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ORIGINAL ARTICLE

Carbon sources and sealing systems affect the *in vitro* cloning of *Eucalyptus pilularis* selected tree

Fontes de carbono e sistemas de trocas gasosas afetam a clonagem *in vitro* de árvore selecionada de *Eucalyptus pilularis*

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ABSTRACT

Carbon sources and porous membranes influence the growth and development of plants grown *in vitro* culture due to their effects on morphogenesis and biomass production. This study aimed to evaluate the effects of carbon sources (sucrose, glucose, fructose, and galactose) on the *in vitro* multiplication, different glucose concentrations (0, 10 and 20 g L^{-1}) and sealing systems (WM - rigid polypropylene caps without membrane, and 1M - rigid polypropylene caps with a 1.0-cm-diameter hole covered with a 1 cm^2 membrane) on *in vitro* elongation and adventitious rooting of *Eucalyptus pilularis* selected tree. Glucose resulted in better responses to *in vitro* multiplication and elongation stages when compared to sucrose, fructose and galactose. Sucrose resulted in highest levels of photosynthetic pigment content. Glucose at a concentration of 20 g L^{-1} combined with 1M sealing system resulted in improved response on *in vitro* elongation stage, with the highest shoots length. Supplementation at culture medium with 20 g L^{-1} of glucose and 1M sealing system resulted in better adventitious rooting, as well as in the best results of *in vitro* culture of *E. pilularis* for 390 days, and favoured the clonal micro plant production.

Keywords: *Plant cloning; Vegetative propagation; In vitro culture; Adventitious rooting.*

RESUMO

A fonte de carbono e membranas porosas podem influenciar o crescimento e desenvolvimento de plantas cultivadas *in vitro* devido aos seus efeitos na morfogênese e na produção de biomassa. Este estudo teve como objetivo avaliar os efeitos de fontes de carbono (i.e., sacarose, glicose, frutose e galactose) na multiplicação *in vitro*, diferentes concentrações de glicose (0, 10 e 20 g L^{-1}) e sistemas de vedação (WM - tampas rígidas de polipropileno sem membrana, e 1M - tampas rígidas de polipropileno com furo de 1,0 cm de diâmetro e cobertas por membrana de 1 cm^2) no alongamento e enraizamento *in vitro* de tecidos coletados de árvore selecionada de *Eucalyptus pilularis*. A glicose resultou em melhores respostas às etapas de multiplicação e alongamento in vitro quando comparada à sacarose, frutose e galactose. A sacarose favoreceu o maior conteúdo de pigmentos fotossintéticos. A glicose na concentração de 20 g L^{-1} combinada ao sistema de vedação 1M proporcionou melhor resposta na fase de alongamento *in vitro*, com maior comprimento de brotações. A suplementação em meio de cultura com 20 g L^{-1} de glicose e sistema de vedação 1M resultou no melhor enraizamento adventício, bem como nos melhores resultados para o cultivo *in vitro* de *E. pilularis* em 390 dias, favorecendo a produção de microplantas clonais.

Palavras-chave: *Planta clonal; Propagação vegetativa; Cultivo in vitro; Enraizamento adventício.*

1. INTRODUCTION

The globalized market demands quality gains of its products proportional to the productivity of forest plantations, coupled with a reduction in production costs. Therefore, the search for species that meet these objectives is inherent to the expansion of the forest production chain. In this context, wood from species of *Eucalyptus* and *Corymbia* genera as a source of raw material for solid wood products

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has stood out (Hornburg et al., 2012). *Eucalyptus pilularis* Smith is a species belonging to native forests in subtropical regions along the east coast of Australia (West, 2023). This species is recommended for the formation of commercial plantations in tropical regions due to its frost tolerance (Cassidy et al., 2012) and has shown productive potential for its wood for use in sawmills and laminating. Thus, the definition of methodologies for cloning of *E. pilularis* is important for the expansion of breeding programs and future commercial forest plantations.

Conventional *in vitro* culture environment present differences from the external environment, leading to various physiological and morphological disturbances in plants. These disturbances encompass insufficient chlorophyll production, leading to the inability to sustain growth via photosynthesis, stomatal disorders, lack of the leaf cuticle layer, abnormal parenchyma, and hyperhydricity (Tisarum et al., 2018). Gas exchange used for *in vitro* plant propagation consists of a cultivation method that maintains the carbohydrate source and adds a ventilation system to the flasks (Miranda et al., 2024; Souza et al., 2024). This allows gas exchanges, improving the transpiration rate and, consequently, nutrient absorption (Souza et al., 2019); and may represent a feasible methodology for obtaining micro-propagated plants.

Various techniques have been developed to enhance the photosynthetic capacity of micro-propagated plants (Souza et al., 2020a, 2024). These methods include lids with gas-permeable filters or culture flasks with increased ventilation (Saldanha et al., 2013). The addition of carbohydrates as a carbon and energy source can facilitate the plant to perform metabolic functions, *in vitro* morphogenesis including induction, shoot proliferation, and root emission (Cheong & An, 2015; Tormen et al., 2018; Esquivel et al., 2024; Miranda et al., 2024).

The method of flask sealing employs polyethylene caps as barriers, which inevitably restrict the flow of photosynthetically active photons and gas exchange. As a result, the relative humidity inside the container increases, the concentration of ethylene rises, the concentration of CO_2 decreases, transpiration rates decline, water and nutrient absorption become impaired, and growth and development are reduced (Souza et al., 2020a), and high mortality during *ex vitro* acclimatization are reported (Tisarum et al., 2018).

The kind of carbon source used on *in vitro* culture is important as it impacts plant metabolism, growth, yield, and antioxidant properties. Carbohydrates serve as energy sources and provide carbon for biosynthesis, regulating gene expression and growth. External carbohydrate sources like sucrose and glucose are essential, influencing plant development and hormonal signalling networks (Yaseen et al., 2013), and there is evidence of a strong interaction between carbohydrate content and endogenous plant growth regulator levels; with these effects directly related to the morphogenic process (Xu et al., 2020).

The success of micro-propagation as a tool for plant propagation of superior genotypes is directly associated with the expansion of knowledge regarding plant responses to *in vitro* culture. Recent advances in photo-autotrophic micropropagation methods provide a new platform with various successful results for *in vitro* optimization of the culture environment for efficient protocols (Yaseen et al., 2013). These adaptations include the emission and development of roots, regulation of transpiration through functional stomata, enhancement of CO_2 assimilation to increase photosynthetic rate, production of leaves, expansion of leaf area, and shoot and root biomass accumulation (Silva et al., 2022).

Given the limited number of studies on micropropagation of *Eucalyptus* adult genotypes, the aim of study was to evaluate the effect of carbon sources on *in vitro* multiplication and elongation stages; and glucose concentration and sealing systems on *in vitro* elongation and adventitious rooting stages of a selected *E. pilularis* tree.

2. MATERIALS AND METHODS

2.1. Study site and experimental material

Tissues used in the experiments were obtained from the *in vitro* multiplication stage through three successive subcultures, performed in the same culture medium described for the experiment. This procedure aimed to increase the number of shoots available for the subsequent experimental stages (Avelar et al., 2020, 2022). Buds were derived from nodal segments collected from epicormic shoots of pruned branches of a 49-year-old *Eucalyptus pilularis* Smith tree (Avelar et al., 2020). The selected tree was grown and tested in Lavras, Minas Gerais, Brazil (21°14'S, 44°59'W, and 919 m of altitude) (Instituto de Pesquisas e Estudos Florestais, 1984).

2.2. *In vitro* multiplication – effect of carbon sources

Standardized explants (i.e., clusters of buds with 0.25 cm^2) were inoculated under aseptic conditions in glass test tubes (25 × 150 mm) containing 10 mL of WPM culture medium (Lloyd & McCown, 1980). The medium was supplemented with 2.22 µM 6-benzylaminopurine (BAP) and 0.27 µM α-naphthaleneacetic acid (NAA), 6 g L^{-1} agar (Molinari et al., 2021), and 20 g L^{-1} carbon sources (i.e., treatments: sucrose, glucose, fructose, and galactose). Subculturing was performed every 30 days.

The experiment was conducted in a completely randomized design, using four carbon sources with thirty-six replicates. Each replicate corresponded to one test tube containing a single explant.

After 120 days of *in vitro* culture, tissue oxidation (Figure 1A) and vigour (Figure 1B) for each treatment were evaluated using a scoring scale (score_1, 2 and 3). The number of buds per explant, chlorophyll *a*, chlorophyll *b*, total chlorophyll (*a* + *b*), ratio chlorophyll (*a*/*b*) and carotenoids contents were measured.

2.3. *In vitro* elongation – effect of carbon sources

Explants from *in vitro* multiplication stage were standardized (i.e., bud cluster with 0.25 cm^2) and inoculated under aseptic conditions in glass test tubes (25 × 150 mm) containing 10 mL WPM culture medium. The culture medium was supplemented with 2.68 µM NAA and 0.22 µM BAP (Molinari et al., 2021), 6 g L^{-1} of agar, and 20 g L^{-1} of carbon sources (i.e., treatments: sucrose, glucose, fructose, and galactose). Subculturing was performed every 30 days.

The experiment was conducted in a completely randomized design, using four carbon sources with thirty replicates. Each replicate corresponded to one test tube containing a single explant.

After 110 days of *in vitro* culture, tissue oxidation (Figure 1A) and vigour (Figure 1B) for each treatment were evaluated using a scoring scale. The number of shoots per explant (shoot > 0.5 cm) and length of shoots (cm) were evaluated.

2.4. *In vitro* elongation – effect of glucose concentration and sealing systems

Explants from *in vitro* elongation stage (i.e., effect of carbon sources) with 110 days were sub-cultured under aseptic conditions in glass flasks (65 × 95 mm) containing 40 mL WPM culture medium. The culture medium was supplemented with 2.68 µM NAA, 0.22 µM mg L^{-1} BAP (Molinari et al., 2021), 6 g L^{-1} of agar, and glucose concentrations × sealing systems (i.e., treatments).

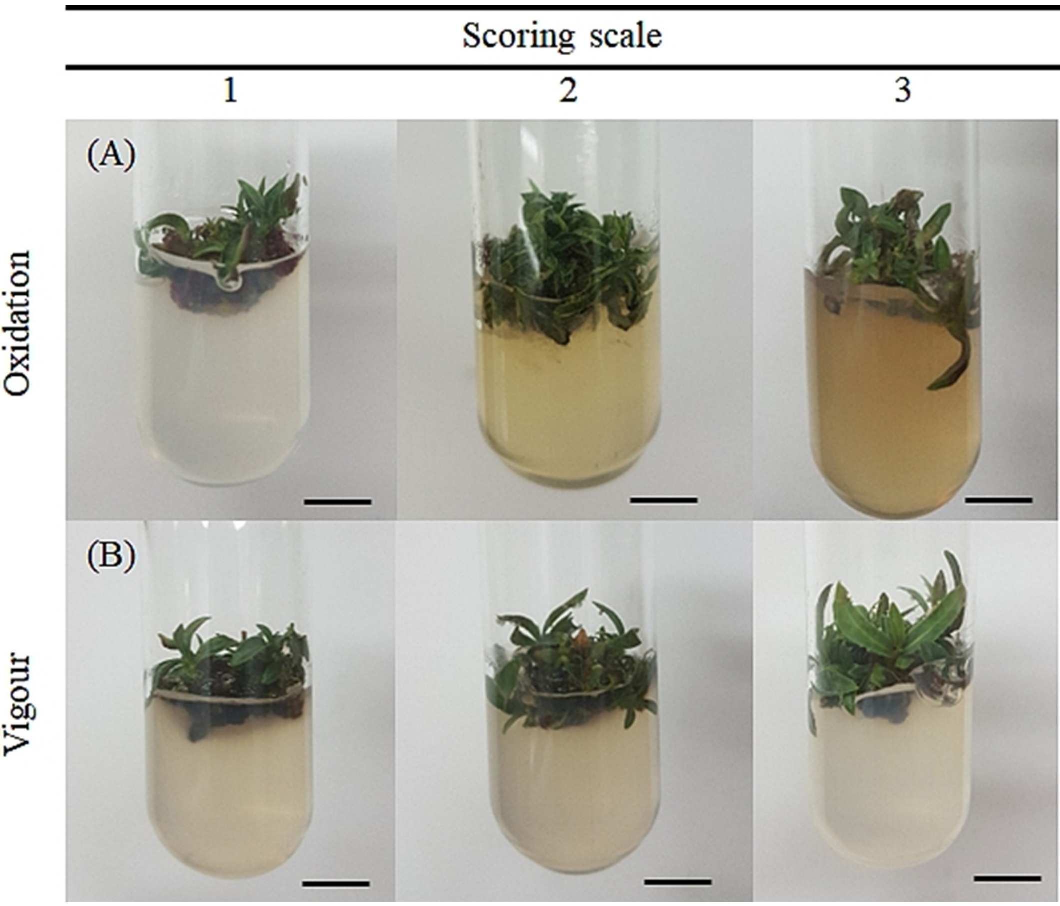

Figure 1. Details of tissue oxidation and vigour of *Eucalyptus pilularis* explants according to the scoring scale. (A) Oxidation: Score_1 = Null: no oxidation; Score_2 = Medium: reduced oxidation at the base of explants and/or culture medium; Score_3 = High: complete oxidation of bud/shoot and culture medium; (B) Vigour: Score_1 = Low: absence of bud/shoot induction and/or presence of senescence and death; Score_2 = Good: Bud/shoot induction, but with reduced leaves; Score_3 = Excellent: bud/shoot induction with active growth. Bar = 1 cm.

The experiment was conducted in a completely randomized design in a 3 × 2 factorial arrangement, with six treatments: three glucose concentrations (0, 10, and 20 g L^{-1}) combined with two sealing systems (WM - rigid polypropylene caps without membrane, and 1M - rigid polypropylene caps with a 1.0-cm-diameter hole covered with a 1.0 cm^2 membrane, according to Molinari et al., 2021). Each experimental unit replicated eight times consisted of one glass flask containing two explants. The combination of glucose concentrations (0, 10, and 20 g L^{-1}) and sealing systems (WM: without membrane; 1M: with membrane) generated six treatments: WM_0, WM_10, WM_20, 1M_0, 1M_10, and 1M_20.

After 40 days of *in vitro* culture, tissue oxidation (Figure 1A) and vigour (Figure 1B) for each treatment were evaluated using a scoring scale. The number of shoots per explant (shoot > 0.5 cm) and length of shoots (cm) were evaluated.

2.5. *In vitro* adventitious rooting – effect of glucose concentration and sealing systems

Elongated shoots obtained from the best performing treatment of the elongation (shoot > 1.0 cm) were transferred to glass flasks (65 × 95 mm) containing 40 mL WPM culture medium supplemented with 1.07 µM NAA, 0.98 µM indole-3-butyric acid (IBA), and 0.22 µM BAP (Molinari et al., 2021), 6 g L^{-1} of agar, and glucose concentrations × sealing systems (i.e., treatments). Subculturing was performed every 30 days.

The experiment was conducted in a completely randomized design in a 3 × 2 factorial arrangement, with six treatments: three glucose concentrations (0, 10, and 20 g L^{-1}) and two sealing systems (WM - rigid polypropylene caps without membrane, and 1M - rigid polypropylene caps with a 1.0-cm-diameter hole covered with a 1.0 cm^2 membrane, according to Molinari et al., 2021) with eight replicates, each glass flask containing two explants.

After 120 days of *in vitro* culture, tissue oxidation (Figure 1A) and vigour (Figure 1B) for each treatment were evaluated using a scoring scale. The adventitious rooting percentage, number of roots per explant, and length of roots (cm) were evaluated. The micro-propagation stages described above are illustrated in Figure 2.

2.6. Incubation conditions and culture medium preparation

The pH of the culture medium solution was adjusted to 5.8 (± 0.05) with NaOH (0.1 M) and/or HCl (0.1 M) before autoclaving and the agar (Merck S.A.) addition. The culture medium was autoclaved at a temperature of 121°C (1.0 kgf cm^{-2}) for 20 minutes. Explants were cultured in a growth room with a temperature of 24°C (±1°C), 16 h-photoperiod, and an irradiance of 40 µmol m^{-2} s^{-1} quantified by a radiometer (LI-COR®, LI-250A Light Meter).

2.7. Photosynthetic pigment contents

Leaf content of photosynthetic pigments [(i.e., chlorophyll *a*, chlorophyll *b*, total chlorophyll (*a* + *b*), ratio chlorophyll (*a*/*b*) and

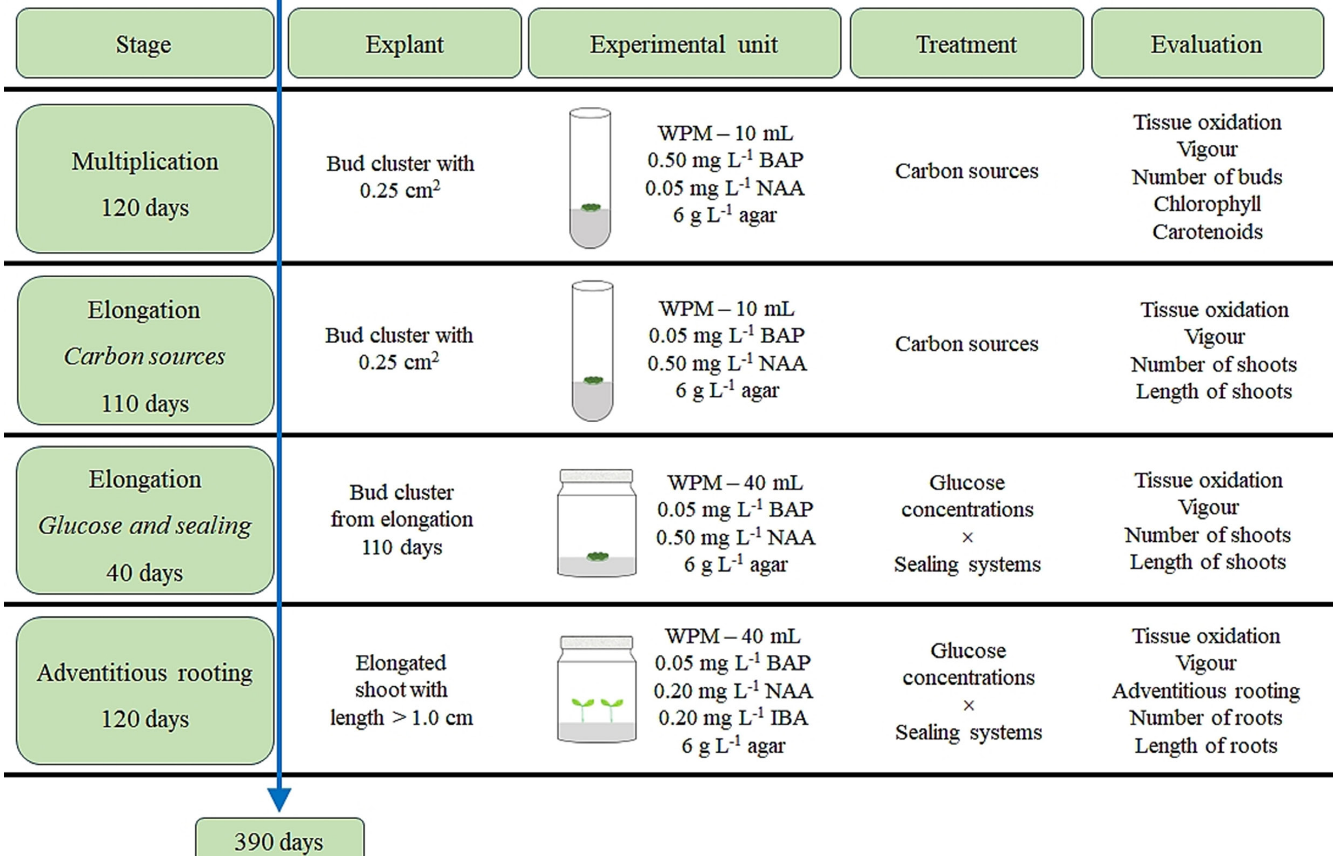

Figure 2. Flowchart including the stages for the micropropagation of *Eucalyptus pilularis* from *in vitro* multiplication until adventitious rooting, amounting to 390 days.

carotenoids)] were evaluated in the multiplication stage at 120 days. The extraction was performed using a methodology adapted from Lichtenthaler (1987). In a partially dark environment, 25 mg of fresh leaves were collected from three explants and placed in 5-mL Eppendorf tubes. Then, 2.5 mL of dimethylsulfoxide (DMSO) solution was pipetted into each sample and stored in the dark for 48 hours. The pigment was extracted from the leaves, and an absorbance (A) reading was performed at 480 nm, 649 nm, and 665 nm with a spectrophotometer (KASUAKI). The readings were performed in triplicate. Chlorophyll and carotenoid contents were calculated following the equations described by Wellburn (1994): chlorophyll $a = [(12.19 \times A_{665}) - (3.45 \times A_{649})]$; chlorophyll $b = [(21.99 \times A_{649}) - (5.32 \times A_{665})]$ and carotenoids = $(1.000 \times A_{480} - 2.14 \times Ca - 70.16 \times Cb)/220$, with the results expressed in µg of pigment per mg of fresh leaf tissue (µg mg^{-1}), C*a* = chlorophyll *a* and, C*b* = chlorophyll *b* contents.

2.8. Statistical analyses

Statistical analyses were conducted with R software, version 4.2.1 (R Core Team, 2022), ExpDes package, version 1.1.2 (Ferreira et al., 2013). The variables that did not have a normal distribution according to Shapiro-Wilk's test ($P > 0.05$) and/or did not show homogeneity of variances according to Bartlett's test ($P > 0.05$) were arc-sin-transformed. Number of shoots per explant, length of shoots, adventitious rooting percentage, number of roots per explant, length of roots, photosynthetic pigment contents [(i.e., chlorophyll *a*, chlorophyll *b*, total chlorophyll (*a* + *b*), ratio chlorophyll (*a*/*b*) and carotenoids)] were subjected to analysis of variance (ANOVA, $P < 0.05$) and compared by Tukey's test ($P < 0.05$). Tissue oxidation and vigor data were analyzed by principal component analysis (PCA) with the R software (R Core Team, 2022), using the "factoextra" R package (version 1.0.7) (Kassambara & Mundt, 2020).

3. RESULTS

3.1. *In vitro* multiplication – effect of carbon sources

At the 120th day of *in vitro* culture the tissue oxidation showed reduced correlation between carbon sources, and the mean values were 76.4% for score_1, 19.4% for score_2, and 4.2% for score_3 (Figure 3A). Glucose resulted the highest correlation and frequency of score_3 for vigour (38.9%); and sucrose (5.6%), galactose (11.1%) and fructose (16.7%) resulted in the lowest means (Figure 3B). Sucrose (80.6%) and galactose (80.6%) were more correlated to score_1 for vigor (Figure 3B).

Number of buds per explant (13.9) was higher with glucose supplementation to the culture medium, differing significantly from other carbohydrate sources (Figure 4A). Significant variations were observed in the levels of all evaluated photosynthetic pigments (Figures 4B-F), including chlorophyll *a*, chlorophyll *b*, total chlorophyll (*a* + *b*), ratio chlorophyll (*a* / *b*), and carotenoids, at 120 days of *in vitro* multiplication of *E. pilularis*.

The inclusion of sucrose in the culture medium resulted the highest levels of chlorophyll *a* (0.182 µg mg^{-1}) (Figure 4B), chlorophyll *b* (0.093 µg mg^{-1}) (Figure 4C), total chlorophyll (0.268 µg mg^{-1}) and carotenoids (0.046 µg mg^{-1}) (Figure 4F). Glucose and fructose supplementation to the culture medium showed intermediate results for chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids, while galactose resulted in the lowest contents (Figures 4B-D and 4F). However, the

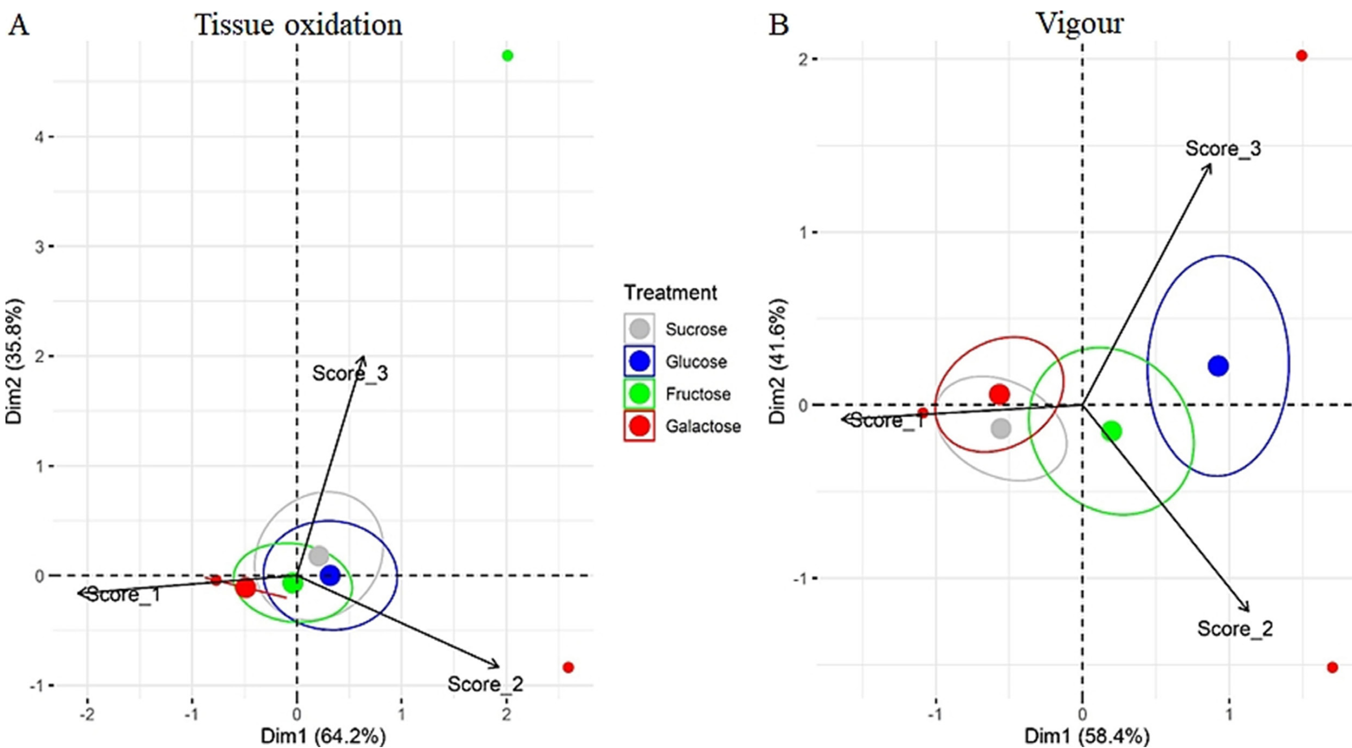

Figure 3. Principal component analysis (PCA) of frequency of tissue oxidation (Figure 1A) and vigour (Figure 1B), based on the scoring scale of *Eucalyptus pilularis* in different carbon sources supplementation at 120 days *in vitro* multiplication. (A) PCA for tissue oxidation (Figure 1A); (B) PCA for vigour (Figure 1B). Dim1=Principal component 1 (PC1), Dim2=Principal component 2 (PC2).

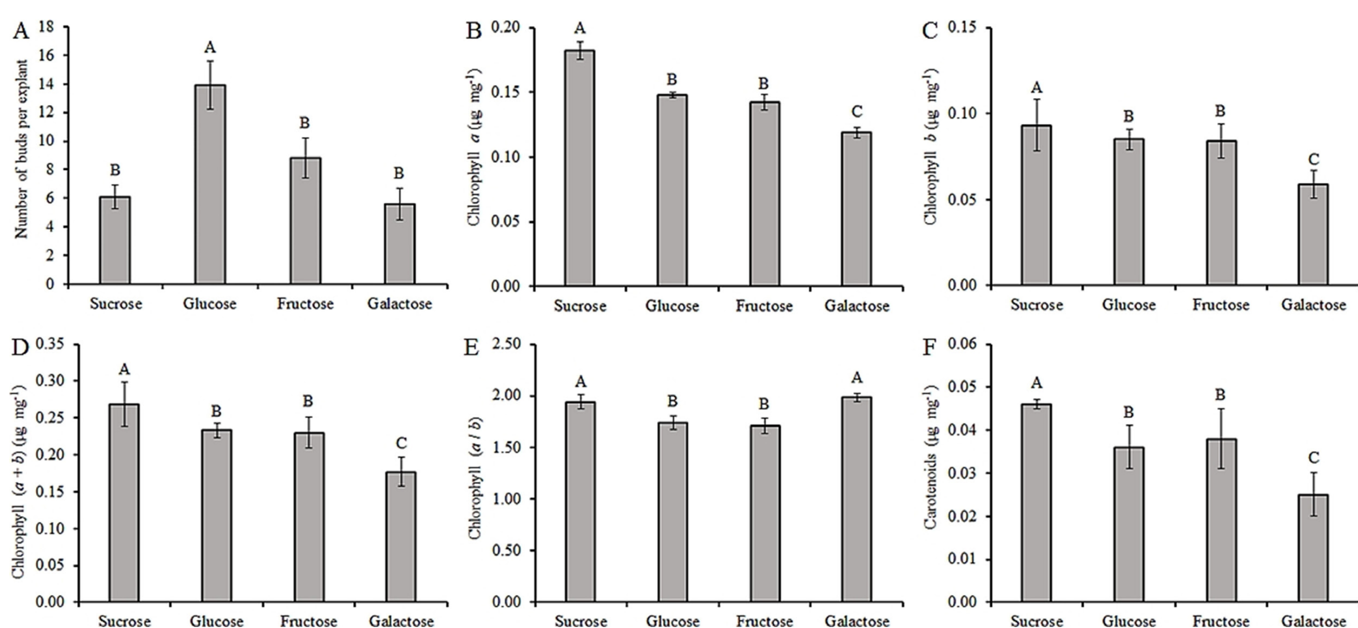

Figure 4. Features of *Eucalyptus pilularis* explant on *in vitro* multiplication stage according to the carbon sources supplemented to the culture medium at 120 days. (A) Number of buds per explant; (B) Chloropyll *a* content; (C) Chloropyll *b* content; (D) Chloropyll (*a*+*b*) content; (E) Chloropyll (*a*/*b*) content; and (F) Carotenoid content. Means with the same letters were not significantly different according to Tukey's test ($P < 0.05$). Data presented as mean ± standard error.

ratio of chlorophyll (*a* / *b*) did not differ significantly from sucrose (1.940) and galactose (1.983) supplementation to the culture medium, which showed the highest relationship when compared to glucose (1.739) and fructose (1.710) (Figure 4E).

3.2. *In vitro* elongation – effect of carbon sources

Significant variations were observed after 110 days of *in vitro* cultivation among all evaluated treatments regarding to different

carbon sources in the elongation stage of *E. pilularis*. The results showed that glucose (65.4% of frequences) and fructose (71.4% of frequences) were associated with score_1, i.e., the lowest means for tissue oxidation (Figure 5A). In contrast, sucrose (55.0%) and galactose (31.8%) presented the lowest frequencies of observations for score_1 (Figure 5A).

Glucose (42.3% of frequences) and fructose (42.9% of frequencies) resulted the highest averages for shoot vigour in score_3, differing from sucrose (5.0% of frequencies) and galactose (22.7% of frequencies) (Figure 4B).

Glucose (7.8 shoots per explant), Fructose (8.7 shoots per explant) and galactose (6.8 shoots per explant) supplementation at culture medium resulted in the highest means for the number of shoots, which were significantly different from the means for sucrose (5.7 shoots per explant) at 110 days of *in vitro* culture (Figure 6A). In contrast, glucose (1.17 cm) and fructose (0.98 cm) produced the highest means for shoot length, which differed significantly from the means obtained with sucrose (0.58 cm) (Figure 6B). Galactose (0.75 cm) supplementation showed intermediate behaviour in relation to shoot length (Figure 6B).

3.3. *In vitro* elongation – effect of glucose concentration and sealing systems

Glucose concentrations and the flask sealing systems tested during the *in vitro* elongation of *E. pilularis* influenced the morpho-physiological responses. WM_0 (100.0% of frequencies), WM_10 (87.5% of frequencies), and 1M_20 (87.5% of frequencies) resulted in the

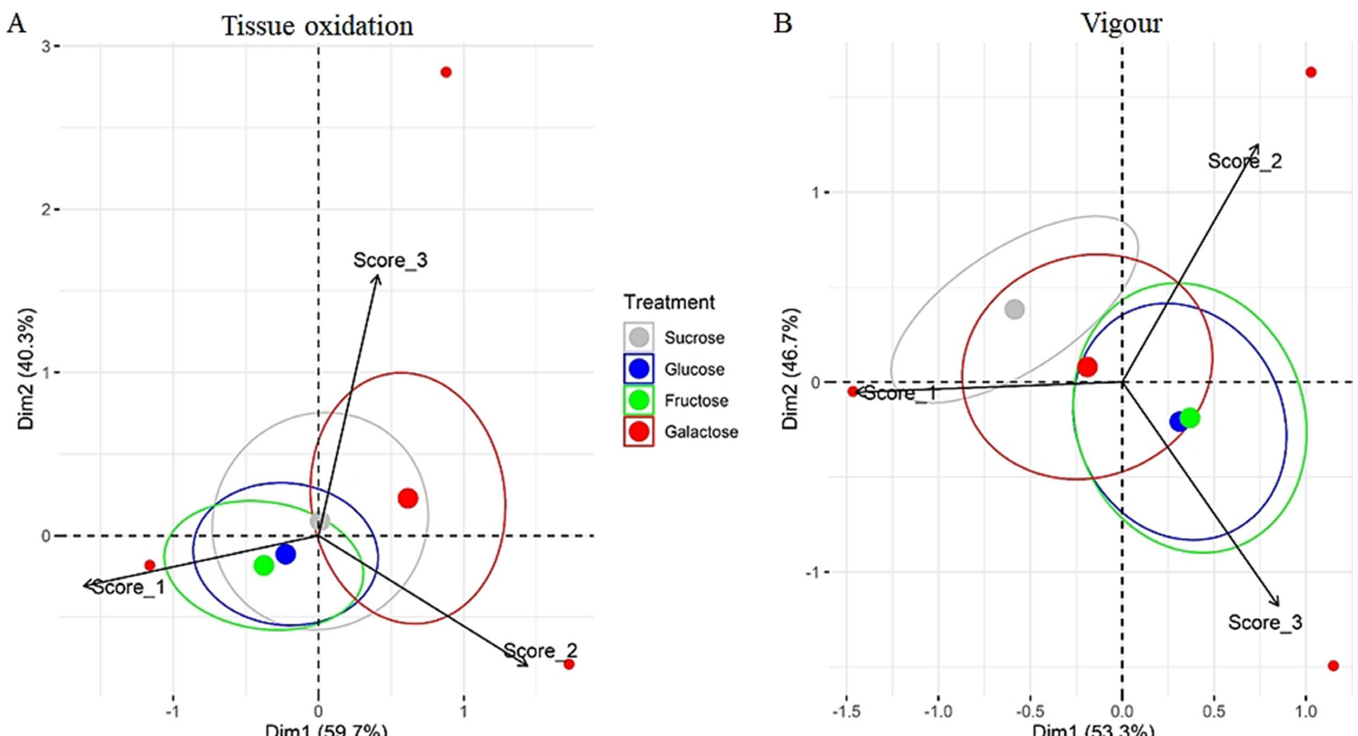

Figure 5. Principal component analysis (PCA) of frequency of tissue oxidation (Figure 1A) and vigour (Figure 1B) based on the scoring scale of *Eucalyptus pilularis* in different carbon sources supplementation at 110 days *in vitro* elongation. (A) PCA for tissue oxidation (Figure 1A); (B) PCA for vigour (Figure 1B). Dim1=Principal component 1 (PC1), Dim2=Principal component 2 (PC2).

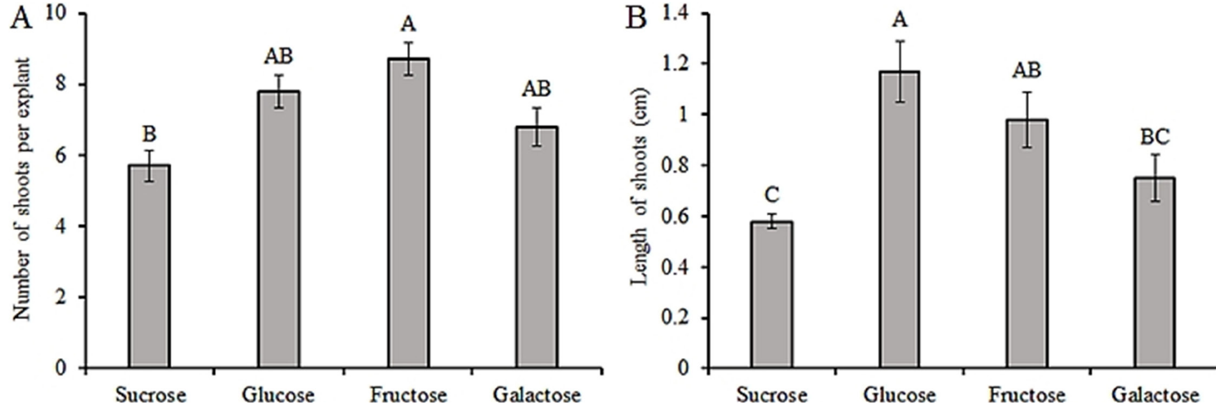

Figure 6. Features of *Eucalyptus pilularis* explant on *in vitro* elongation stage according to the carbon sources supplemented to the culture medium at 110 days. (A) Number of shoots per explant; (B) Length of shoots. Means with the same letters were not significantly different according to Tukey's test ($P < 0.05$). Data presented as mean ± standard error.

reduced oxidation level (Figure 7A). On the other hand, flasks with polyethylene caps without a membrane (WM) at 20 g L^{-1} of glucose (WM_20) resulted in 25.0% of frequencies in score_1 (Figure 7A). 1M_0 (62.5% of frequencies) and 1M_10 (50.0 of frequencies) showed intermediate behaviour in relation to score_1 (Figure 7A).

Supplementation of the culture medium with 20 g L^{-1} of glucose resulted in the highest value for the vigour of the explants (65.5% of frequencies in score_3) when using the 1M sealing system (1M_20) (Figure 7B). The absence of glucose supplementation to the culture medium (WM_0 and 1M_0) was associated with the lowest vigour values (score_1), regardless of the type of sealing (Figure 7B). WM_10, WM_20, and 1M_10 were more associated with scores_1 and 2 for vigour (Figure 7B).

A concentration of 20 g L^{-1} of glucose added to the culture medium resulted in the better number of shoots per explant in the sealing system without a membrane (WM_20), with 9.8 shoots per explant, which was significantly different from the other concentrations (Figure 8A). On the other hand, in the sealing system with a membrane (1M_0,

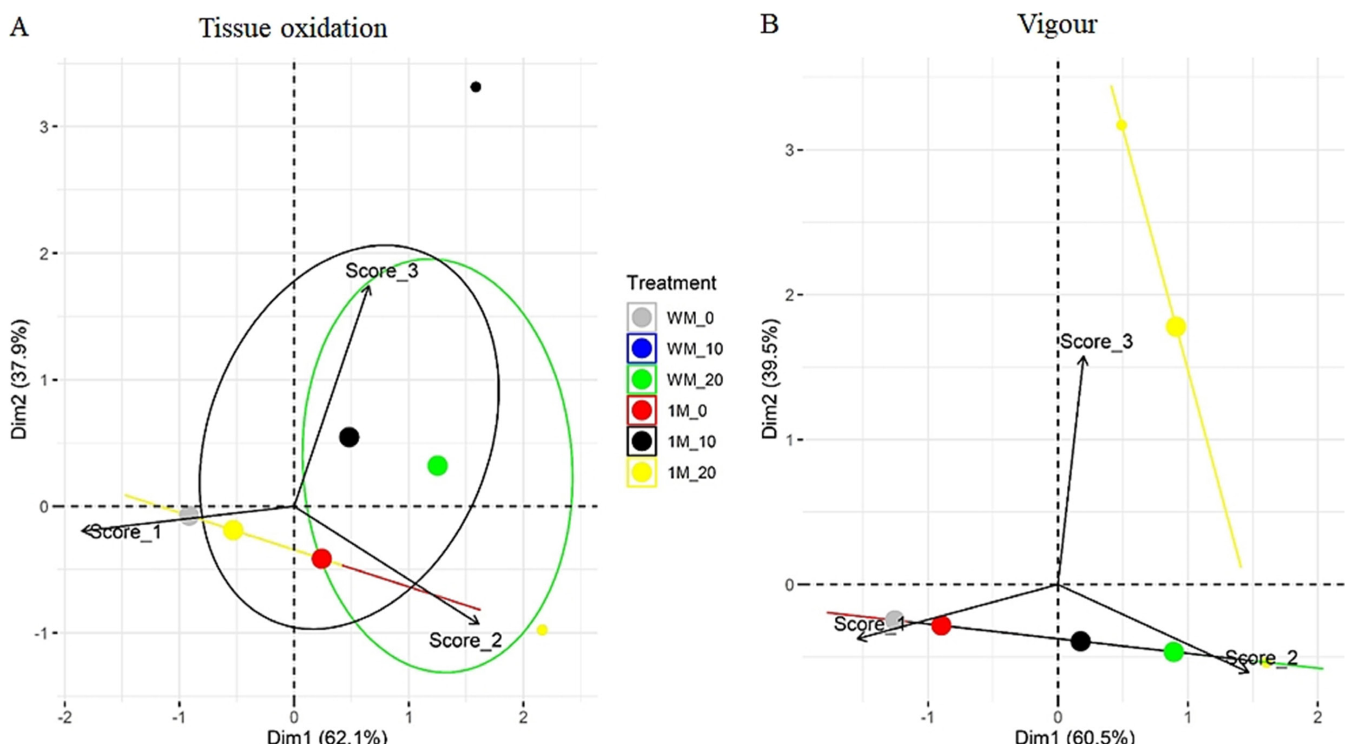

Figure 7. Principal component analysis (PCA) of frequency of tissue oxidation (Figure 1A) and vigour (Figure 1B) based on the scoring scale of *Eucalyptus pilularis* in different carbon sources supplementation at 40 days *in vitro* elongation. (A) PCA for tissue oxidation (Figure 1A); (B) PCA for vigour (Figure 1B). WM_0: rigid polypropylene caps without membrane and absence of glucose supplementation; WM_10: rigid polypropylene caps without membrane and 10 g L^{-1} of glucose supplementation; WM_20: rigid polypropylene caps without membrane and 20 g L^{-1} of glucose supplementation; 1M_0: rigid polypropylene caps with a 1.0-cm-diameter hole covered with a 1.0 cm^2 membrane and absence of glucose supplementation; 1M_10: rigid polypropylene caps with a 1.0-cm-diameter hole covered with a 1.0 cm^2 membrane and 10 g L^{-1} of glucose supplementation; 1M_20: rigid polypropylene caps with a 1.0-cm-diameter hole covered with a 1.0 cm^2 membrane and 20 g L^{-1} of glucose supplementation. Dim1=Principal component 1 (PC1), Dim2=Principal component 2 (PC2).

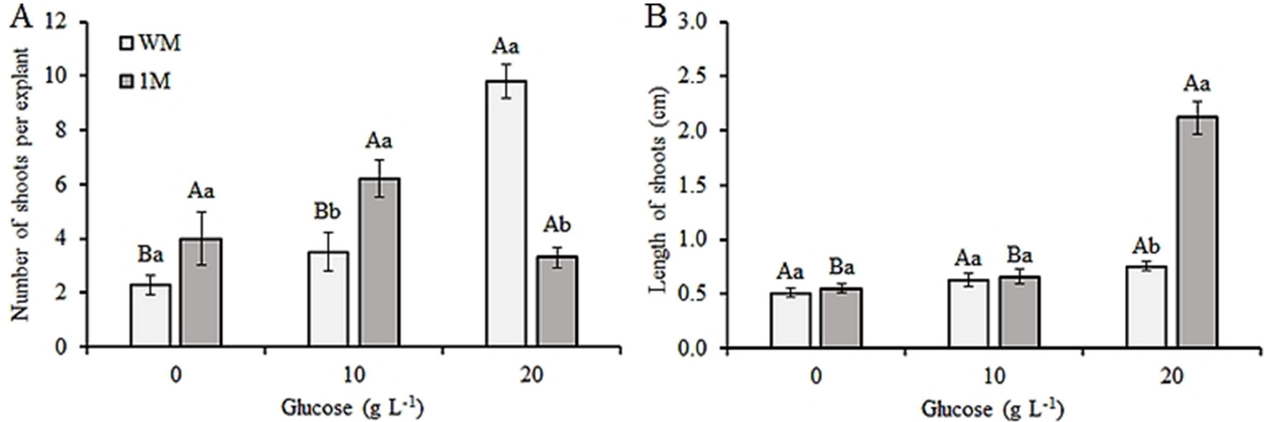

Figure 8. Features of *Eucalyptus pilularis* explants on the *in vitro* elongation stage according to the carbon sources supplemented to the culture medium at 40 days. (A) Number of shoots per explant; (B) Length of shoots. Means followed by the different uppercase letters represent significant differences when comparing different glucose concentration given the same sealing system; and different lowercase letters represent significant differences when comparing different sealing system within the same glucose concentration according to Tukey's test ($P < 0.05$). Data presented as mean ± standard error.

1M_10 and 1M_20), number of shoots per explant was not significantly influenced, regardless of glucose concentration (Figure 8A).

Using the 1M sealing system and adding 20 g L^{-1} of glucose (1M_20) to the culture medium resulted in the longer shoot length per explant (2.1 cm), with significant difference from the of the other concentrations and sealing system tested (Figure 8B).

3.4. *In vitro* adventitious rooting – effect of glucose concentration and sealing systems

In vitro rooting of *E. pilularis* was influenced by the concentrations of glucose and the type of flask sealing systems tested. Flask sealing system without porous membrane (WM) in conjunction with glucose concentrations of 20 g L^{-1} (WM_20) and flasks with one porous membrane (1M) with 10 g L^{-1} of glucose (1M_10) showed greater correlation with score_1 for tissue oxidation (Figure 9A).

The better frequencies for vigour (i.e., score_3) were found in flasks containing 1M associated with 10 g L^{-1} (1M_10 = 25.0% of frequencies) and 20 g L^{-1} (1M_20 = 37.5% of frequencies) of glucose supplemented in culture medium (Figure 9B). WM_0, WM_10, WM_20 and 1M_0 showed a correlation with a low vigour (score_1) (Figure 9B).

There was no interaction between glucose concentrations and sealing system for the percentage of adventitious rooting (Figures 10A-B) and root length per explant (Figures 10C-D), at 120 days of *in vitro* cultivation. However, there was an interaction between factors glucose and sealing system for the number of roots per explant (Figure 10E).

The supplementation of 20 g L^{-1} of glucose at culture medium resulted in the highest means for the rooting percentage (28.8% of rooting) and length of roots per explant (1.0 cm), differing significantly in relation to other treatments (Figures 10A and C).

Adventitious rooting percentage (Figure 10B) and length of roots (Figure 10D) were favoured with the use of flasks with the 1M of sealing system, resulting in 23.1% of rooting and 1.0 cm of length, respectively, and it's mean were significantly different from the observed in flasks without a membrane.

Number of roots per explant, when using a sealing system without a membrane (WM), roots were only observed (1.0 roots per explant) when supplemented with 20 g L^{-1} of glucose (Figure 10E). However, when using one membrane (1M), the supplementation with 10 g L^{-1} (1.0 roots per explant) and 20 g L^{-1} (1.3 roots per explant) of glucose resulted in a better number of roots per explant, denoting high dependence on a carbohydrate source for root production according to the type of sealing (Figure 10E).

4. DISCUSSION

In vitro growth and development of different species during the stages of micro-propagation are affected by several factors, including the type and concentrations of the carbon source (Tormen et al., 2018), the rate of gas exchange and the flask sealing systems (Silva et al., 2017; Souza et al., 2019).

Carbohydrates are included in culture media to maintain the osmotic potential and serve as a source of energy and carbon for various processes, including cell growth, and induction of the *in vitro* organogenesis (e.g., proliferation of shoots and emission of roots), which are dependent on genotype and growth stage (Yaseen et al., 2013; Emara et al., 2018).

While sucrose is a commonly used carbohydrate on *in vitro* studies for shoot induction and development in woody species, its effectiveness

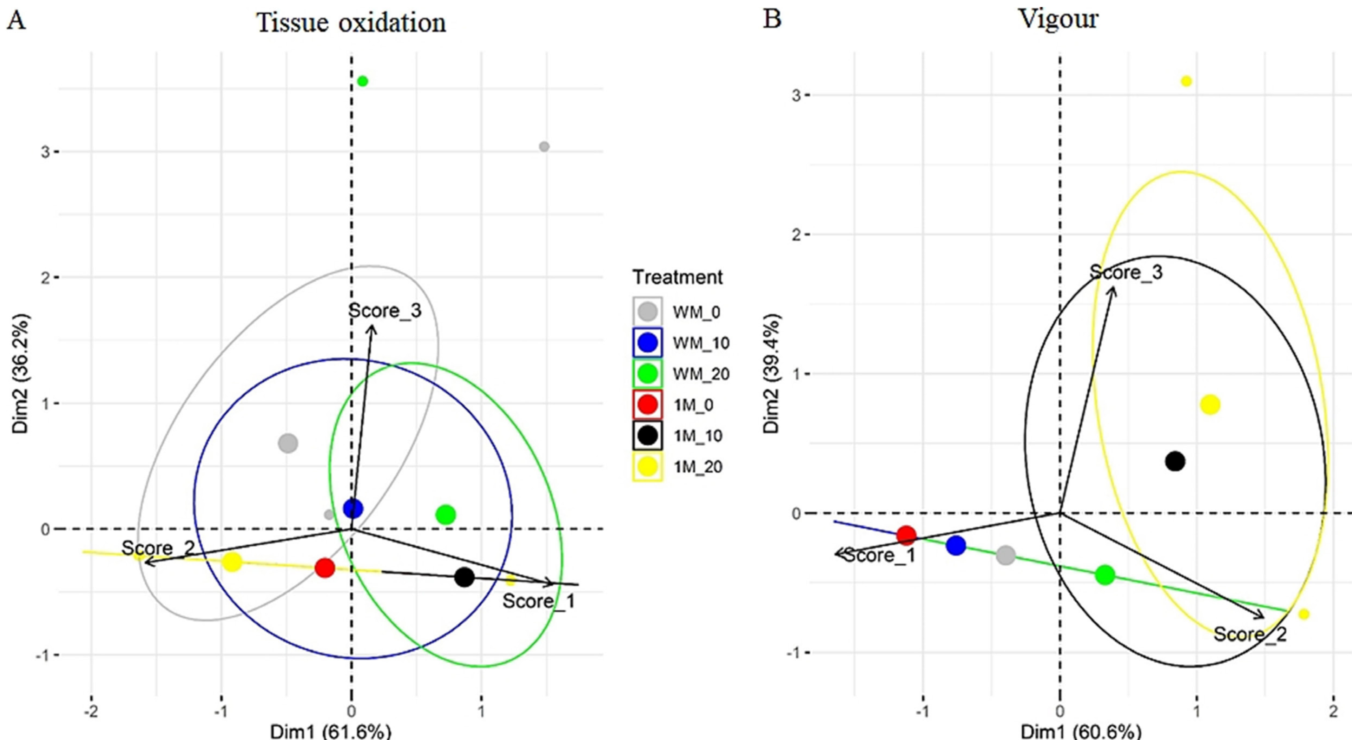

Figure 9. Principal component analysis (PCA) of frequency of tissue oxidation (Figure 1A) and vigour (Figure 1B) based on the scoring scale of *Eucalyptus pilularis* with different carbon sources supplementation at 120 days *in vitro* rooting. (A) PCA for tissue oxidation (Figure 1A); (B) PCA for vigour (Figure 1B). WM_0: rigid polypropylene caps without membrane and absence of glucose supplementation; WM_10: rigid polypropylene caps without membrane and 10 g L^{-1} of glucose supplementation; WM_20: rigid polypropylene caps without membrane and 20 g L^{-1} of glucose supplementation; 1M_0: rigid polypropylene caps with a 1.0-cm-diameter hole covered with a 1.0 cm^2 membrane and absence of glucose supplementation; 1M_10: rigid polypropylene caps with a 1.0-cm-diameter hole covered with a 1.0 cm^2 membrane and 10 g L^{-1} of glucose supplementation; 1M_20: rigid polypropylene caps with a 1.0-cm-diameter hole covered with a 1.0 cm^2 membrane and 20 g L^{-1} of glucose supplementation.Dim1=Principal component 1 (PC1), Dim2=Principal component 2 (PC2).

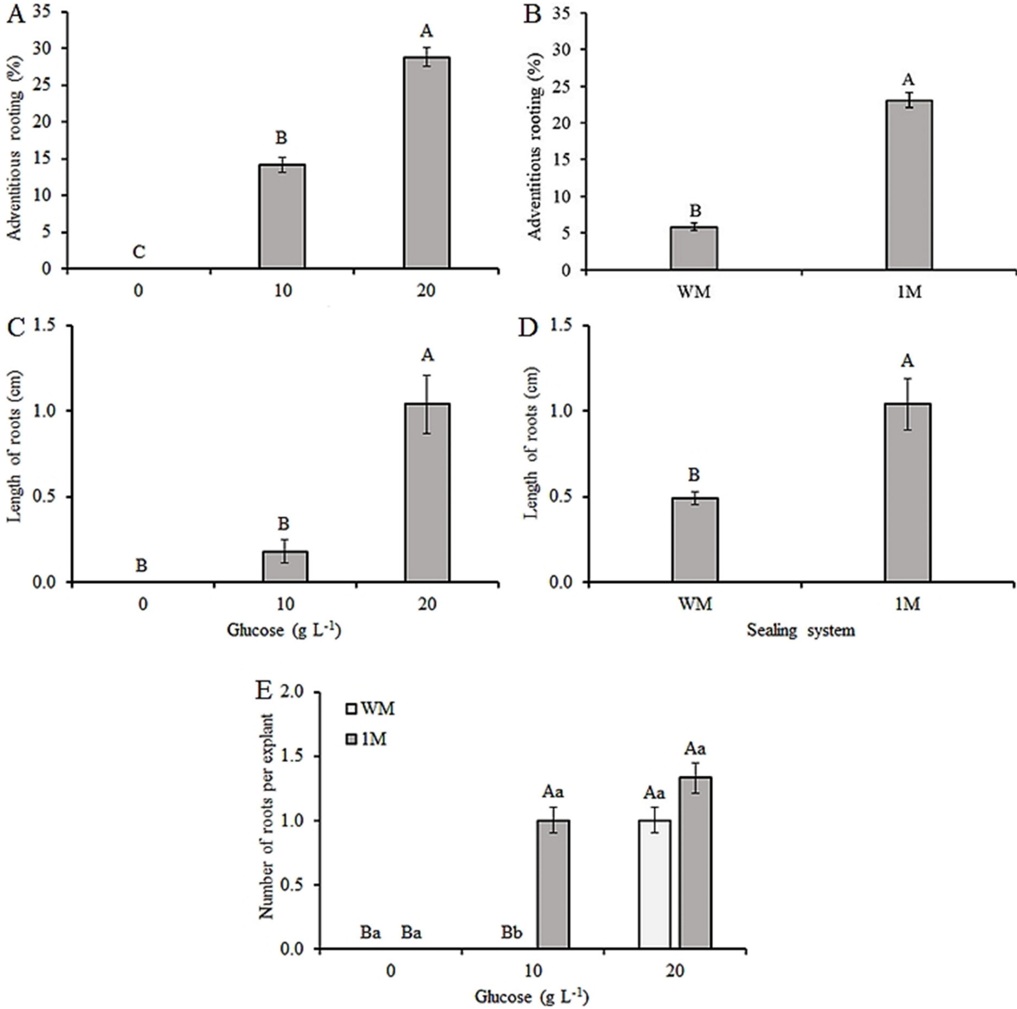

Figure 10. Features of *Eucalyptus pilularis* explants on *in vitro* rooting stage according to the carbon sources supplemented to the culture medium at 120 days. (A) Adventitious rooting according to glucose concentration; (B) Adventitious rooting according to sealing system (WM - rigid polypropylene caps without membrane, and 1M - rigid polypropylene caps with a 1.0-cm-diameter hole covered with a 1.0 cm^2 membrane); (C) Length of roots according to glucose concentration; (D) Adventitious rooting according to sealing system; and (E) Number of roots per explant according glucose concentration and sealing system. In (A-D): Means with the same letters were not significantly different according to Tukey's test ($P < 0.05$). In (E): Means followed by the different uppercase letters represent significant differences when comparing different glucose concentration given the same sealing system; and different lowercase letters represent significant differences when comparing different sealing system within the same glucose concentration according to Tukey's test ($P < 0.05$). Data presented as mean ± standard error.

may vary (Cheong & An, 2015). This fact is justified because sucrose is a nonreducing sugar, which is less reactive than reducing sugars (Carvalho et al., 2013). Our study found that nonreducing sugars resulted in the lowest means for shoot vigour and the number and length of shoots.

In addition, sucrose, a carbohydrate with 12 carbons in its chemical structure, is partially hydrolysed into glucose and fructose, both with 6 carbons, when passing through the autoclave, and the presence of these compounds is essential for plant growth since plant or explant photosynthesis is limited (Bettencourt et al., 2016).

Carbohydrates other than sucrose have been found to be successful as a carbon source on *in vitro* culture; however, their effectiveness varies between different plant species. Glucose, for example, is a reducing sugar easily absorbed by explants and provides a better carbon source for tissue growth by diffusing across excised tissue surfaces and being metabolized during *in vitro* culture (Hartmann et al., 2011). Tormen et al. (2018) found that using a 15 g L^{-1} of glucose concentration in *Eucalyptus cloeziana* resulted in higher means for the number and length of shoots. Although these results were not significantly different from those obtained with a concentration of 15 g L^{-1} of sucrose, there was a reduction in tissue oxidation, as was also observed in *E. pilularis*.

Fructose, a monosaccharide, is another carbohydrate that can produce positive results on *in vitro* cultivation of some species. Preethi et al. (2011) reported that fructose yielded better results than sucrose, maltose, and glucose in *Stevia rebaudiana*.

The use of a culture medium containing 16 g L^{-1} of fructose or glucose instead of a concentration of 30 g L^{-1} of sucrose resulted in the best results in terms of elongation and fresh weight for *Prunus* species (Cheong & An, 2015). Similarly, Harathi et al. (2016) observed the highest means for the number of shoots in *Sphaeranthus indicus* in response to a fructose medium, which supports the results observed for *E. pilularis*. Orlova et al. (2021) also reported a positive effect on *in vitro* multiplication of *Lonicera coerulea* cultivars when sucrose was replaced by glucose at concentrations ranging from 20 to 40 g L^{-1}, as observed in the present study.

Carbohydrates are known to have a repressive effect on the expression of genes involved in photosynthesis and can lead to a decrease in the accumulation of chlorophyll, as reported by Rolland et al. (2006). Sucrose suppression in the culture medium has been shown

to significantly reduce the total chlorophyll levels in *Handroanthus impetiginosus* and *Jacaranda brasiliana* (Souza et al., 2020a).

In vitro multiplication of *E. pilularis* showed higher levels of photosynthetic pigments when using a culture medium supplemented with sucrose; however, sucrose can have negative feedback effects on photosynthesis, reducing the amount and activity of rubisco (Badr et al., 2015). This can affect the plant's ability to store the energy necessary for metabolic activities (Emara et al., 2018) and increase the osmotic potential of the culture medium, making it difficult for plants to capture water *in vitro* (Pérez et al., 2015). As a result, reduced vigour and shoot length may occur during multiplication, and reduced vigour, lower shoot number, and length may occur during *in vitro* elongation.

Conventional *in vitro* cultivation environment differs from the external environment, which can result in physiological and morphological disturbances in the plant (Mendes et al., 2015; Miranda et al., 2024; Souza et al., 2024). Therefore, morphological and physiological adaptations are crucial for ensuring high survival rates and rapid growth of seedlings before *ex vitro* acclimatization. These adaptations include the emission and development of roots, the prevention of water loss through functional stomata (resulting in a low transpiration rate), the improvement of CO_2 assimilation (resulting in a high photosynthetic rate), the production of new leaves and increases in leaf area, and an increase in shoots and root biomass (Hoang et al., 2017; Silva et al., 2022).

Mixotrophic conditions, which involve the use of both organic carbon sources and inorganic carbon dioxide (CO_2), have been shown to be efficient in improving *in vitro* elongation and rooting in photo-autotrophic plants (plants that can obtain their carbon solely from CO_2 through photosynthesis). Several studies have reported the benefits of photo-mixotrophic or mixotrophic conditions on *in vitro* cultivation (Emara et al., 2018; Souza et al., 2019, 2020b). For example, Souza et al. (2019) found that the use of flasks with membranes that allow gas exchange was an efficient alternative for improving *in vitro* elongation and rooting of *Eucalyptus dunnii*, and the absence of carbohydrates in the culture medium was found to be inefficient during these stages. These findings agree with previous studies on *E. pilularis*.

The utilization of flask lids with porous membranes has been found to provide advantageous effects, such as an increase in chlorophyll contents (*a*, *b* and total), as well as enhancements to the photosynthetic system in seedlings of the 'Perolera' pineapple cultivar, as demonstrated by Mendes et al. (2015). Additionally, Fernandes et al. (2013) reported improved shoot length, number of internodes, and shoot dry mass observed in *Tectona grandis* when a sucrose concentration of 18 g L^{-1} and flasks with plastic caps containing a filter were employed.

Micro-environment within the flasks during *in vitro* culture can vary depending on the sealing system employed (Freitas et al., 2021). The conventional method of sealing on *in vitro* propagation involves the use of polyethylene caps that act as barriers, limiting the flow of photosynthetically active photons and restricting gas exchange, thereby increasing relative humidity inside the container (Krisantini & Wiendi, 2018). This, in turn, elevates the concentration of ethylene (Silva et al., 2017) and reduces the concentration of CO_2 (Chen et al., 2019), which can hinder plant growth.

Furthermore, the use of porous membranes in the sealing system can also improve the physical and physiological conditions of the plantlets. The higher gas exchange facilitates the removal of excess water vapor (Miranda et al., 2024), which can reduce the occurrence of fungal and bacterial contamination (Silva et al., 2022). The decrease in relative humidity inside the flask can also contribute to a reduction in hyperhydricity, a common physiological disorder on *in vitro* culture characterized by excessive water absorption and reduced shoot elongation (Miranda et al., 2024; Souza et al., 2024). Overall, using sealing systems with porous membranes can improve the growth and development, resulting in healthier and more vigorous plants.

The use of sealing systems that allow for increased gas exchange has been shown to affect the *in vitro* cultivation of various plant species positively. For example, Ribeiro et al. (2019) observed improved growth and development of *Dendrobium bigibbum* when using a sealing system with porous membranes, which allowed for increased CO_2 concentration and reduced ethylene levels. Similarly, Souza et al. (2019) reported enhanced *in vitro* elongation and rooting of *Eucalyptus dunnii* when using flasks with membranes that allow gas exchange. Molinari et al. (2021) also found that the use of a sealing system with a porous membrane improved shoot growth, biomass production, and photosynthetic activity in *Eucalyptus grandis* × *Eucalyptus urophylla*.

Utilizing a membrane system with one or two filters to promote natural ventilation has resulted in improved rooting of *Plectranthus amboinicus*. Furthermore, for better growth, apical segments of this species should be cultured under natural ventilation with a filter, while nodal segments should be cultured with two filters (Silva et al., 2017).

In the case of *E. pilularis*, the absence of carbohydrates did not lead to *in vitro* rooting. In contrast, the utilization of sealing systems with lids containing 1M facilitated the emission of adventitious roots, which corroborates the findings in the *in vitro* rooting of *Eucalyptus dunnii* micro-cuttings (Souza et al., 2019).

5. CONCLUSION

Micro-propagation technique was efficient for the vegetative propagation of a *E. pilularis* selected tree in 390 days, considering that the source of carbohydrates, glucose concentration, and gas exchange system play determining roles in obtaining clonal microplantlets.

Glucose resulted in better responses to *in vitro* multiplication and elongation when compared to sucrose, fructose and galactose.

Supplementation at culture medium with 20 g L^{-1} of glucose on the *in vitro* elongation stage, combined with sealing system of 1M, resulted in the better means for the evaluated features.

Supplementation at culture medium with 20 g L^{-1} of glucose and sealing system of 1M favoured the rhizogenic process.

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AUTHOR CONTRIBUTIONS

MLMA: conducting the experiment, statistical analysis, writing, and discussion; KIRS, DML, DMSCS, LSO: statistical analysis, writing, discussion, review, and interpretation of results; and GEB: supervisor, was responsible for the obtention of funds and the administration of project, writing, review, and discussion. All authors revised the manuscript and approved the final version.