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In vitro cloning of a selected *Eucalyptus pilularis* tree and genetic stability analysis of micropropagated plants

Clonación in vitro de un árbol seleccionado de *Eucalyptus pilularis* y análisis de estabilidad genética de plantas micropropagadas

Maria Lopes Martins Avelar [a], Denys Matheus Santana Costa Souza [a], Letícia Vaz Molinari [b], Sérgio Bruno Fernandes [a], Júlio Cézar Tannure Faria [c], Dulcinéia de Carvalho [d], Gustavo Leal Teixeira [e], Gilvano Ebling Brondani [a]*

* Corresponding author: [a] Federal University of Lavras, Laboratory of *In vitro* Culture of Forest Species, Department of Forestry Sciences, Lavras, Minas Gerais, Brazil, gilvano.brondani@ufla.br

[b] Federal University of Lavras, Laboratory of Forest Pathology, Department of Phytopathology, Lavras, Minas Gerais, Brazil.

[c] Federal University of Espírito Santo, CCAE/Ufes, Department of Forest Sciences and Wood, Jerônimo Monteiro, Espírito Santo, Brazil.

[d] Federal University of Lavras, Laboratory of Forest Improvement and Genetic Resources, Department of Forestry Sciences, Lavras, Minas Gerais, Brazil.

[e] Federal University of Minas Gerais, University Campus, Institute of Agricultural Science, Montes Claros, Minas Gerais, Brazil.

SUMMARY

Establishing vegetative propagation techniques to promote the rejuvenation/reinvigoration of genotypes is essential for the rescue of adult trees of the *Eucalyptus* genus used in species and provenance tests. The aim of this study was to evaluate the induction of epicormic buds and shoots in pruned branches and the *in vitro* establishment, multiplication, genetic fidelity, and elongation of three 44-year-old *Eucalyptus pilularis* selected plants. M1, M2 and M3 represent selected plants. M1 produced the highest number of epicormic buds, number of shoots, and with the lowest rate of tissue oxidation and non-reactive explants. In the establishment stage, M3 exhibited the lowest contamination percentage, number of shoots, and shoot length. The emission of shoots occurred only for M3, and it was the unique genotype subjected to the other stages (*i.e.* multiplication, elongation, and rooting). In the multiplication stage, the highest vigour and shoot length values of were found in the 15th subculture. Phenolic oxidation had its highest value in the 12th subculture, decreasing from the 13th. The highest value for number of shoots was found in the 11th subculture. No polymorphism was observed in the selected plant (M3) and the clonal plants obtained in the 15th subculture. For shoots elongation, the use of culture medium containing 0.10 mg L^{-1} BAP and 1.00 mg L^{-1} NAA provided the lowest means for oxidation, and the highest for vigour, number, and length of shoots. The emission of adventitious roots was observed, demonstrating that, through micropropagation, it was possible to induce the competence to root the material, even at an advanced ontogenetic age.

Keywords: cloning, adventitious rooting, genetic fidelity, *in vitro* propagation, rejuvenation.

RESUMEN

Establecer técnicas de propagación que promuevan el rejuvenecimiento/revigorización de genotipos es fundamental para el rescate de árboles adultos del género *Eucalyptus*. El objetivo de este estudio fue evaluar la inducción de yemas y brotes epicórmicos en ramas podadas y el establecimiento, multiplicación, elongación y fidelidad genética de tres plantas seleccionadas de *Eucalyptus pilularis* de 44 años de edad. M1, M2 y M3 representan las plantas seleccionadas. M1 fue el mejor en número de yemas epicórmicas, número de brotes, baja tasa de oxidación del tejido y explantes que no responden. En la fase de establecimiento, M3 mostró baja contaminación, número de brotes y longitud de brotes. La emisión de brotes ocurrió para M3, y fue el único genotipo sometido a las demás fases (multiplicación, elongación y enraizamiento). En la fase de multiplicación, los mayores valores de vigor y longitud de brotes se encontraron en el subcultivo 15. La oxidación fenólica tuvo su mayor valor en el subcultivo 12, disminuyendo a partir del 13. El valor más alto para el número de brotes se encontró en el subcultivo 11. No se observó polimorfismo entre la planta seleccionada M3 y los brotes obtenidos en el subcultivo 15. En elongación, el uso de medio de cultivo que contenía 0,10 mg L^{-1} de BAP y 1,00 mg L^{-1} de ANA proporcionó las medias más bajas para la oxidación, y los más altos para vigor, número y longitud de brotes por explante. Se observó la emisión de raíces adventicias, demostrando que, a través de la micropropagación, era posible inducir la competencia para enraizar el material, incluso en una edad ontogenética avanzada.

Palabras clave: clonación, enraizamiento adventicio, fidelidad genética, propagación *in vitro*, rejuvenecimiento.

INTRODUCTION

The success of forest stands comprising species of the genus *Eucalyptus* in Brazil stand out for their rapid growth, productivity and environmental adaptation in various soil and climate conditions (Costa *et al*. 2019). The interest in *Eucalyptus* species began in the 1960s with tax incentives and intensified in the mid-1970s, when they were planted for species and provenance tests to identify potential species for industrial applications, aiming at the possible establishment of future forest plantations (IPEF 1984).

Such tests were done through the Forest Development and Research Project, together with the Brazilian Institute of Forest Development and EMBRAPA (IPEF 1984). One of the last ongoing experimental tests is in Lavras, Minas Gerais State (IPEF 1984).

Among the species tested, *Eucalyptus pilularis* Smith is one with great value in Australia, due to great commercial importance in both native and planted forests in the subtropical areas eastern in this country. It is found mainly in coastal plains and mountainous areas off the coast of New South Wales to southern Queensland (between latitudes 25°50′ and 37°50′) (West *et al.* 2020).

In Brazil, it is considered as one of the species with the greatest potential for Southeast region, particularly for sawmills and rolling mills (Gomes *et al*. 1977). According to Castellano *et al*. (2013), evaluating species and provenances of centenary eucalypts trees in Rio Claro, in São Paulo State (collection implemented in 1919), *Eucalyptus pilularis* was one of the species that presented the highest mean diameter (> 80 cm), the highest volume of wood and the highest commercial heights (> 42 m). In spite of that, there are important genetic variations within the species, making it possible and important to explore this variation to obtain superior materials, rescue and clone them on a large scale in the industrial context, aiming to form extensive clonal plantations (Silva *et al.* 2015).

Some rescue techniques cannot be used due to the impossibility of felling or girdling selected trees for subsequent cloning (Baccarin *et al.* 2015). Therefore, the induction of epicormic shoots from pruned branches of selected trees is a way to obtain juvenile tissues (Wendling *et al.* 2013, Oliveira *et al.* 2015a, Baccarin *et al.* 2015, Nascimento *et al.* 2018). The increase in ontogenetic age causes a reduction in the rooting capacity of tree propagules, and tissue rejuvenation can favor the cellular competence in the rhizogenic process (Hartmann *et al*. 2011, Wendling *et al.* 2013, Baccarin *et al.* 2015, Trueman *et al.* 2018).

In adult trees with advanced ontogenetic age, rescue via epicormic shoots and the use of micropropagation are ways to promote the rejuvenation of the tissues to improve adventitious rooting capacity (Avelar *et al.* 2020). However, the technique involves the possibility of somaclonal variation in such material *in vitro* grown, mainly due to the number of subcultures to promote tissue rejuvenation. Thus, it is important to conduct genetic fidelity tests to confirm cloning.

The aim of this work is to evaluate the induction of epicormic buds and shoots in pruned branches, the *in vitro* establishment, multiplication, elongation, and genetic fidelity of three 44-year-old *E. pilularis* selected plants.

METHODS

Study site and experimental material. The experiments were carried out at the Forest Nursery and at the Laboratory of *in vitro* Culture of Forest Species, which belongs to the Department of Forest Sciences (DCF) of the Federal University of Lavras (UFLA), Brazil.

The plant tissues used to obtain the explants came from epicormic shoots of branches pruned in September 2018 from three 44-year-old *Eucalyptus pilularis* selected plants (M1, M2, and M3-tree, figure 1A), that were part of a species and provenance test of *Eucalyptus* and *Corymbia*, set up in 1974 at the Forest Nursery (IPEF 1984) (table 1), located in Lavras, Minas Gerais State, Brazil (21º 22’ 75’’ S, 44º 96’ 98’’ W; 919 m a.s.l., and Cwa Köppen Climate Classification).

Epicormic buds and shoots. The trees were visually selected, and the selection criteria included a straight trunk without pathogen attacks and with branches in the lower portion of the canopy, according to the indication reported by Avelar *et al.* (2020), to reduce the effects of ontogenetic age as to facilitate pruning of the branches.

Pruned branches, with mean diameters ranging from 3.0 to 6.3 cm (three measurements on each branch: upper, middle, and lower portion), were cut down to 0.50 m in length, stored in a greenhouse (relative air humidity > 80 % and temperature of 20-35 °C) and they were placed upright inside 5-L polyethylene pots filled with washed sand to induce epicormic shoots (figures 1B and 1C).

The experiment had a completely randomized design. Three selected plants (M1, M2, and M3-tree) were tested, with three replicates containing two branches per pot. After 45 days in the greenhouse, the number of epicormic buds and shoots of the branches were evaluated.

In vitro establishment. Shoots with 4-5 cm of length were collected after 45 days in a greenhouse. Before the collection (48 hours before), a fungicide solution with 0.5 g L^{-1} dimethyl 4,4′-(*o*-phenylene) bis(3-thioallophanate) was applied in the shoots. After the collection, the shoots were immersed in sterile deionized water and taken to the tissue culture laboratory.

Standardized 2-3-cm-long nodal segments, with two axillary buds and no leaves were used as explants (figure 2D). The explants were washed under running water for 5 minutes, then were disinfected for 30 seconds in a 70 % ethanol solution (v/v) and for 10 minutes in a NaOCl solution with 1.00-1,25 % active chlorine at a horizontal laminar flow hood. After this step, the nodal segments were washed in sterile deionized water three times after each immersion in ethanol or NaOCl.

Thus, the explants were placed in test tubes (25 mm × 150 mm) with 10 mL of MS culture medium (Murashige and Skoog 1962) supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar. The pH was adjusted to 5.8 ± 0.05 before autoclaving at 127 °C and 1.5 kgf cm^{-2} for 20 minutes and agar addition. After inoculation, the explants were maintained at 25 ± 2 °C under a 16-hour light (40 µmol m^{-2} s^{-1}) photoperiod.

The experiment was conducted in a completely randomized design. Three selected plants (M1, M2, and M3-tree) were tested, with 30 replicates of one explant each. At 30 days (figure 1E), the mean percentages of tissue oxi-

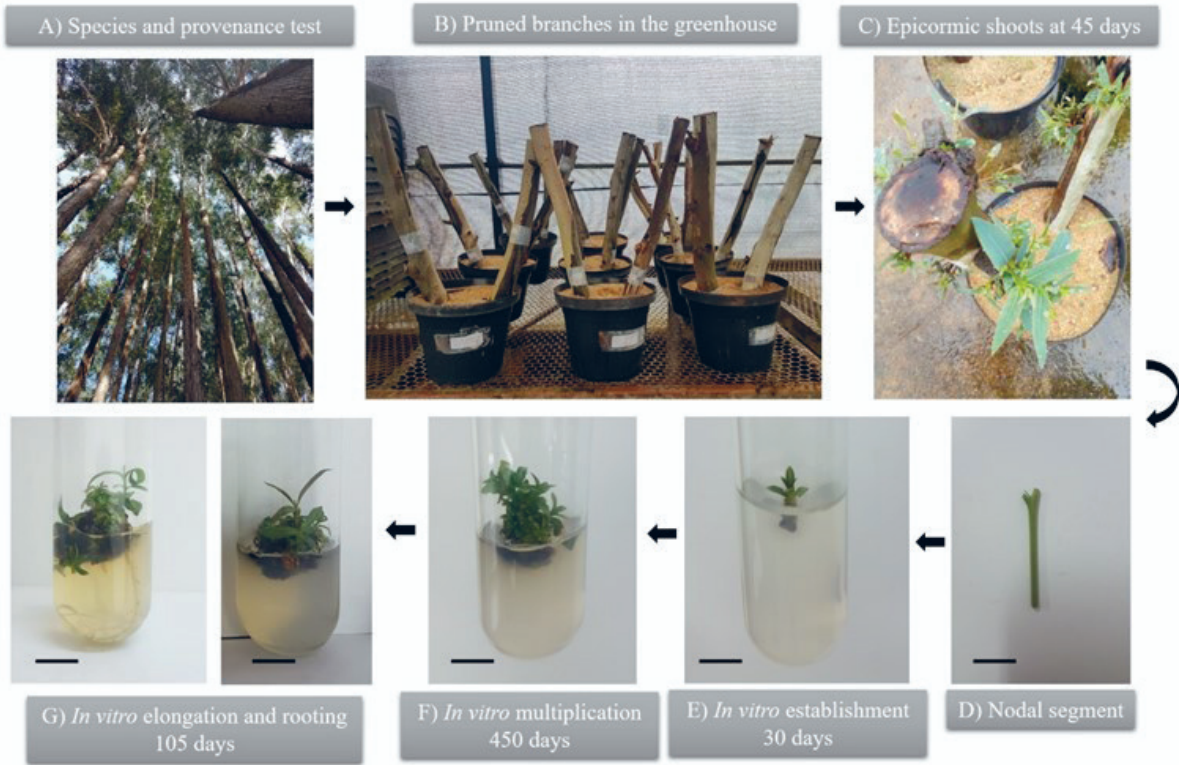

Figure 1. Steps from the selection and pruning of the branches of the selected trees located in the species and provenance test to the *in vitro* elongation/rooting. A) *Eucalyptus pilularis* trees located in the species and provenance test; B) Branches placed in polyethylene vases (5 L), filled with washed sand and placed in a greenhouse; C) Epicormic shoots on branches of M1 at 45 days in a greenhouse; D) Nodal segment for *in vitro* inoculation; E) Explant established *in vitro* at 30 days; F) Shoots emitted in the *in vitro* multiplication stage in the 15th subculture (450 days); G) Elongated shoots and adventitious root emission in *E. pilularis* in a culture medium containing 0.05 mg L^{-1} of BAP and 0.5 mg L^{-1} of NAA at 105 days. Bar = 1 cm.

Pasos desde la selección y poda de las ramas de los árboles seleccionados ubicados en el ensayo de especie y procedencia hasta la elongación/enraizamiento *in vitro*. A) *Eucalyptus pilularis* árboles ubicados en el ensayo de especies y procedencias; B) Ramas colocadas en vasijas de polietileno (5 L), llenas de arena lavado y colocadas en invernadero; C) Brotes epicórmicos en ramas de M1 a los 45 días en invernadero; D) Segmento nodal para inoculación *in vitro*; E) Explante establecido *in vitro* a los 30 días; F) Brotes emitidos en la fase de multiplicación *in vitro* en el subcultivo 15 (450 días); G) Emisión de brotes alargados y raíces adventicias en *E. pilularis* en medio de cultivo con 0,05 mg L^{-1} de BAP (bencilaminopurina) y 0,5 mg L^{-1} de ANA (ácido naftalenoacético) a los 105 días. Barra = 1 cm.

Table 1. Provenance and location of the *Eucalyptus pilularis* selected adult plants in the species and provenance test.

Procedencia y ubicación de las plantas adultas seleccionadas de *Eucalyptus pilularis* en el ensayo de especie y procedencia.

Selected tree	Identification	Provenance	Latitude (S)	Longitude (E)	Altitude (m)
M1	9492	Gallangowan QLD	26°30’	152°20’	580
M2	6183	Mte. Glorius – W Brisbane QLD	27º15’	152°40’	600
M3	9463	SW Casino NSW	28°52’	153°03’	549

dation, contamination (*i.e.* bacterial and/or fungal), non-responsive explants (*i.e.* absence of shoots), *in vitro* establishment (*i.e.* absence of tissue oxidation, contamination and non-responsive explants) and the number and shoot length per explant were evaluated.

In vitro multiplication. Standardized 0.5-cm-long shoots, obtained in the *in vitro* establishment stage, were transferred to glass test tubes (25 mm × 150 mm) with 10 mL of WPM culture medium (Lloyd and McCown 1980) supplemented with 1.0 mg L^{-1} 6-benzylaminopurine (BAP), 0.05 mg L^{-1} α-naphthalene acetic acid (NAA), 20 g L^{-1} sucrose and 6 g L^{-1} agar. In total, 50 replications of one explant each were used.

A total of 15 subcultures were performed every 30 days (figure 1F), in which oxidation and vigour were evaluated on the scale proposed by Oliveira *et al.* (2016), as were the number and shoot length per explant. After obtaining the data, regression curves relating the subcultures and the variables evaluated were generated. In addition, the genetic fidelity of the experimental material was tested in the 15th subculture in comparison to the respective selected plant.

Genetic fidelity. DNA extractions and amplifications were performed according to the protocol adapted from Doyle and Doyle (1990). Universal primers (ISSR) were used to evaluate the fidelity of the genetic materials. The PCR reactions were performed using a total amount of 13 µL having 30 ng of DNA template and 10 µL of PCR mix [1.5 mM of PCR buffer Phoneutria®, 1.5 mM dNTP, 1 U Taq polymerase Phoneutria® (5 U μL^{-1}) and 0.2 mM of each primer and sterile ddH_2O]. Amplifications were performed in a thermocycler (GeneAmp PCR System 9700) with an initial denaturation of DNA at 94 °C for 5 min, followed by 30 s denaturation at 94 °C, and 45 s annealing at 42 °C and 1 min extension at 72 °C, the final extension of 10 min at 72 °C and a holding temperature of 4 °C. The amplified products were separated in agarose gel (1.5 %) and stained with GelRedTM (Uniscience). The amplified products were compared with a marker of known molecular weight (Ladder 1 Kb Plus). The gel images were photographed using the gel doc system (Bio-Rad, USA). The occurrence of polymorphism in each sample was assessed using only well-defined bands.

In vitro elongation and adventitious rooting. Shoots obtained in the *in vitro* multiplication stage (*i.e.* 0.5-cm of length), were transferred to glass test tubes (25 mm × 150 mm) with 10 mL of WPM culture medium (Lloyd and McCown 1980) supplemented with 20 g L^{-1} sucrose and 6 g L^{-1} agar and different concentrations of BAP and NAA.

The experiment was carried out in a completely randomized design, in a 2 × 2 factorial arrangement, being tested two BAP concentrations (0.05 mg L^{-1} and 0.10 mg L^{-1}) and two NAA concentrations (0.5 mg L^{-1} and 1.0 mg L^{-1}) with 15 replications of one explant each.

Three subcultures were performed every 35 days (figure 1G), in which each explant was evaluated for oxidation and vigour on the scale proposed by Oliveira *et al.* (2016), as were the number of shoots, shoot length and adventitious rooting.

Statistical analyses. Analyses were processed in R software, version 3.0.3. The means of the treatments were taken to perform the statistical analyses and to fit the regression equations. The variables that did not show a normal distribution according to the Shapiro-Wilk ($P > 0.05$) were arcsin-transformed. After performing ANOVA ($P < 0.05$), for the significant variables, the Tukey's test ($P < 0.05$).

RESULTS

Epicormic buds and shoots. Significant differences were found between the three *E. pilularis* selected plants in the variables evaluated in the epicormic bud and the shoot induction stage in the pruned branches. The technique employed is thus considered feasible, since all selected plants produced shoots.

The highest mean number of buds at 45 days was observed for M1 (18.0 buds), which differed significantly from M2 (1.7 buds) and M3 (4.7 buds) at 5 % significance (figure 2A). M1 also had the most epicormic shoots (67.0 shoots), differing significantly from M3 (23.7 shoots) and M2 (6.0 shoots) (figures 2B).

In vitro establishment. The response variables evaluated (percentages of oxidation, contamination, non-responsive explants, and *in vitro* establishment and number and shoot length per explant) in the *in vitro* establishment stage showed significant differences) between the clones studied (figure 3).

M1 showed the lowest tissue oxidation (*i.e.* absence of tissue oxidation), differing significantly from M2 (33.3 %) (figure 3A). The lowest mean percentual of contamination by fungi and bacteria was observed for M3 (50.0 %), which was significantly different from the other treatments (figure 3B).

The lowest mean percentages of non-responsive explants were observed in M3 and M1, both at 6.7 %, while it was 66.7 % in M2 (figure 3C). The highest results for the number and shoot length were observed in M3 (1.5 shoots per explant and 0.6 cm, respectively) (figure 3D and 3E). Regarding *in vitro* establishment, M3 was the only clone that established (33.3 %) (figure 3F).

In vitro multiplication. From the polynomial regression curves of the second and third degrees along the 15 subcultures, it was found that explant vigour increased up to the 11th subculture (1.9), with a drop in the two subsequent subcultures (1.8 and 1.7) and growing again from the 14th subculture (2.1), reaching the highest value in the 15th subculture (2.3) (figure 4A). As for oxidation, there

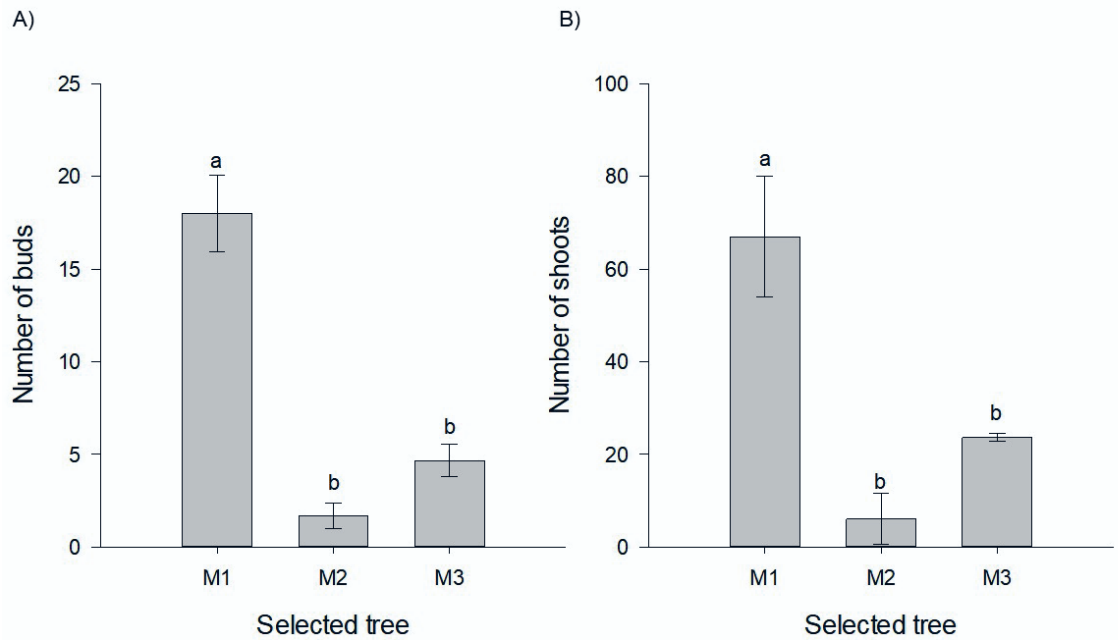

Figure 2. Variables evaluated at 45 days in a greenhouse on branches from three *Eucalyptus pilularis* selected plants (M1, M2, and M3-tree). A) Number of epicormic buds. B) Number of shoots. Different lowercase letters (a, b) above the bars represent significant differences between treatments (selected plants) according to Tukey′s test ($P < 0.05$). Data presented as mean ± standard error. Number of replicates = three replicates containing two branches per pot.

Variables evaluadas a los 45 días en invernadero sobre ramas de tres plantas seleccionadas de *Eucalyptus pilularis* (árbol M1, M2 y M3). A) Número de yemas epicórmicas. B) Número de brotes epicórmicos. Las letras minúsculas diferentes (a, b) arriba de las barras representan diferencias significativas entre tratamientos (plantas seleccionadas) según la prueba de Tukey ($P < 0,05$). Datos presentados como media ± error estándar. Número de repeticiones = tres repeticiones que contienen dos ramas por maceta.

was an increase in the release of phenolic compounds in the culture medium up to the 12th subculture (1.6), with a reduction from the 13th on (figure 4B).

Regarding the number of shoots, in the 11th subculture the highest number of sprouts emitted per explant was observed (12.5 shoots), with a drop in the 12th (9.1 sprouts) and stabilising up to the 15th (8.8 shoots) (figure 4C). For shoot length, it was found that the highest value was found in the 15th subculture (0.9 cm) (figure 4D).

Genetic fidelity. In the genetic fidelity analyses, amplicons were observed for all ISSR primers used. No polymorphism was found between the selected tree and the plants obtained in the 15th subculture of the *in vitro* multiplication stage (figure 5).

In vitro elongation and adventitious rooting. The response variables (oxidation and vigour, through a grading scale, number and shoot length per explant) in the *in vitro* elongation presented a significant interaction in face of the NAA and BAP concentrations (table 2 and figure 6). There were no significant differences between treatments for *in vitro* rooting.

The use of 0.10 mg L^{-1} BAP combined with 1.00 mg L^{-1} NAA resulted in the lowest means for oxidation (1.0), the highest for vigour (2.1), number of shoots per explant (8.0 shoots) and shoot length (1.0 cm) (table 2).

The general mean for *in vitro* rooting was 3.4 %, although there were no significant differences between the factors studied, demonstrating that, through micropropagation technique, it was possible to induce competence to root the material (*i.e.* complete micropagated plant), even with advanced ontogenetic age of M3 genotype (figure 6).

DISCUSSION

Several studies used the technique and confirmed success in the emission of epicormic shoots in pruned branches and in the vegetative propagation of adult trees of forest species, as observed in 44-year-old species of the genera *Eucalyptus* and *Corymbia* (Avelar *et al.* 2020), 26-year-old *Eucalyptus cloeziana* trees (Oliveira *et al.* 2015a), 20-year-old *Araucaria angustifolia* trees (Wendling *et al.* 2009), 19-year-old *Ilex paraguariensis* trees (Wendling *et al.* 2013) and 13-year-old *Eucalyptus benthamii* trees (Baccarin *et al.* 2015).

The use of this technique is important mainly for the conservation of germplasm and for the selection of superior individuals, in which the rescue is usually carried out in the adult stage, where the propagation can be limited

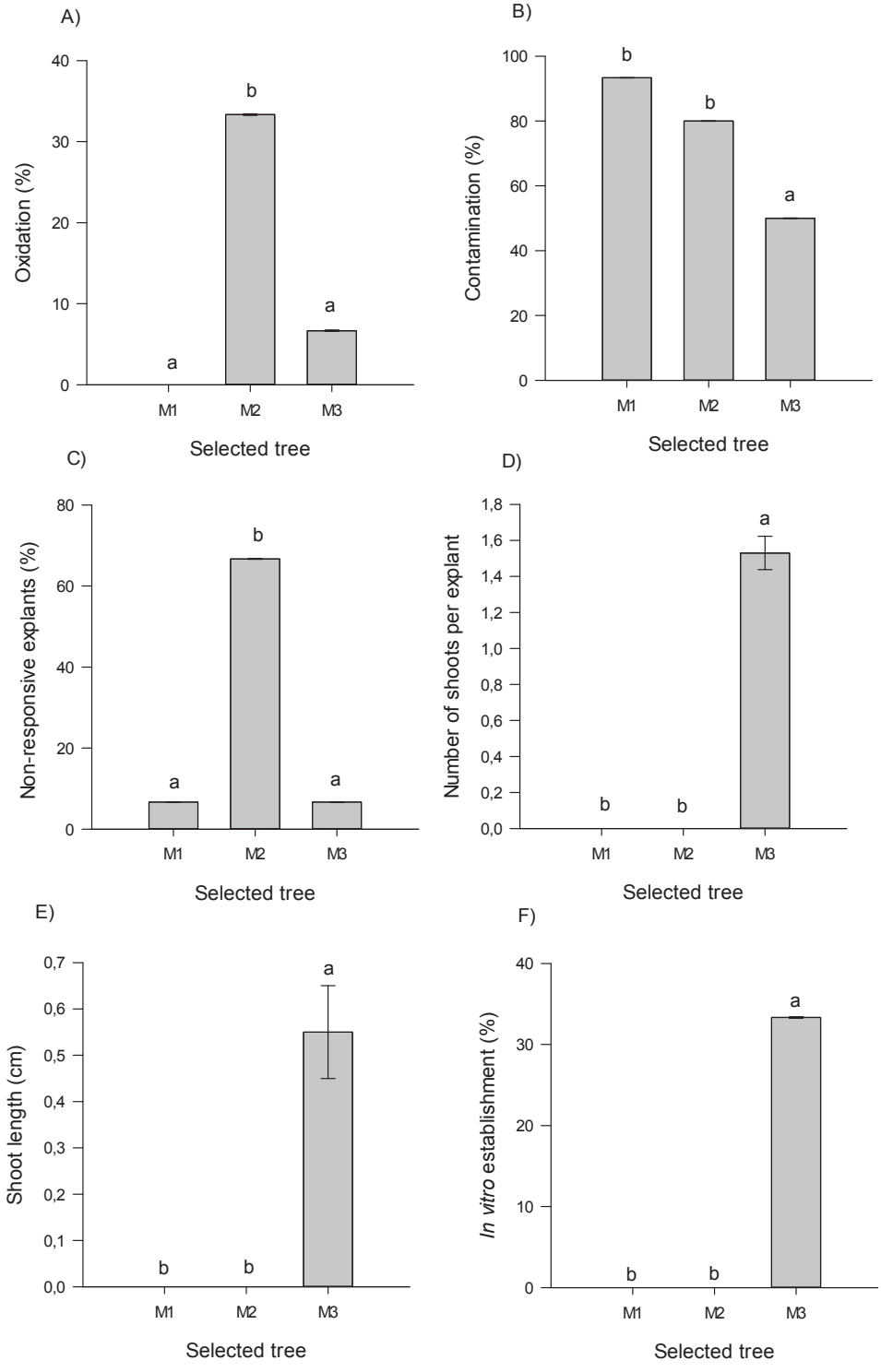

Figure 3. Variables evaluated in the *in vitro* establishment stage of nodal segments from three *Eucalyptus pilularis* selected plants (M1, M2, and M3-tree). A) Shoot oxidation (%). B) Contamination by fungi and bacteria (%). C) Nonresponsive explants (%). D) Number of shoots per explant. E) Shoot length (cm). F) *In vitro* establishment (%). Different lowercase letters (a, b) above the bars represent significant differences between treatments (selected plants) according to Tukey′s test ($P < 0.05$). Data presented as mean ± standard error. Number of replicates = 30 replicates of one explant each.

Variables evaluadas en la fase de establecimiento *in vitro* de segmentos nodales de tres plantas seleccionadas de *Eucalyptus pilularis* (árbol M1, M2 y M3). A) Oxidación de brotes (%). B) Contaminación por hongos y bacterias (%). C) Explantes no respondedores (%). D) Número de brotes por explante. E) Longitud del brote (cm). F) Establecimiento *in vitro* (%). Las letras minúsculas diferentes (a, b) arriba de las barras representan diferencias significativas entre tratamientos (plantas madres) según la prueba de Tukey ($P < 0,05$). Datos presentados como media ± error estándar. Número de repeticiones = 30 réplicas de un explante cada una.

due to the ontogenetic age (Wendling *et al.* 2013, Oliveira *et al.* 2015b). Avelar *et al*. (2020) evaluated the epicormic shoots technique for the rescue of selected eucalypts trees, including *E. pilularis* species. The authors observed the viability of the epicormic shoots technique to obtain propagules aimed at *in vitro* establishment, however reinforcing that *in vitro* morphophysiological responses can be genotype-dependent.

The induction of buds and the emission of epicormics shoots in pruned branches is linked to the plant physiology,

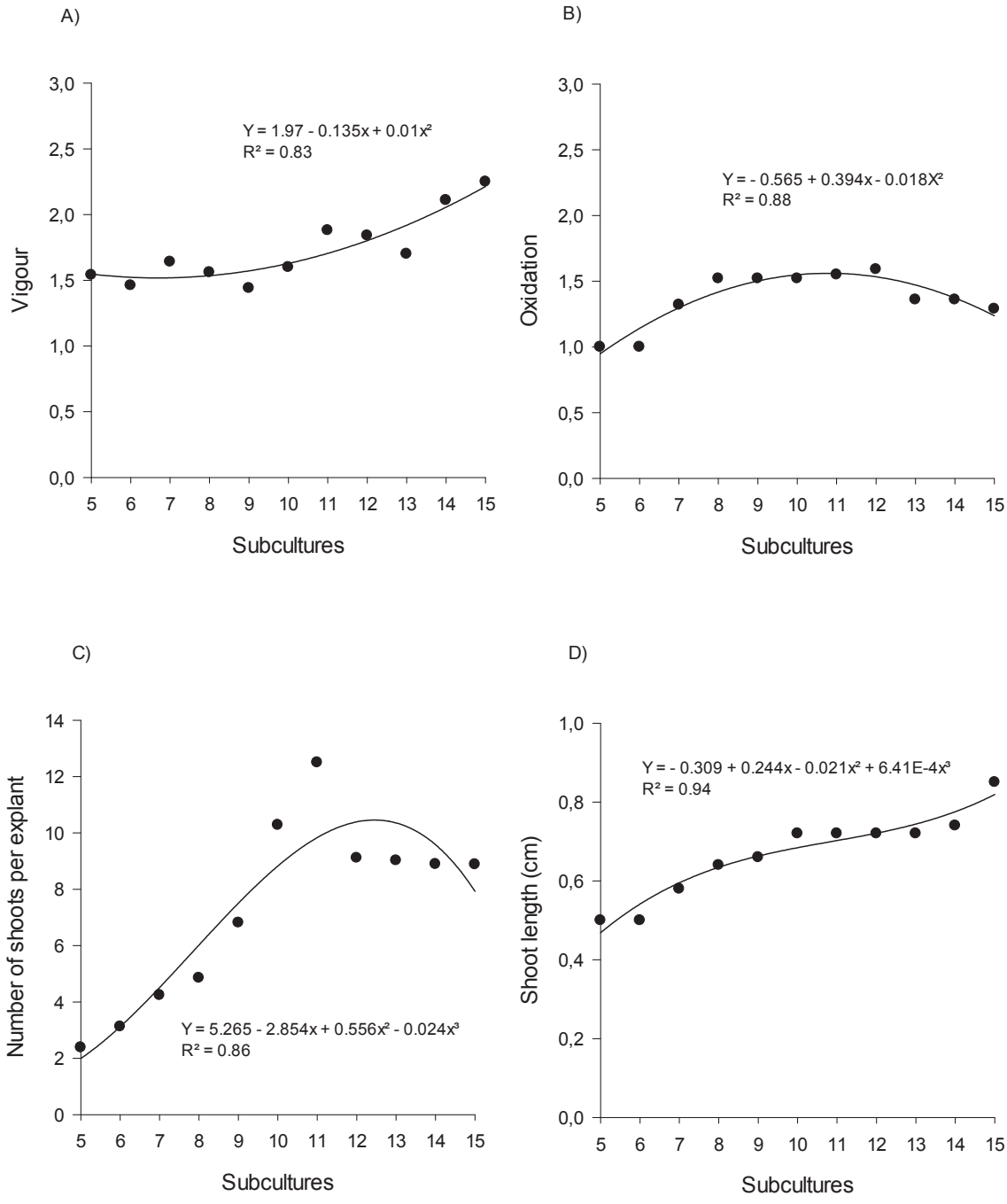

Figure 4. Second- and third-order regression curves of the variables evaluated in the *in vitro* multiplication stage of the M3-tree *Eucalyptus pilularis* selected plant over the subcultures. A) Shoot vigour on a scoring scale. B) Shoot oxidation on a scoring scale. C) Number of shoots per explant. D) Shoot length (cm). * $P < 0.05$. Number of replicates = 50 replications of one explant each.

Curvas de regresión de segundo y tercer orden de las variables evaluadas en la fase de multiplicación *in vitro* de la planta seleccionada de *Eucalyptus pilularis* del árbol M3 sobre los subcultivos. A) Vigor del brote en una escala de puntuación. B) Oxidación en una escala de puntuación. C) Número de brotes por explante. D) Longitud del brote (cm). * $P < 0,05$. Número de repeticiones = 50 réplicas de un explante cada una.

in which there is an alteration in the balance between auxins and cytokinins, favouring the emergence of the aerial part (Wendling *et al.* 2013). This change may promote a higher shoot production by favouring rejuvenation in the region opposite the base of the aerial part of the tree branches.

In addition, the species show anatomical and morpho-physiological differences in the meristem and related tissues, in which epicormic shoots develop. Thus, even in selected plants of the same species, differences are observed that affect the number of induced shoots, as observed by Oliveira *et al.* (2015b) in *Eucalyptus cloeziana*.

Oxidation of phenolic compounds, in addition to promoting the darkening of the medium, can lead to explant death and has been observed mainly in the *in vitro* culture of woody species, such as species of *Eucalyptus* (Navroski *et al.* 2014, Baccarin *et al.* 2015, Oliveira *et al.* 2015a).

The observed oxidation values in M1 corroborate those reported by Baccarin *et al.* (2015), who studied oxidation in the *in vitro* establishment stage of explants from epicormic shoots induced in branches of *Eucalyptus benthamii* selected trees (0.0 %). Navroski *et al.* (2014) also found differences in oxidation percentage between different *Eucalyptus dunnii* genotypes, ranging from 13.3 to 73.3 %.

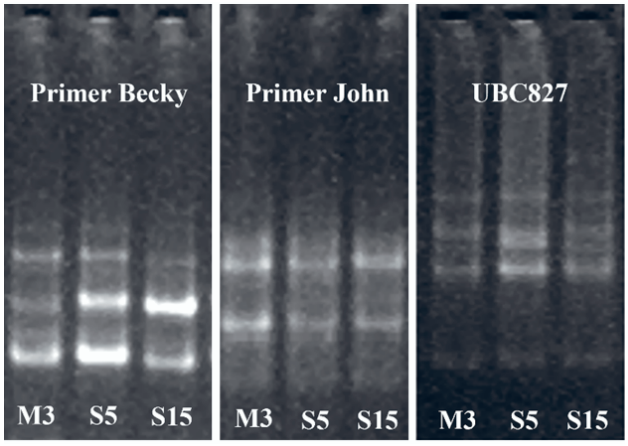

Figure 5. Agarose gels and primers used (Becky, John and UBC827) in the genetic fidelity test comparing the selected M3-tree, the 5th and the 15th subcultures (S5 and S15).

Geles de agarosa y cebadores utilizados (Becky, John y UBC827) en la prueba de fidelidad genética que compara el árbol M3 seleccionado, los subcultivos n° 5 y n° 15 (S5 y S15).

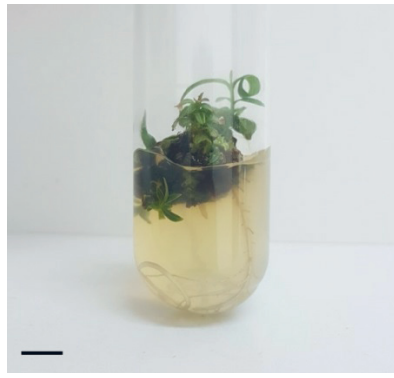

Figure 6. Emission of adventitious roots in elongated shoots of *Eucalyptus pilularis* in culture medium containing 0.05 mg L^{-1} BAP and 0.5 mg L^{-1} NAA, at 105 days. Bar = 1 cm.

Emisión de raíces adventicias en brotes alargados de *Eucalyptus pilularis* en medio de cultivo con 0,05 mg L^{-1} de BAP y 0,5 mg L^{-1} de ANA, a los 105 días. Barra = 1 cm.

Table 2. Evaluated variables (oxidation, vigour, number of shoots per explant and shoot length) on *in vitro* elongation of *Eucalyptus pilularis* as a function of BAP and NAA concentrations. The letters (A, a, B, b) represent statistical differences between treatments according to the Tukey's test ($P < 0.05$). Capital letters (on the line) represent the differences in NAA concentrations within the BAP concentration. Lowercase letters (in the column) represent the differences in BAP concentrations within the NAA concentration.

Se evaluaron variables (oxidación, vigor, número de brotes por explante y longitud de brotes) sobre la elongación *in vitro* de *Eucalyptus pilularis* en función de las concentraciones de BAP y ANA. Las letras (A, a, B, b) representan diferencias estadísticas entre tratamientos según la prueba de Tukey ($P < 0,05$). Las letras mayúsculas (sobre la línea) representan las diferencias en las concentraciones de ANA dentro de la concentración de BAP. Las letras minúsculas (en la columna) representan las diferencias en las concentraciones de BAP dentro de la concentración de ANA.

BAP (mg L^{-1})	Oxidation		Vigour		NS		SL (cm)	
	NAA (mg L^{-1})		NAA (mg L^{-1})		NAA (mg L^{-1})		NAA (mg L^{-1})	
	0.50	1.00	0.50	1.00	0.50	1.00	0.50	1.00
0.05	$1.6^{Aa} \pm 0.19$	$1.7^{Aa} \pm 0.15$	$1.6^{Aa} \pm 0.16$	$1.2^{Ab} \pm 0.11$	$5.8^{Aa} \pm 0.62$	$3.6^{Bb} \pm 0.21$	$0.6^{Aa} \pm 0.03$	$0.5^{Ab} \pm 0.01$
0.10	$1.5^{Aa} \pm 0.17$	$1.0^{Bb} \pm 0.00$	$1.7^{Aa} \pm 0.18$	$2.1^{Aa} \pm 0.19$	$5.6^{Ba} \pm 0.79$	$8.0^{Aa} \pm 0.72$	$0.7^{Ba} \pm 0.06$	$1.0^{Aa} \pm 0.11$

The letters (A, a, B, b) represent statistical differences between treatments according to the Tukey test at 5 % significance. Capital letters (on the line) represent the differences in NAA concentrations within the BAP concentration. Lowercase letters (in the column) represent the differences in BAP concentrations within the NAA concentration. NS = Number of shoots per explant; SL = Shoot length. Data presented with mean ± standard error. Number of replicates = 15 replications of one explant each.

In addition, to be successful in micropropagation, it is necessary that a few explants emit shoots free of contamination, since the *in vitro* establishment stage is the most limiting of *in vitro* propagation (Trueman *et al.* 2018). The different contamination percentages between the studied selected trees show that the metabolic pathway of microorganisms can be defined by the genotype due to differences in gene expression, which can influence carotenoid biosynthesis and hyphal aggregation (Postemsky and Curvetto 2016).

The highest percentages of contamination (figure 3B) in explants from M1 and the high percentages of oxidation and contamination (figures 3A and 3B) in explants from M2 may have caused tissue death *in vitro*, making establishment impossible. This response differs from that reported in Avelar *et al*. (2020), where it was possible to obtain *in vitro* established shoots from selected M1 and M2 selected plants, however, the author did not study the other micropropagation stages. In *Eucalyptus cloeziana*, Oliveira *et al.* (2015a) observed differences in the *in vitro* establishment percentage of explants from epicormic shoots emitted in branches pruned in December from different 26-year-old selected plants, ranging from 26.4 to 51.2 %.

In the *in vitro* multiplication of explants from parent trees of *Eucalyptus cloeziana* at 26 years of age, whose plant material was subcultured monthly for 19 months, the concentrations of 1.03 mg L^{-1} BAP and 0.06 mg L^{-1} NAA resulted in the highest sprout proliferation for one of the selected plants (Oliveira *et al.* 2015a), concentrations close to those used for *E. pilularis*.

The reduction in vigour and number of shoots emitted per explant on the 12th and 13th subcultures may have occurred due to the increase in phenolic oxidation on the 11th and on the 12th, which resulted in a higher value for this evaluated characteristic (1.6) (figure 4B).

The absence of polymorphism in *E. pilularis* demonstrate that the protocols used did not affect the genetic stability of the material and, consequently, they were faithful to the parent plant, contrary what was observed by Teixeira *et al.* (2020) when testing genetic fidelity in populations of *Eucalyptus urophylla* and *Eucalyptus microcorys*, using the same protocol.

The success of micropropagation depends on several factors, including the concentrations of plant growth regulators, since they are the main responsible for the stimuli of responses for cell differentiation, growth, and elongation (Hartmann *et al.* 2011). Among the most used phytoregulators in tissue culture, cytokinins promote cell division and are linked to cell differentiation in the process of formation of aerial parts and roots, while auxins control cell growth and elongation, mainly in the promotion of lateral and adventitious roots (Hartmann *et al.* 2011).

BAP is the most used cytokinin, as it has been shown to be more effective in the multiplication of several woody species, in addition to providing the lowest acquisition cost (Aragão *et al.* 2011). As for auxins, NAA has been used in varying concentrations according to species and/or cultivar, combined with cytokinins to stimulate induction, elongation of shoots and rooting in various plant species (Hartmann *et al.* 2011), corroborating the results found for *E. pilularis*.

However, plant growth regulators and plant cells interact in a complex way and, if the tissue is not responsive, it will not respond properly to the exogenous application of these components, regardless of the concentrations and combinations used (Navroski *et al.* 2015). Several studies show different combinations used in the *in vitro* elongation of different plant species (Brondani *et al.* 2009, Navroski *et al.* 2015, Oliveira *et al.* 2016). Navroski *et al.* (2015) observed that the use of 0.5 mg L^{-1} NAA in the *in vitro* elongation of *Eucalyptus dunnii* explants, obtained from shoots from the felling of three-year-old trees, resulted in the highest averages for number and shoot length at 30 days of *in vitro* culture.

NAA concentrations between 0.25 and 0.75 mg L^{-1} resulted in the highest number of elongated shoots at 60 days and the use of 1.0 mg L^{-1} of NAA resulted in a negative effect for elongation, promoting callus induction in the *in vitro* cultivation of explants from a clone of *Eucalyptus benthamii* × *E. dunnii*, conducted in a clonal mini-hedge (Brondani *et al.* 2009), which was not observed for *E. pilularis*.

In 26-year-old *Eucalyptus cloeziana* selected trees, the number of elongated shoots was higher in treatments where NAA plant growth regulator was not added to the culture medium, regardless of BAP concentration (Oliveira *et al.* 2015a), also differing of the results found for *E. pilularis*.

The different morphological responses observed in relation to the application of plant growth regulators may be linked to the genotype (*e.g.* genotype-dependent response) and physiological/ontogenetical ages of the selected tree (Brondani *et al.* 2009, Navroski *et al.* 2015, Oliveira *et al.* 2016, Wang *et al*. 2023). Despite low adventitious rooting, it was possible to obtain a complete *in vitro* plant of the M3 genotype, and it is necessary to develop studies to improve the cloning of the genetic material in commercial scale (*e.g.* tissue reinvigoration and rejuvenation studies). This technique can be tested to other species of *Eucalyptus* and *Corymbia*, especially when the purpose of micropropagation is to rescue adult trees with advanced ontogenetic age and induce competence for adventitious rooting through successive subcultures that aim to reinvigorate and/or rejuvenate tissues. The micropropagated plants can be used to form clonal mini-hedge aiming at the continuous clone production of the selected tree.

CONCLUSIONS

Epicormic shoot technique was efficient to obtain shoots for the *in vitro* introduction of the three adult *Eucalyptus pilularis* trees (M1, M2, and M3). *In vitro* establishment was feasible from the M3 selected tree, demonstrating that

there is a genotype-dependent effect. Highest values for vigour and shoot length were observed in the 15th subculture of *in vitro* multiplication stage. No polymorphism was found between the parent plant and the 15th subculture of the *in vitro* multiplication stage, demonstrating genetic stability of the M3 genotype. The use of 0.10 mg L^{-1} BAP combined with 1.00 mg L^{-1} NAA in the *in vitro* elongation stage resulted in the lowest means for oxidation and the highest for vigour, number, and shoot length per explant of the M3 genotype. Through micropropagation technique, it was possible to induce competence to root (*i.e.* complete microplant), even at an advanced ontogenetic age, demonstrating the possibility of cloning the selected tree.

AUTHORS CONTRIBUTION

MLMA designed the study and the experimental design; MLMA, DMSCS, LVM, SBF, JCTF conducted the field data collection; MLMA and DC analyzed the results; GLT review of text (English Language); GEB supervised the study, contributed to the discussion and interpretation of the results, and obtaining and managing resources. All authors prepared the manuscript.

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