant multiplication in laboratory and biofactory environments (Máximo et al. 2018). This type of system optimizes plant cultivation through automating processes (Mendonça et al. 2016), enables biomass gain and shortens propagation time (Máximo et al. 2018), as well as increases plant production per area (Regueira et al. 2017). Accordingly, TIBs are capable of improving nutrient supply and gas transfer processes, as well as of minimizing physiological disturbances in plants, thus improving the development of micropropagated cultures (Carvalho et al. 2019).

Recent studies have reported the influence of wavelength types such as 450–495 nm (blue light), 620–750 nm (red), 750–850 nm (extreme red), and 495–570 nm (green) (Batista et al. 2018) on plants morphophysiological processes since light is used as an energy source for photosynthesis (Heringer et al. 2017). However, despite the economic importance of eucalypts (e.g., *Eucalyptus* and *Corymbia*), and of its hybrids, studies focused on investigating the effect of different spectral qualities on plant growth and development, based on the use of TIB in the in vitro multiplication, remain incipient.

Our aim was to evaluate the in vitro multiplication phase of *E. grandis* × *E. urophylla*, based on spectral quality use and on cultivation in TIB system.

Material and methods

Study site and tissues source

The experiment was conducted in the Laboratory of In Vitro Culture of Forest Species at the Department of Forestry Sciences of the Federal University of Lavras (UFLA), Lavras, Minas Gerais, Brazil. The tissues used to generate the explants (i.e., nodal segment with 1 cm of length and axillary bud without leaves) was previously in vitro established and multiplied; it derived from ministumps of the A211 hybrid clone of *Eucalyptus grandis* Hill ex Maiden × *Eucalyptus urophylla* ST Blake grown at “Viveiro Esteio Produção de Mudas”, São João Del Rei, Minas Gerais, Brazil. Ministumps were established in a clonal mini-garden under semi-hydroponic system of the sand-bed channel type. Plants received dripping nutrient solution, which was applied four times a day, at total daily flow of 4 L m⁻².

Shoot multiplication in temporary immersion bioreactor (TIB)

Shoots produced at the in vitro establishment stage were isolated, standardized in length (0.5 cm and two buds), and inoculated in glass flasks (250 mL) under aseptic conditions. To the glass flasks were added 150 mL of WPM liquid culture medium (Lloyd and McCown, 1980), which was supplemented with 20 g L⁻¹ of sucrose (Vetec®), 0.5 mg L⁻¹ of 6-benzylaminopurine (BAP—Sigma®), and 0.01 mg L⁻¹ of naphthaleneacetic acid (NAA—Sigma®). Plant tissues were immersed in TIB for 30 s, at 3 h intervals between immersion cycles, throughout 90 culture days. Subcultures were carried out every 30 days for culture medium renewal purposes. The liquid culture medium was prepared with deionized water and its pH was adjusted to 5.8 ± 0.05, before autoclaving. The culture medium was autoclaved at a temperature of 127 °C and pressure of 1.5 kgf cm⁻², for 20 min.

Spectral quality

The experiment has followed a completely randomized design based on five spectral qualities [fluorescent lamp (F/L), red (R/E), green (G/R), blue (B/L), and yellow (Y/E) cellophane], with twenty replications comprising one explant, each. Treatments with red, green, blue, and yellow spectral quality were applied by filtering the light output of fluorescent lamps through double cellophane sheets, which were used to wrap the TIB flasks. SPECTRA PEN Z850 portable spectroradiometer (Qubit Systems-Kingston, Ontario-USA) was used to analyze variations in absolute irradiance (μW cm⁻² nm⁻¹) and the wavelength (nm) of light emitted by different spectral qualities. Spectral of each treatment used in this experiment are shown in Fig. 1.

The experiment was carried out in growth room at 24 ± 1 °C, under 16 h of photoperiod. Two cold white fluorescent tubes (Philips T10, 0.60 m long, 20 W and color 6400–6500 K) were used on each shelf. Photodiometer model QSO-S Procheck + Sensor-PAR Photon Flux (Decagon Devices, Pullman, Washington-USA) was used to measure the irradiance of the two lamps (40 μmol m⁻² s⁻¹).

Plant vigor (VIG) and hyperhydricity (HYP) were evaluated at 90 days of cultivation, based on the scale of notes by Oliveira et al. (2016), as shown in Fig. 2. Number of shoots per explant (> 0.5 cm) (NSE) and shoot length (> 0.5 cm) per explant (SL), as well as photosynthetic pigment content (PPC) were evaluated.

Fig. 1 Variations in absolute irradiance ($\mu\text{W cm}^{-2} \text{ nm}^{-1}$) and wavelength (nm) of light emitted for in vitro cultivation of *E. grandis* × *E. urophylla*. **a** Fluorescent lamp; **b** red cellophane; **c** green cellophane; **d** blue cellophane; **e** yellow cellophane

Photosynthetic pigment analysis

Leaf discs (i.e., 25 mg of fresh matter) were sampled after 90 days of exposure to different spectral qualities. Samples were inoculated in 5 mL of DMSO solution (Sigma aldrich) and stored in the dark for 48 h, based on the methodology by Lichtenthaler (1987). Sample absorbance was

determined in triplicate with the aid of a quartz cuvette with 10 mm of optical path, in Genesys 10UV spectrophotometer (ThermoScientific, USA). The wavelengths (665, 649 and, 480 nm) and equations used to calculate chlorophylls *a*, *b*, *a* + *b* and total carotenoid concentration were based on the method by Wellburn (1994).

Fig. 2 Scale of notes used to assess vigor and hyperhydricity in *E. grandis* × *E. urophylla* tissues. **a** Scale 1-VIG: low shoot induction, senescence and death; **b** scale 2-VIG: shoots induction, although with reduced leaves; **c** scale 3-VIG: shoot induction and active growth, without apparent nutritional deficiency; **d** scale 1-HYP: hypertrophy in leaf tissues and explant internodes; **e** scale 2-HYP: reduced leaf tissue hypertrophy; **f** scale 3-HYP: no hyperhydricity. Bar = 0.5 cm

Leaf anatomy

Representative leaf samples from each treatment were collected and kept in FAA solution (formaldehyde, acetic acid, 70% of ethanol solution, 1:1:18) for 48 h. Next, they were transferred to 70% ethanol solution and dehydrated at increasing ethanol series concentrations (80, 90, and 100%) in each solution for 30 min (Johansen 1940). Subsequently, they were soaked in historesin (Biosystems, Nussloch, Germany) at the ratio of 1:1, in a hot oven (overnight). Blocking was processed with pure hydroxyethyl methacrylate resin and cross-sections (7 µm of thick) were obtained with the aid of manual rotating microtome and razor. Tissues were stained with toluidine blue (Vetec Química Fina Ltda, Rio de Janeiro, Brazil), mounted on Entellan histological slides (Merck KGaA, Darmstadt, Germany) and photomicrographed with a digital camera (AxionCam ERc5s) at micrometric and objective scale: 20× and 40×, respectively. Adaxial (ADE) and abaxial epidermis (ABE), mesophyll (ME), palisade parenchyma (PP), and spongy parenchyma (SP) thicknesses were measured in three fields of organ view, with leaf area corresponding to 0.04 mm²; measurements comprised fifteen replications (5 anatomical sections × 3 fields of organ view) with one leaf, each.

Data analysis

Analyses were processed in the R software, version 3.0.3 (R Development Core Team 2018), using the ExpDes package, version 1.1.2 (Ferreira et al. 2013). Variables that did not show the normal distribution in the Shapiro–Wilk test, at 5% of significance level, were transformed by arcsen. Treatment means

were subjected to analysis of variance (ANOVA) in Tukey's test at 5% of probability.

Results and discussion

The visual appearance of *E. grandis* × *E. urophylla* clone explants can be seen in Fig. 3a–e and morphological features in Fig. 4a–e. Different spectral qualities used in explant of *E. grandis* × *E. urophylla* (A211 clone) under in vitro culture have influenced the plant vigor, depending on the scale of notes, at 90 days after inoculation (Fig. 4a). The highest mean recorded for VIG was associated with the use of Y/E (2.75), the closest being the scale of notes 3 represented in Fig. 2c, which was significantly different from that recorded for F/L (Fig. 4a) ($p < 0.05$). These results are close to the ones reported by Souza et al. (2018) in *Corymbia citriodora* (Hook.) KD Hill & LAS Johnson × *C. torelliana* (F. Muell.) and by Miranda et al. (2020) in *Eucalyptus urophylla*, who observed good vigor in explants subjected to spectral qualities at peak wavelengths of 450 and 600 nm. On the other hand, Smirnakou et al. (2016) recorded the best vigor results for *Abies borisii-regis* explants subjected to fluorescent lamp light source.

HYP is an undesirable aspect of in vitro multiplication, and have shown different HYP responses according to spectral qualities. Based on the scale of notes 3 represented in Fig. 2f, R/E and G/R recorded the best HYP results (2.75, on average), which were significantly different ($p < 0.05$) from that recorded for B/L, as shown in Fig. 4b. Silva et al. (2017) conducted a study with *Plectranthus amboinicus* (Lour.) Spreng explants grown under red light; results have shown good vegetative vigor and lack of hyperhydricity. Studies have evidenced the influence of red and green light on low stomatal conductance, which is an ecological adaptation to avoid excessive leaf water loss (Aasamaa and Aphalo 2016; Batista et al. 2018).

HYP has been associated with the micropropagation of woody species, which can influence crop development. This outcome may be linked to cultivation factors such as the use of a liquid medium in bioreactor systems, which tend to increase water and mineral salts' absorption by plants. In addition, plants can be affected by light irradiation, air temperature and relative humidity (Xiao et al. 2011). Thus, methodologies aimed at overcoming or mitigating HYP in tissues are important strategies to be adopted in propagation systems.

Explants subjected to B/L presented the best SL results (2.26 cm, on average), which were significantly different from that recorded for R/E and G/R light spectra ($p < 0.05$) (Fig. 4c). The use of blue LED lamps enabled the shoots length per explant in *Acer saccharum* Marsh (Singh et al.

Fig. 3 Details of *E. grandis* × *E. urophylla* shoots in TIB under different spectral qualities on in vitro multiplication phase. **a** Fluorescent lamp (F/L); **b** red (R/E); **c** green (G/R); **d** blue (B/L); **e** yellow (Y/E). Bar = 0.5 cm

2017) and *Bambusa oldhamii* Munro (Silveira et al. 2020); this outcome highlights the important role played by wavelength (400–500 nm) in photomorphological responses of propagules grown in controlled environments—it appears to be a useful technology capable of optimizing shoot development and yield.

There is variation in plant morphophysiological responses to blue light (400–500 nm), such as changes in plant growth and development to allow adaptations to changes in environmental conditions (Lazzarini et al. 2017). However, plant species, such as *Rehmania glutinosa* (Gaertn) Steud. (Manivannan et al. 2015), *Plectranthus amboinicus* (Lour.) Spreng (Noguchi and Amaki 2016), *Solanum lycopersicum* L., *Cucumis sativus* L. and *Capsicum annum* L. (Snowden et al. 2016) grown under wavelengths of 400–500 nm recorded reduced growth.

With respect to NSE, there was a significant difference in shoot proliferation, depending on the adopted spectral quality. Y/E enabled higher NSE (7.70 shoots, on average) than R/E, G/R, and F/L (Fig. 4d). Data described in the literature corroborate the one found in the current study since greater

peaks in the spectrum of wavelengths 450 and 600 nm induced a larger number of shoots in *Platycodon grandiflorum* (Jacq.) A. DC. (Liu et al. 2014), *Populus euramericana* Moench. (Kwon et al. 2015) and *Phalaenopsis amabilis alba* Blume (Massaro et al. 2018).

The application of white or yellow LED light to crops can help increasing plant growth and yield, since it enables deeper light penetration in leaves and maximizes photosynthetic rates, in comparison to monochromatic blue and red lights, as observed in *Lactuca sativa* hydroponics (Lin et al. 2013). These studies have shown that plants need a broad light spectrum to optimize photosynthetic processes and that this needs changes from species (Lazzarini et al. 2017). Thus, the application of yellow spectral quality to *E. grandis* × *E. urophylla* has proved to be suitable for shoot multiplication processes in large scale production systems based on TIB.

The best PPC (chlorophyll *a*, *b* and carotenoids) results were compared to explants subjected to Y/E spectral quality (Fig. 4e). Chlorophyll *a*, *b* and carotenoid biosynthesis is influenced by the quality and intensity of the emitted light;

Fig. 4 Morphological features observed in *E. grandis* × *E. urophylla* on in vitro multiplication under different spectral qualities [Fluorescent lamp (F/L), Red (R/E), Green (G/R), Blue (B/L) and Yellow (Y/E) cellophane]. **a** Scale of notes—Vigor (VIG); **b** scale of notes—Hyperhydricity (HYP); **c** shoot length (SL); **d** number of shoots per

explant (NSE); **e** Photosynthetic PIGMENT contents (PPC). *Means followed by equal letters did not differ from each other, according to the Tukey's test at 5% of probability. Bars represent the standard deviation from the mean value

the best efficiency in photosynthetic processes prevails at wavelengths ranging from 450 to 600 nm (Gupta and Kar-makar 2017).

In vitro multiplication of *Gerbera jamesonii* Bollus ex Hook. F. presented higher chlorophyll *a* and *b* contents under wavelength peaks at 450 and 600 nm (red/blue LEDs) than under monochromatic light sources of 450 nm (blue LEDs) and 600 nm (red LEDs) (Pawlowska et al. 2018). Carotenoid concentrations observed in *Stevia rebaudiana* (Bertoni.) shoots exposed to wavelengths of 450 nm and 600 nm (red/blue LEDs) were higher than that of shoots exposed to 450 nm (blue LEDs) (Ramírez-Mosqueda et al. 2017).

Broad light spectrum absorption promotes greater energy state (Silveira et al. 2020). The excitation energy in the chlorophyll molecule can be used in the photochemical step, be lost in the form of heat or even cause damage to the photosynthetic apparatus such as blue free radical formation (Neto et al. 2020). Thus, knowledge about the association between wavelengths and plant growth patterns on in vitro cultivation can contribute to the understanding of the micropropagation protocols to be applied to the species investigated in the present study.

The leaf anatomy (Fig. 5a–e) evidenced different responses between spectral qualities adopted on in vitro multiplication based in TIB system (Fig. 6a–e). *E. grandis* × *E. urophylla* clone subjected to treatment based on Y/E spectral quality recorded the highest ADE mean (14.64 µm), which was significantly different ($p < 0.05$) from that recorded for the R/E treatment (Fig. 5a). Schuerger et al. (1997) have found thicker leaves and a larger number of chloroplasts per cell in *Capsicum annum* plants subjected to blue LEDs than in plants treated with red LEDs in association with infrared (735 nm) and fluorescent lamp.

On the other hand, the highest ABE values were observed for plants grown under R/E spectral quality (9.94 µm, on average); they were significantly different ($p < 0.05$) from values recorded for the G/R treatment (Fig. 5b). According to Li et al. (2018), yellow and blue LEDs were more advantageous for the morphogenesis of *Solanum tuberosum* plants than green LEDs. Different spectral qualities have influenced the growth and development of plant cells, tissues, and organs, as well as triggered different morphological and anatomical responses (Macedo et al. 2011; Hoge-woning et al. 2012).

Results recorded for ME and SP presented the same trend, the highest means were recorded for plants grown under spectral qualities Y/C (150.16 µm and 109.63 µm) and B/C (121.93 µm and 90.04 µm), which differed from the other treatments ($p < 0.05$) (Fig. 5c, e). Kwon et al. (2015)

reported a great number of parenchymal cells undergoing cellular division process in *Populus euramericana* plants subjected to treatment with red/blue light, and it enabled greater shoot and leaf development. In addition, Macedo et al. (2011) reported that the 450 nm wavelength in the blue light treatment led to the thicker epidermis and mesophyll cells in *Alternanthera brasiliana* (L.) Kuntze on in vitro cultivation.

Changes in the structural organization of leaf cells can be observed at the anatomical level in plants exposed to different environments, including different light spectra (Fig. 6a–e). Plants subjected to broad light spectrum (450–600 nm) presented larger adaxial epidermis, palisade parenchyma, and mesophyll (Fig. 6e) dimensions, the opposite outcome was observed in plants subjected to fluorescent (Fig. 6a), red (Fig. 6b) and green (Fig. 6c) light. These results are similar to the ones reported by Miranda et al. (2020) for *Eucalyptus urophylla* explants grown under a broad light spectrum. Each spectrum is capable of changing the expression levels of specific genes involved in the structural organization of plant cells, as well as of photosynthetic pathway genes (Fan et al. 2013).

Based on the results, Y/E spectral quality was efficient in enabling the organized development of adaxial and mesophyll epidermis cells, including substantial chlorophyll concentrations in the spongy parenchyma (Fig. 6e). This anatomical development is causally linked to the more SL and NSE recorded in the current study. *E. grandis* × *E. urophylla* explants subjected to G/R treatment recorded the highest PP means (32.55 µm), although there was not significant difference between treatments ($p > 0.05$) (Fig. 5d).

Morphological and anatomical results have implications in the optimization of TIB systems used to produce clonal plants of eucalypts species, based on the micropropagation technique. Broad yellow light spectrum has proved to be viable and important for explant in vitro multiplication in controlled environments.

Conclusions

- The current study provided evidences that wavelength specificity influences the morphology and anatomy of *E. grandis* × *E. urophylla* plants grown in controlled environment; thus, it emerges as a useful technology to optimize clonal plant production at large scale.
- The shoots can be obtained in explants in a temporary immersion bioreactor (TIB) system. Yellow and blue spectral quality can be used on in vitro cultivation, without harming the development of the shoots.
- Yellow and blue spectral qualities are the most suitable to be used in *E. grandis* × *E. urophylla* on in vitro multipli-

Fig. 5 Leaf anatomy features observed in *E. grandis* × *E. urophylla* on in vitro multiplication under different spectral qualities [Fluorescent lamp (F/L), Red (R/E), Green (G/R), Blue (B/L) and Yellow (Y/E) cellophane]. **a** Adaxial Epidermis (ADE); **b** abaxial epidermis

(ABE); **c** mesophyll (ME); **d** palisade parenchyma (PP); and **e** spongy parenchyma (SP) thickness. *Means followed by equal letters did not differ from each other, according to the Tukey's test at 5% of probability. Bars represent the standard deviation from the mean value

Fig. 6 Cross-sections of leaf blade observed in *E. grandis* × *E. urophylla* on in vitro multiplication under different spectral qualities. **a** Fluorescent lamp (F/L); **b** red (R/E); **c** green (G/R); **d** blue (B/L); **e**

yellow (Y/E). * ADE adaxial epidermis; ABE abaxial epidermis; ME mesophyll; PP palisade parenchyma; SP spongy parenchyma. Bar = 200 µm

cation, since they enabled lesser hyperhydricity, greater number of shoots per explant, length of shoots, as well as thicker mesophyll and spongy parenchyma.

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Availability of data and materials All data presented in this study are available for consultation.

Code availability Not applicable' for that section.

Compliance with ethical standards

Conflicts of interest/Competing interests Not applicable' for that section.

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