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Ontogenetic age and inoculation methods for the *in vitro* establishment of *Eucalyptus pilularis* Smith

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ABSTRACT: We aimed to evaluate the *in vitro* establishment of nodal segments of *Eucalyptus pilularis* Smith considering two origins of tissues (Or_1 - epicormic shoots collected from pruned branches of selected adult trees; Or_2 - shoots collected from seminal mini-stumps) and four inoculation methods (Me_1 - culture medium supplemented with 0.5 g L^{-1} activated charcoal; Me_2 - culture medium supplemented with 800 mg L^{-1} PVP30; Me_3 - exposure to light for 30 days; Me_4 - exposure to dark for 7 days). At 30 days after the *in vitro* inoculation of tissues, there was no establishment of tissues from epicormic shoots (Or_1). Or_2 resulted in lower percentages of tissue oxidation and contamination by microorganisms, in addition to having presented establishment and formation of shoots. Me_1 resulted in a lower mean tissue oxidation, although it differed statistically only from Me_4. An origin of the tissues of ontogenetic age was a determining factor for the successful *in vitro* establishment of *E. pilularis*. The use of the Or_2 origin and the Me_1, Me_2, and Me_3 methods are recommended to reduce phenolic oxidation of tissues in the *in vitro* establishment.
Keywords: antioxidant agents; exposure to light; epicormic shoots; tissue oxidation.

Idade ontogenética e métodos de inoculação para o estabelecimento *in vitro* de *Eucalyptus pilularis* Smith

RESUMO: Objetivou-se avaliar o estabelecimento *in vitro* de segmentos nodais de *Eucalyptus pilularis* considerando duas origens de tecidos (Or_1 - brotos epicórmicos coletados em galhos podados de matrizes adultas e Or_2 - brotos coletados de minicepas seminais) e quatro métodos de inoculação (Me_1 - meio de cultura suplementado com 0,5 g L^{-1} de carvão ativado, Me_2 - meio de cultura suplementado com 800 mg L^{-1} de PVP30, Me_3 - exposição à luminosidade por trinta dias e Me_4 - exposição em ambiente com ausência de luminosidade por sete dias). Aos 30 dias após a inoculação *in vitro* dos tecidos, constatou-se que não houve estabelecimento de tecidos oriundos da Or_1. A utilização de segmentos nodais provenientes da Or_2 resultou em menores percentuais de oxidação e de contaminação por microrganismos, além de ter apresentado estabelecimento e emissão de brotos. O uso do Me_1 resultou em menor média de oxidação, embora tenha diferido estatisticamente somente do Me_4. A origem dos tecidos associada à idade ontogenética foi um fator determinante para o sucesso do estabelecimento *in vitro* de *E. pilularis*. Recomenda-se a utilização da Or_2 e dos métodos Me_1, Me_2 e Me_3 a fim de reduzir a oxidação fenólica dos tecidos durante o estabelecimento *in vitro*.
Palavras-chave: agentes antioxidantes; exposição à luminosidade; brotos epicórmicos; oxidação de tecidos.

1. INTRODUCTION

Eucalyptus pilularis Smith is a tree species that stands out in Australia for its rapid growth and its excellent-quality wood (MOURA, 2001). It is found mainly in the coastal plains and mountainous coastal areas of the state of New South Wales to the south of Queensland (latitudes between 25°50’ and 37°50’ (FONSECA et al., 2010).

In Brazil, despite the potential that the species has for sawmilling and laminating, especially in the south-eastern region of the country (Moura et al. 1980), there are few planted areas of it (CASTELLANO et al., 2013), and studies on its forestry and vegetative propagation are still scarce. However, genetic variation is observed within the species, so it is possible to explore this variation to obtain superior genotypes and, later, rescue them for cloning in order to create artificial forests (SILVA et al., 2015).

Several techniques have been used for the vegetative propagation of eucalypts species, including micropropagation, which allows the *in vitro* conservation of germplasm, the acceleration of breeding programmes through mass production of selected genotypes, clonal cleaning, and the possibility of rejuvenating and reinvigorating tissues of selected plants in the adult phase (WENDLING et al., 2014). The success of *in vitro* culture depends on several factors, including those related to the tissue, the degree of juvenility of the vegetative propagule (ontogenetic age), the level of contamination control, and the vigour (physiological age) of the plant from which the tissues

originate (WENDLING et al., 2014; BACCARIN et al., 2015; OLIVEIRA et al., 2015). *In vitro* establishment is one of the limiting steps of micropropagation, which seeks to obtain contamination-free tissues to continue with the other stages through the induction of new meristems (TRUEMAN et al., 2018). Phenolic oxidation is another recurrent challenge to *in vitro* propagation, especially of woody species, and may reduce growth and development or even kill the tissue, thus preventing or hindering establishment (OLIVEIRA et al., 2015; BACCARIN et al., 2015).

The use of antioxidants in the culture medium, such as activated charcoal and polyvinylpyrrolidone (PVP), and different incubation conditions regarding lighting are ways to reduce tissue oxidation and contamination by microorganisms, making a successful *in vitro* establishment more likely (FAGUNDES et al., 2017; LENCINA et al.; 2018; GOLLE et al., 2020). However, there is a need to define the ideal asepsis conditions for each species according to the origin of the tissues.

Thus, the objective of the present study was to evaluate the *in vitro* establishment of nodal segments of *Eucalyptus pilularis* considering two tissue origins and four inoculation methods.

2. MATERIALS AND METHODS

2.1. Study site and experimental material

The experiment was conducted at the Forest Nursery and the Laboratory of *In Vitro* Culture of Forest Species, both belonging to the Department of Forestry Sciences of the Federal University of Lavras (UFLA), Lavras, Minas Gerais, Brazil.

The experimental materials used to obtain the explants (1.5-cm-long nodal segments containing an axillary bud and no leaves) were derived from two tissue sources: Or_1 - epicormic shoots collected from pruned branches of 46-year-old *Eucalyptus pilularis* mother plants (Figure 1A); and Or_2 - shoots collected from 1-year-old seminal mini-stumps established in a mini-garden (Figure 1B), both from a test of *Eucalyptus* and *Corymbia* species, established in 1974, at the UFLA Forest Nursery (IPEF, 1984).

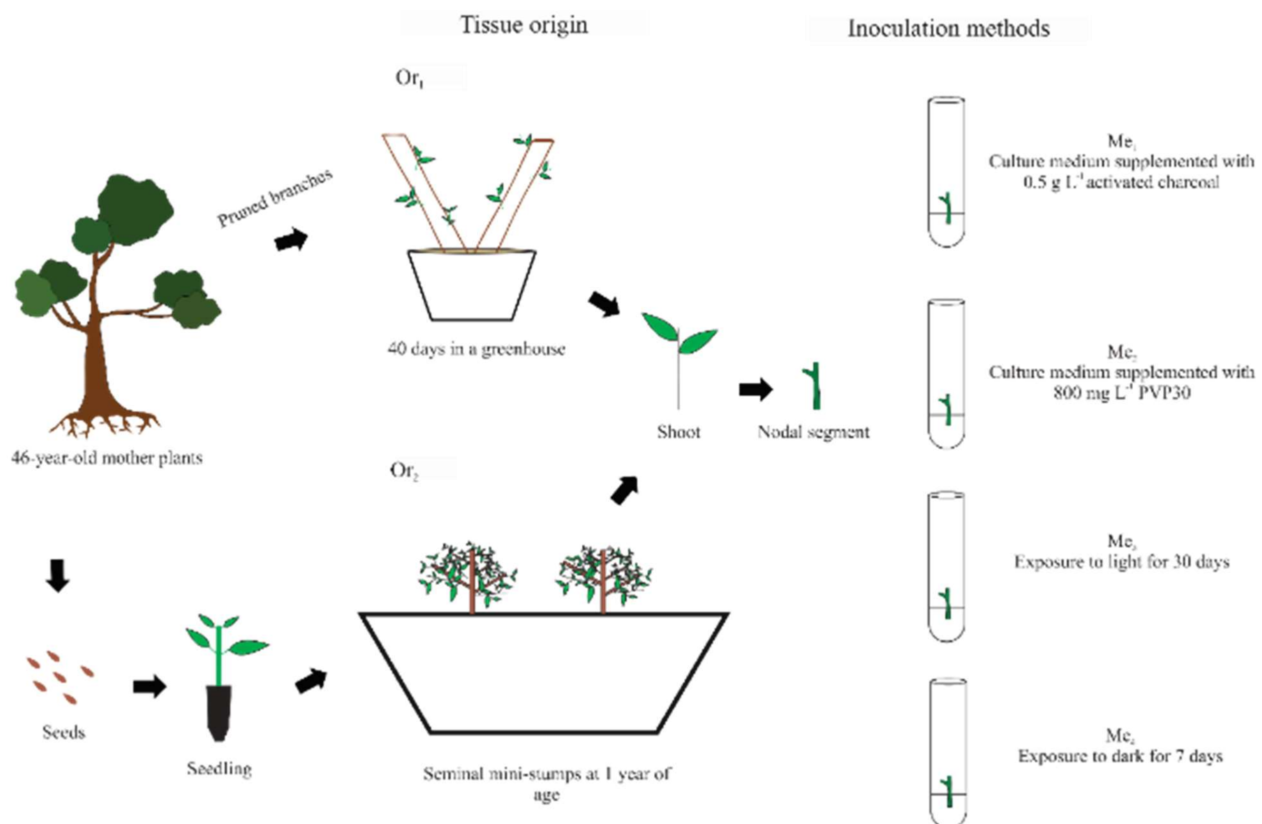

Figure 1. Flowchart of the process for obtaining propagules of different origins, and the different inoculation methods used for the *in vitro* establishment of nodal segments of *Eucalyptus pilularis*.

Figura 1. Fluxograma do processo para obtenção de propágulos de diferentes origens e diferentes métodos de inoculação utilizados para o estabelecimento *in vitro* de segmentos nodais de *Eucalyptus pilularis*.

2.2. Collection and preparation of branches for inducing the formation of epicormic shoots

The mother plants used for pruning the branches were selected based on visual criteria. We preferred the straightest stem possible, free of pathogen attacks, and with branches located in the lower portion of the canopy to minimize the effects of tissue maturity and facilitate the cutting and collection of branches. At the end of September 2019, the branches were sectioned to 0.50 m length and placed in a climate-controlled greenhouse with relative air humidity higher than 80% and air temperature between 20 °C and 35 °C. The plants were humidified by an intermittent nebulization system with a high-pressure and low-flow nebulizer, automatically controlled by a humidistat. The branches were arranged vertically in polyethylene pots (5 L) that were filled with washed sand, without fertilization, to induce the formation of epicormic shoots (Figure 1A). After 40 days of permanence of the branches in a greenhouse,

epicormic shoots of 3 to 5 cm were collected, immersed in autoclaved deionized water, and transported to the laboratory.

2.3. Collection of shoots from seminal mini-stumps

The mini-stumps were obtained from seedlings produced from seeds that were collected from the test of *Eucalyptus* and *Corymbia* species located in the Forest Nursery of the Federal University of Lavras (IPEF, 1984) and were established in a seminal mini-garden under a semi-hydroponic system in raised beds filled with sand at the Forest Nursery of the Federal University of Lavras (UFLA), Lavras, Minas Gerais. Shoots were collected from the mini-stumps and were used to prepare explants for *in vitro* establishment.

The nutrient solution, composed of calcium nitrate (0.920 g L^{-1}), potassium chloride (0.240 g L^{-1}), potassium nitrate (0.140 g L^{-1}), monoammonium phosphate (0.096 g L^{-1}), magnesium sulfate (0.364 g L^{-1}), water-soluble iron (0.040 g L^{-1}), boric acid (2,800 mg L^{-1}), zinc sulfate (0.480 mg L^{-1}), manganese sulfate (1,120 mg L^{-1}), copper sulfate (0.100 mg L^{-1}), and sodium molybdate (0.040 mg L^{-1}), was applied by dripping, four 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 mS m^{-2}.

Shoots 10 cm in length were collected from the mini-stumps in a seminal mini-garden (Figure 1B) 15 days after pruning performed below the terminal meristem, aiming to break the apical dominance and form axillary shoots. Then, they were immersed in autoclaved deionized water and transported to the laboratory.

2.4. *In vitro* establishment

For both the pruned branches and the seminal mini-stumps, 48 h before the collection of shoots, a dimethyl 4,4'-(*o*-phenylene)bis(3-thioallophanate) fungicide was applied at a concentration of 0.5 g L^{-1}. From the shoots collected (Or_1 and Or_2), the nodal segments were standardized to two axillary buds, without leaves and 1.5 cm length. The nodal segments (explants) were washed under running water for 5 min and then immersed in 70% alcohol solution (v/v) for 30 s with constant agitation inside a horizontal laminar flow hood. Then they were immersed in NaOCl solution (1.00-1.25% active chlorine) for 15 min. After each immersion in alcohol or NaOCl, the nodal segments were washed three times with autoclaved deionized water. Then they were immersed in Orthocide 500® fungicide solution (50% captan as the active ingredient; 0.5 g L^{-1}) before inoculation. After asepsis, the explants were inoculated vertically under aseptic conditions in 15 cm × 2.5 cm glass test tubes containing 10 mL of WPM culture medium (LLOYD; MCCOWN, 1981). The time from the collection of shoots under field conditions to inoculation in culture medium was less than 2 hours. During collection, transport, and the intervals between disinfection and inoculation, the explants were kept immersed in autoclaved deionized water to avoid dehydration and preserve the turgidity of the tissues.

The culture medium was supplemented with 20 g L^{-1} sucrose and 6 g L^{-1} agar and was prepared using deionized water. The pH of the culture medium was adjusted to 5.8 ± 0.05 with NaOH (0.1 M) and HCl (0.1 M), before autoclaving and adding agar. Autoclaving of the culture medium was performed at a temperature of 127 °C and pressure of 1.5 kgf cm^{-2} for 20 min.

After inoculation, the explants were kept in a growth room at 24 °C ± 1 °C under a 16-h photoperiod and 40 µmol m^{-2} s^{-1} irradiance (quantified by a LI-250A Light Metre radiometer, LI-COR®).

2.5. Experimental design and evaluations

The experiment was conducted in a completely randomized design with a 2 × 4 factorial arrangement. Two tissue origins were tested (Or_1 - epicormic shoots collected from pruned branches of 46-year-old mother plants; Or_2 - shoots collected from 1-year-old seminal mini-stumps) and four inoculation methods (Me_1 - culture medium supplemented with 0.5 g L^{-1} of activated charcoal; Me_2 - culture medium supplemented with 800 mg L^{-1} PVP30; Me_3 - exposure to light for 30 days; Me_4 - exposure to dark for 7 days). At 30 days after inoculation, the percentage of tissue oxidation, percentage of unresponsive explants (explants with green colour and no oxidation, but absence of bud and shoot formation), rate of fungal and/or bacterial contamination, establishment percentage (explants free of contamination and oxidation and that formed shoots), and the number of shoots formed per explant were calculated (Figure 2A-D).

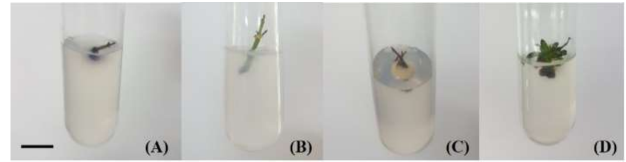

Figure 2. Details of the characteristics evaluated during the *in vitro* establishment of explants (nodal segments) from shoots of *E. pilularis* seminal mini-stumps. A) Explant representing oxidized tissue; B) Unresponsive explant; C) Contaminated explant; D) Explant *in vitro* established. Bar = 1 cm.

Figura 2. Detalhes das características avaliadas durante o estabelecimento *in vitro* de explantes (segmentos nodais) provenientes de brotações de minicepas seminais de *Eucalyptus pilularis*. A) Explante oxidado; B) Explante não-responsivo; C) Explante contaminado; D) Explante estabelecido *in vitro*. Barra = 1 cm.

2.6. Data analysis

The analyses were performed in R Core Team software (2018). The variables that did not have a normal distribution according to the Shapiro-Wilk test ($p > 0.05$) but did not show homogeneity of variances according to the Bartlett test ($p > 0.05$) were arcsin-transformed. The means of the treatments were subjected to analysis of variance (ANOVA, $p < 0.05$) and compared by the Duncan test ($p < 0.05$).

3. RESULTS

The ontogeny of the tissues and the inoculation methods tested in the *in vitro* establishment of nodal segments of *Eucalyptus pilularis* influenced the morphophysiological responses of the tissues in the evaluated characteristics (Figure 3). The two inputs showed no interaction.

Comparing phenolic oxidation between the two tissue origins, the shoots from seminal mini-stumps (Or_2) had the lowest means (27.9%), differing statistically from epicormic shoots (66.2%) (Figure 3A). Regarding the inoculation methods, incubation for 7 days in the dark (Me_4) resulted in significantly higher means of tissue oxidation (67.7%) than the other methods (Figure 3B).

The rate of unresponsive explants (Figure 3C) was affected only by inoculation method. Exposure to the dark for 7 days (Me_4) and the presence of PVP30 in the culture medium (Me_2) resulted in the lowest means (26.5%), differing statistically from Me_3 (55.9%).

The origin of the tissues significantly affected in fungal and/or bacterial contamination, *in vitro* establishment, and number of shoots per explant. The inoculation of Or_2 shoots resulted in a lower mean contamination rate (54.4%) (Figure 3D) and higher mean establishment percentage (33.8%) (Figure 3E) and mean number of shoots per explant (0.6 shoots) (Figure 3F). It was not possible to establish nodal segments from epicormic shoots with high ontogenetic age (Or_1).

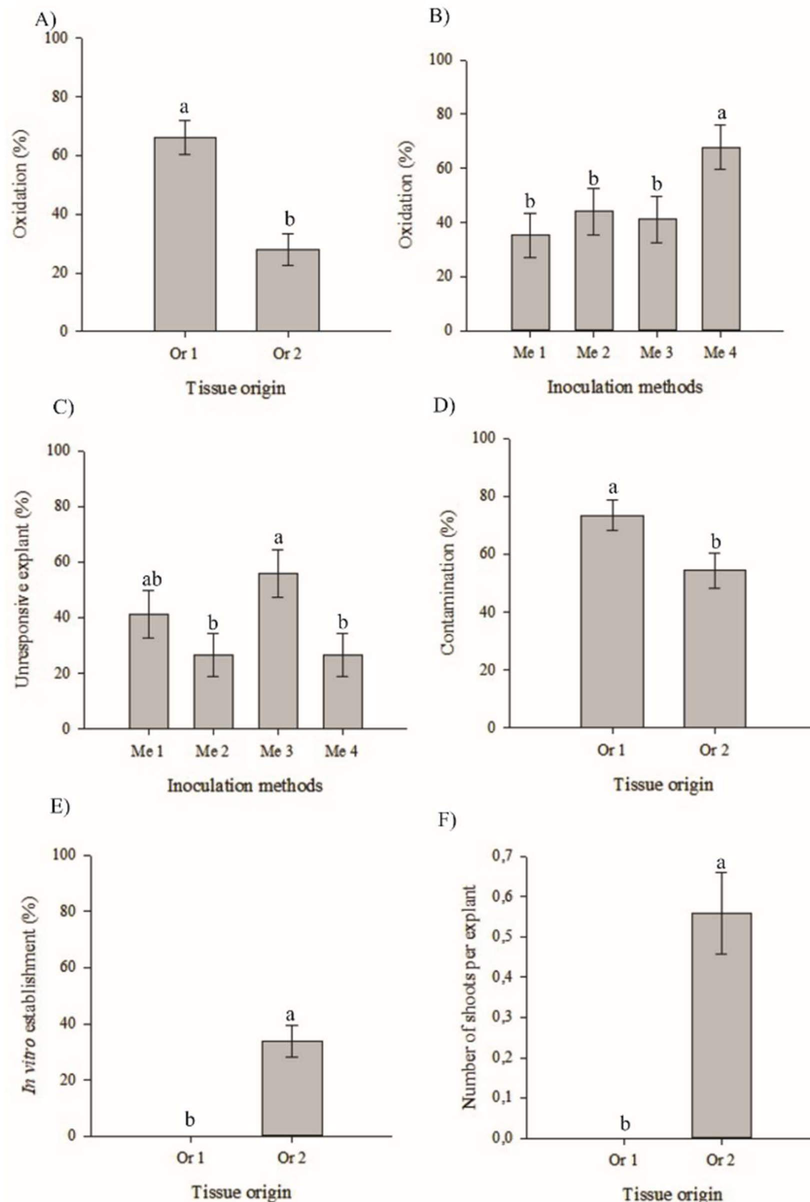

Figure 3. Characteristics evaluated in the *in vitro* establishment of nodal segments of *Eucalyptus pilularis* from two tissue sources (Or_1 and Or_2) and subjected to four inoculation methods (Me_1, Me_2, Me_3, and Me_4). A) Oxidation (%) as a function of the origin of the tissues; B) Oxidation (%) as a function of inoculation method; C) Unresponsive explants (%) as a function of inoculation method; D) Fungal and/or bacterial contamination (%) as a function of tissue origin; E) *In vitro* establishment (%) as a function of tissue origin; F) Number of shoots per explant as a function of the origin of the explants. The lowercase letters (a, b) above the bars represent significant differences between treatments according to the Duncan test at 5% significance.

Figura 3. Características avaliadas no estabelecimento *in vitro* de segmentos nodais de *Eucalyptus pilularis* provenientes de duas origens de tecidos (Or_1 e Or_2) e submetidos a quatro métodos de inoculação methods (Me_1, Me_2, Me_3, e Me_4). A) Oxidação (%) em função da origem dos tecidos; B) Oxidação (%) em função do método de inoculação; C) Explantes não-responsivos (%) em função do método de inoculação; D) Contaminação fúngica e/ou bacteriana (%) em função da origem dos tecidos; E) Estabelecimento *in vitro* (%) em função da origem dos tecidos; F) Número de brotos por explante em função da origem dos explantes. As letras minúsculas (a, b) acima das barras representam diferenças significativas entre os tratamentos de acordo com o teste de Duncan a 5% de significância.

4. DISCUSSION

The use of antioxidants, such as PVP and activated charcoal, is one of the methods indicated for the control of tissue oxidation, especially in woody species (AHMAD et al., 2013). In this context, the use of activated charcoal (Me_1) or PVP30 (Me_2) in the culture medium or exposure to light for

30 days (Me_3) reduced the oxidation of *E. pilularis* tissues. The adsorption of phenolic compounds and toxic substances by activated charcoal during the *in vitro* culture of woody species has the advantage of reducing tissue oxidation, which can improve and regulate the *in vitro* growth under certain conditions (FAGUNDES et al., 2017; LENCINA et al., 2018), corroborating the results of the present study. However, few studies have reported its effects according to ontogenetic age. Here, tissue with a more advanced ontogenetic age probably exuded more phenolic compounds in the culture medium, which may have favoured oxidation, since younger explants are less prone to oxidation than more mature explants (Paiva; Paiva, 2001).

PVP is a polyamide that prevents oxidation and polymerization of phenolic compounds and is selective for this type of substance (ZHOU et al., 2010). The use of the antioxidant effectively reduced phenolic oxidation in *E. pilularis*, as also observed by Silveira et al. (2016) in *Calophyllum brasiliense* (Cambess.). However, Sartor et al. (2013) observed that the use of PVP as an antioxidant for *Dalbergia nigra* (Vell.) led to greater oxidation of explants. These differences show that the responses found for a given trait are influenced by the antioxidant agent and by the species studied.

Miranda et al. (2019), studying the *in vitro* establishment of *Eremanthus incanus*, observed that PVP and activated charcoal had non-significant effects on the oxidation percentage of the explants, and values of 50-60% were observed, which were higher than those found in the present study for *E. pilularis*. In *Rubus idaeus* L., there was an increase in oxidation in the cultivars as the dose of activated charcoal in the culture medium increased (FAGUNDES et al., 2017). The increase in the concentration of an antioxidant can be harmful by adsorbing other substances from the nutrient medium, causing undesirable effects on *in vitro* culture (GALDIANO-JÚNIOR et al., 2010). Some studies report the efficiency of supplementation of the culture medium with activated charcoal and PVP or the combination of both in reducing phenolic oxidation, as observed in nodal segments of *Eugenia pyriformis* (ASSIS et al., 2017) and *Psidium guajava* (AGUILAR et al., 2016), corroborating the results found for *E. pilularis*.

Alfenas et al. (2009) indicate that, in addition to the use of antioxidants, incubation of explants in the dark for 5-7 days may reduce oxidation. According to Termignoni (2005), plants with high tissue oxidation should be kept in the dark immediately after inoculation in the culture medium, where they should remain for one week before being exposed to light. The absence of light during the culture period of *Eugenia involucrata* DC. reduced phenolic oxidation and favoured the development of callogenic structures (GOLLE et al., 2020). However, the inoculation method of exposure of the tissues to the dark for 7 days (Me_4) was not efficient in reducing the phenolic oxidation in the tissues of nodal segments, resulting in the highest percentage of oxidation (67.6%), corroborating the results observed by Miranda et al. (2019) in *Eremanthus incanus*.

Although Me_4 resulted in the lowest percentages of unresponsive explants (26.5%), i.e., explants that showed green colour, absence of oxidation, but absence of bud and shoot formation, this does not imply that the other explants produced buds and did not oxidize, given that the treatment generated many oxidized explants. Therefore, the high percentages of phenolic oxidation in the group left in the dark for 7 days may have resulted in tissue death.

Regarding the contamination percentages, Fagundes et al. (2017) observed that the largest losses due to fungal contamination in *Rubus idaeus* L. were found in culture medium without the addition of activated charcoal, but the use of antioxidant doses did not promote significant differences. Regarding the number of shoots, in *Apuleia leiocarpa*, activated charcoal increased the length of shoots and the number of microcuttings per shoot and per micro-stump (LENCINA et al., 2018).

Here, in *E. pilularis*, the inoculation method did not influence the *in vitro* responses of contamination, *in vitro* establishment, or number of shoots per explant. However, the differences found between the two tissue origins for these traits may be related to the degree of tissue juvenility, the origin of the explants, and the physiological and phytosanitary state of the mother plant (WENDLING et al., 2014; OLIVEIRA et al., 2015; BACCARIN et al., 2015).

The mother plant exerts great influence on the exudation of phenolic compounds by the *in vitro* tissues, which is dependent on the genotype, the development phase of the plant, and the season of the year in which the tissues are collected (WERNER et al., 2009). The ontogenetic age of the tissues can be altered by *ex vitro* and *in vitro* methods that accelerate or delay maturation or induce juvenility (WENDLING et al., 2014) and the nutritional, water, phytosanitary, and light conditions under which the plants are grown may alter the physiological age of the tissues (WENDLING et al., 2014).

In this context, the complete reversal of maturation that occurs through meiosis, gametogenesis, and formation of the zygotic embryo, induction of juvenility in mature clones by cultural treatments and reduction of ontogenetic age characterize tissue rejuvenation (WENDLING et al., 2014). On the other hand, the reduction in physiological age, i.e., the increase in tissue vigour, characterizes reinvigoration (WENDLING et al., 2014). The ideal management of a mini-garden in terms of mineral nutrition, for example, can make propagules more predisposed to rooting, as observed by Lopes et al. (2016) for *Eucalyptus urophylla*. A plant with a balanced nutritional status generates propagules with carbohydrates, auxins, and metabolic compounds that are essential for the initiation of the rhizogenic process and formation of adventitious roots (CUNHA et al., 2009), components that may also favour *in vitro* establishment.

Explants from seminal mini-stumps have a younger ontogenetic age (higher tissue juvenility) than explants from epicormic shoots of 46-year-old trees (lower tissue juvenility) according to the tissue maturation gradient (ontogenetic age) (BACCARIN et al., 2015; OLIVEIRA et al., 2015). In addition, the phytosanitary, nutritional, water, and light conditions (physiological age) to which mini-stumps are subjected can be more easily controlled than the natural conditions, which are a source of microorganisms to which trees in the field are exposed. Thus, the inoculation of explants from mini-stumps can favour the vigour of tissues as linked to physiological age, enabling reinvigoration (WENDLING et al., 2014), a lesser release of phenolic compounds as linked to ontogenetic age, the ability to form shoots, and lower percentages of fungal and/or bacterial contamination, as observed in the present study. For this reason, the *in vitro* establishment of explants from *E. pilularis* epicormic shoots may have been compromised by the high percentages of contamination by microorganisms and tissue oxidation when compared to explants originated from shoots

of seminal mini-stumps. This result indicates that the juvenility factor should be considered for *in vitro* establishment when cloning selected trees by micropropagation.

However, the temperatures and rains accumulated during the pruning of branches and in the collection of epicormic shoots may have influenced the ability to emit shoots and the *in vitro* establishment of tissues (Oliveira et al., 2015). According to Avelar et al. (2020), *Eucalyptus pilularis* emitted the highest number of total epicomic shoots (219) at 45 days in the greenhouse and resulted in one of the highest percentages of *in vitro* establishment (60%). Differences in seasonality at the time of installation of the experiments may have influenced the emission of shoots on the branches and the morphophysiological responses in the *in vitro* establishment, however other combined factors may have induced the results found in the present study.

5. CONCLUSION

The use of nodal segments collected from shoots of *E. pilularis* (Or_2) seminal mini-stumps showed the best results because they had less tissue oxidation and fungal and/or bacterial contamination, in addition to favouring the *in vitro* establishment of tissues. Supplementation of the culture medium with activated charcoal (Me_1) or PVP30 (Me_2) or exposure to light for 30 days (Me_3) was effective at reducing the oxidation of *E. pilularis* tissues.

6. ACKNOWLEDGEMENT

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