

Mini-cutting technique for the propagation of a *Eucalyptus camaldulensis* clone selected in a semiarid region

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Abstract

Aim of study: To assess the effectiveness of the mini-cutting technique in propagating a clone of *Eucalyptus camaldulensis* Dehnh selected in semiarid conditions, test the impact of the growth regulators Indole-3-Acetic Acid (IAA) and Indole-3-Butyric Acid (IBA) on rooting and determine the dynamics of adventitious rooting. At the same time, an anatomical study was conducted to examine the tissues involved in the formation of adventitious roots.

Area of study: Mossoró, located in the state of Rio Grande do Norte, Brazil.

Material and methods: The mini-stumps of the *E. camaldulensis* clone were subjected to five consecutive prunings and the shoots were analyzed for their adventitious rooting capacity by testing the IAA and IBA auxins at four doses (0 - control; 1,000; 2,000; and 4,000 mg.L⁻¹). A temporal analysis of rooting was carried out with evaluations performed every 7 days from the day of staking until day 28 and samples were collected to perform the anatomic analysis.

Main results: The mini-stumps from *E. camaldulensis* clone showed 100% survival, 13 propagules per mini-stump/month and 900 sprouts/m²/month. The adventitious rooting was lower in control (55%) than in IAA (85%) and IBA (80%) at 2.000 mg.L⁻¹. Rhizogenesis of the mini-cuttings started at day 14. The IAA and IBA increased mini-cutting rooting speed. Root formation was direct from phloem or vascular cambium in control and from xylem, phloem or vascular cambium in mini-cuttings treated with IAA and IBA.

Research highlights: The *E. camaldulensis* clone showed strong potential for propagation under semiarid conditions, with rooting and survival rates above 80% when treated with auxins. These results confirm the mini-cutting technique as an efficient and practical solution for large-scale propagation, offering valuable applications in commercial forestry under challenging environmental conditions.

Keywords: adventitious rooting; clonal forestry; clonal mini-garden; plant propagation, rhizogenesis.

Técnica de miniestacas para la propagación de un clon de *Eucalyptus camaldulensis* seleccionado en una región semiárida

Resumen

Objetivo del estudio: Evaluar la efectividad de la técnica de miniestacas en la propagación de un clon de *Eucalyptus camaldulensis* Dehnh. seleccionado en condiciones semiáridas, analizar el impacto de los reguladores del crecimiento ácido indol-3-acético (IAA) y ácido indol-3-butírico (IBA) en el enraizamiento y determinar la dinámica del enraizamiento adventicio. Simultáneamente, se realizó un estudio anatómico para investigar el tejido relacionado con el desarrollo del enraizamiento adventicio.

Área de estudio: Mossoró, ubicado en el estado de Rio Grande do Norte, Brasil.

Material y métodos: Las plantas madres del clon de *E. camaldulensis* fueron sometidas a cinco podas consecutivas y los brotes fueron analizados por su capacidad de enraizamiento adventicio mediante la prueba de auxinas AIA y AIB en cuatro dosis (0 - control; 1,000; 2,000; y 4,000 mg.L⁻¹). Se realizó un análisis temporal del enraizamiento con evaluaciones realizadas cada 7 días desde el primer día del estaquillado hasta el día 28, y se recolectaron muestras para realizar el

análisis anatómico.

Principales resultados: Las plantas madres del clon de *E. camaldulensis* mostraron una supervivencia con valores de 100%, con 13 propágulos por planta madre y mes y 900 brotes por m² y mes. El enraizamiento adventicio fue menor en el control (55%) que en el AIA (85%) y el AIB (80%) a 2,000 mg.L⁻¹. La rizogénesis de las miniestacas comenzó en el día 14. El AIA y el AIB aumentaron la velocidad de enraizamiento de las miniestacas, el número de raíces y la longitud de las raíces. La formación de raíces fue directa desde el floema o el cambium vascular en el control y desde el xilema, floema o cambium vascular en las miniestacas tratadas con AIA y AIB.

Aspectos destacados de la investigación: El clon de *E. camaldulensis* mostró un fuerte potencial de propagación en condiciones semiáridas, con tasas de enraizamiento y supervivencia superiores al 80% cuando se trató con auxinas. Estos resultados confirman que la técnica de miniestacas es una solución eficiente y práctica para la propagación a gran escala, y ofrece valiosas aplicaciones en la silvicultura comercial en condiciones ambientales desafiantes.

Palabras clave: enraizamiento adventicio; silvicultura clonal; minijardín clonal; propagación de plantas; rizogénesis.

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Introduction

Eucalyptus camaldulensis Dehnh. is one of the most widely planted Eucalyptus species for paper pulp, fuelwood, and timber due to its fast growth, high drought tolerance, and adaptability to diverse climatic conditions and soil types (Bindumadhava et al., 2011; Shanthi et al., 2015). Its widespread distribution, economic potential, and ability to survive in rainfall zones ranging from 250 to 1,600 mm make it a crucial species for improvement programs. Furthermore, *E. camaldulensis* has been used in hybrid breeding programs to enhance drought and salinity tolerance (Amrutha et al., 2021).

Clonal propagation is a widely employed strategy to explore the economic potential of *Eucalyptus* species and hybrids by multiplying desirable genotypes. Consequently, the efficient development of vegetative propagation protocols is essential for the large-scale production of *E. camaldulensis* clones (Shanmugam & Seenivasan, 2012). Mass multiplication of *E. camaldulensis* clones has been previously reported using various propagation techniques, including micropropagation by direct organogenesis (Girijashankar, 2012), as well as cutting and mini-cutting methods (Bindumadhava et al., 2011; Shanmugam & Seenivasan, 2012). Among these techniques, mini-cutting has proven to be the most efficient and widely used method for cloning superior genotypes of Eucalyptus within the framework of clonal forestry (Xavier et al., 2013; Kuppasamy et al., 2019).

Clones propagated using the mini-cutting technique generally produce highly uniform plants. However, the success of this method depends on the development of adventitious roots, a process influenced by various factors such as plant genotype, physiological status, nutritional condition, and the juvenile state of vegetative shoots (Wendling et al., 2014; Vilasboa et al., 2022). Juvenile branches in good nutritional condition tend to exhibit better rooting ability (Wendling et al., 2015), which is further

enhanced by the application of plant growth regulators (Wendling et al., 2015; Stuepp et al., 2017; Lima et al., 2022). Environmental conditions, such as the collection season, also play a critical role in propagation success (Batista et al., 2015).

Auxins are widely used as growth regulators to enhance plant propagation from cuttings by promoting the formation of adventitious roots from non-root tissues (Díaz-Sala, 2021). They are essential regulators of adventitious root induction (Díaz-Sala, 2020). An imbalance in auxin levels can adversely affect rooting, leading to low rooting rates, reduced numbers of roots, root system asymmetry, and a reduced root-to-shoot ratio (Stuepp et al., 2017). These features significantly influence tree stability, survival, and trunk volume in nurseries and plantations. When natural auxin production in apical meristems is insufficient to induce adventitious root formation in stem tissues (Stuepp et al., 2017; Díaz-Sala, 2020), exogenous auxins, particularly indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA), are commonly used to induce adventitious rooting during clonal propagation (Hartmann et al., 2011).

The application of IAA and IBA has been shown to accelerate adventitious rooting in *Eucalyptus* mini-cuttings, with optimal concentrations varying by genotype (Brondani et al., 2010). Additionally, the origin of the propagule along the branch can influence rooting success due to ontogenetic and physiological factors (Wendling et al., 2014). The morphoanatomy of adventitious rooting is important for identifying anatomical features, such as lignification and the presence of a sclerenchyma ring, that may negatively affect root induction, initiation, and emergence (Bryant and Trueman, 2015).

Anatomical studies of transverse sections have shown that adventitious roots can originate from various tissues, including the cambium zone (phloem, vascular cambium, or pericycle) or from callus (Bryant and Trueman, 2015;

Vilasboa et al., 2021). However, the anatomy of adventitious rooting remains variable and not fully understood (Bryant and Trueman, 2015), despite its critical role in maximizing rooting rates (Pimentel et al., 2020). While IBA has been reported to accelerate anatomical changes at the bases of cuttings (Vilasboa et al., 2021), its effects on *E. camaldulensis* genotypes remain unexplored.

The emergence of adventitious roots is essential for clonal propagation and the production of high-quality *E. camaldulensis* plants. This process is influenced by genetic and ontogenetic factors, as well as anatomical and physiological characteristics, which contribute to the root development of mini-cuttings. However, further research is needed to establish a solid mini-cutting propagation methodology for *E. camaldulensis* clones, facilitating the clonal propagation of selected genotypes in breeding programs.

Therefore, this study aimed to evaluate the effectiveness of the mini-cutting technique for propagating a *E. camaldulensis* genotype selected for semiarid conditions. Additionally, it aimed to determine the impact of IAA and IBA on the rooting of apical mini-cuttings and included an anatomical evaluation of propagules at different stages of adventitious rooting. The hypotheses tested were as follows: (I) the *E. camaldulensis* genotype has potential for clonal propagation using the mini-cutting technique; (II) IAA and IBA stimulate rooting and enhance the vigor of the mini-cutting root system; (III) low relative humidity and high temperatures in the growing environment affect the productivity and survival of the mini-garden; and (IV) mini-cutting anatomical features are related to rooting speed and regeneration capacity.

Material and methods

Plant Material

A five-year-old genotype of *E. camaldulensis* was carefully selected based on its survival rate (90%) and growth performance (height: 15.3 m; diameter at 1.30 m: 10.4 cm) for cultivation in the semiarid region of Mossoró, located at coordinates 05°03'13.1"S, 37°23'34.9"W, and an altitude of 78 m, in the state of Rio Grande do Norte, Brazil. This region experiences a dry semiarid climate, with an average temperature of 27.8°C and a relative humidity of 68.9%. Precipitation is highly irregular, averaging 740 mm/year and often accompanied by prolonged drought periods (Fig. 1). The experiments were conducted in March, May, June, July, August, and September.

Clonal mini-garden

Following the mini-cutting technique described by Xavier et al. (2013), the establishment of the clonal mini-garden began with the formation of mini-stumps (mini-cutting donor plants) through cutting rooting. In May, after reaching an average height of 15 cm, the clones propagated by cutting were transferred to a semi-hydroponic system in a sand channel. Subsequently, following a 50-day acclimatization period, the tips of the mini-stumps were pruned at a height of 12 cm above the base to stimulate the emergence of shoots. The mini-garden was cultivated using a semi-hydroponic system with a suspended bed and maintained in full sunlight.

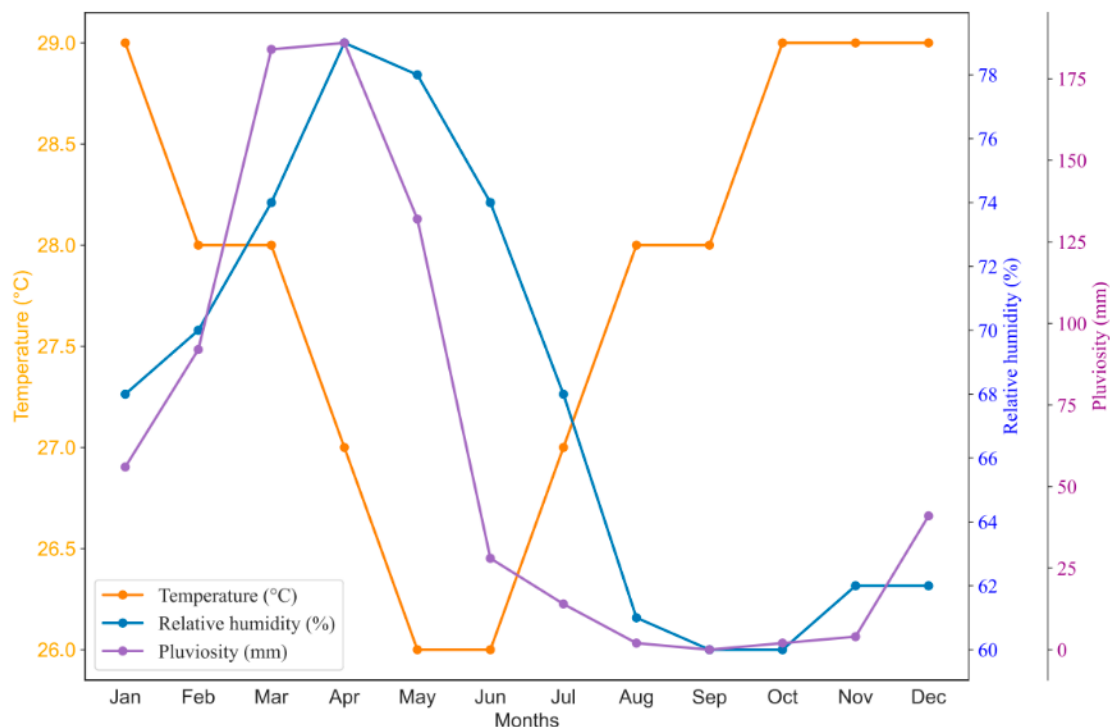


Figure 1. Monthly averages of temperature (°C), relative humidity (%) and pluviosity (mm) for 2018, 2019, 2021 and 2022 in Mossoró, Rio Grande do Norte, Brazil.

The semi-hydroponic system consisted of a masonry trough measuring 7.5 m in length, 0.8 m in width, and 25 cm in depth, filled with medium-grain sand to support the mini-stumps, which were spaced at 12 x 12 cm intervals. Fertigation was provided through drip irrigation. The nutrient solution used in fertigation was composed of the following salt concentrations: (a) 117.0 mg.L⁻¹ of N in the form of nitrate; (b) 15.75 mg.L⁻¹ of N in the form of ammonium; (c) 14.63 mg.L⁻¹ of P; (d) 131.62 mg.L⁻¹ of K; (e) 84.0 mg.L⁻¹ of Ca; (f) 25.21 mg.L⁻¹ of Mg; (g) 73.28 mg.L⁻¹ of S; (h) 0.01 mg.L⁻¹ of B; (i) 0.02 mg.L⁻¹ of Cu; (j) 69.73 mg.L⁻¹ of Fe; (k) 0.03 mg.L⁻¹ of Mn; (l) 0.008 mg.L⁻¹ of Zn; and (m) 0.0016 mg.L⁻¹ of Mo. The electrical conductivity of the nutrient solution was maintained within the range of 1.5 to 2.0 mS m⁻² at a temperature of 27 °C.

Mini-cuttings excision

From the mini-stumps, mini-cuttings measuring between 8 and 10 cm were obtained every 20 days. Mini-cuttings with two pairs of leaves and a length of 8 cm were used for the experiments. Subsequently, the mini-cuttings were cultivated in polypropylene tubes with a volume of 55 cm³, filled with rooting substrate. The substrate composition had the following characteristics: pH in H₂O (5.70), available water (8%), remaining water (25%), total porosity (60%), dry density (878.84 kg.m⁻³), aeration space (30%), and electrical conductivity (0.5 mS.cm⁻¹). Throughout the rooting phase, the mini-cuttings were kept in a greenhouse for 30 days, with relative air humidity above 80% and temperatures maintained between 25–30°C.

Experimental Design

Four experiments were conducted to investigate clonal propagation in *E. camaldulensis*.

Experiment I: Mini-stump survival and production

Mini-stumps were distributed in the sand channel following a completely randomized experimental design with four repetitions of 20 mini-stumps. Five collections of sprouts were performed between June and September, at 20-day intervals, based on the presence of shoots measuring at least 8–10 cm, suitable for preparing mini-cuttings. Every qualifying branch, i.e., those measuring 8–10 cm in length and containing two pairs of leaves, was cut. We assessed mini-stump survival, the number of sprouts per mini-stump, and sprout yield, which was determined as the production of sprouts per square meter per 30 days (sprouts/m²/30 days).

Experiment II: Influence of IAA and IBA

The second experimental strategy evaluated the effect of the auxins IAA and IBA on the adventitious rooting of the selected *E. camaldulensis* clone under high-temperature and water-deficit conditions. The experimental design was

a randomized complete block design in a 2 × 4 factorial arrangement, consisting of two auxins (IAA and IBA) and four concentrations (0 - control, 1,000, 2,000, and 4,000 mg.L⁻¹), with six repetitions, each containing 10 mini-cuttings per plot. After immersion of the basal region in the IAA or IBA solution, the mini-cuttings were immediately placed in plastic tubes pre-filled with rooting substrate and transferred to a rooting greenhouse.

After 30 days in the greenhouse (rooting period), the mini-cuttings were removed from the rooting substrates, washed with water, dried with paper towels, and the following specific characteristics were evaluated: survival, rooting, number of roots at the base of the mini-cuttings, length of the longest root, presence of callus, and oxidation. Survival was determined by the presence of roots and leaves on the propagules. Rooting of mini-cuttings was confirmed when root length was equal to or greater than 0.5 cm. The number of roots was determined by counting those emerging from the base of the mini-cutting. The length of the longest root was measured using a millimeter ruler.

Experiment III: Rotting speed

This experimental strategy aimed to optimize the time required for adventitious rooting of mini-cuttings of the studied clone and to identify the different events involved in adventitious rooting. Sample collection was performed at 0 days (immediately after excision), 7, 14, 21, and 28 days post-excision. During each evaluation, the following characteristics were quantified: the number of live mini-cuttings, rooting, presence of callus, swelling of the base, oxidation, size of the largest root, and number of roots growing from each mini-cutting.

The treatments applied were as follows: control (mini-cutting basal region immersed in distilled water), IBA (mini-cutting basal region immersed in a solution of 2,000 mg.L⁻¹ IBA), and IAA (mini-cutting basal region immersed in a solution of 2,000 mg.L⁻¹ IAA). All mini-cuttings were immersed to a height of 2 cm for 10 seconds in their respective treatment solutions. For each sampling, 50 mini-cuttings were evaluated per treatment, distributed across five repetitions of 10 mini-cuttings per plot, using a randomized block design. During the evaluations, the mini-cuttings were removed from the substrate, washed with water, analyzed, and stored for histological analyses.

Experiment IV: Histological analyses

The fourth experimental strategy aimed to morphoanatomically characterize adventitious rhizogenesis in mini-cuttings of a *E. camaldulensis* clone. Samples from the bases of mini-cuttings (approximately 3 cm) were collected from each treatment—control, IAA, and IBA—and stored in 70% ethanol until further analysis. Transverse sections (10 µm) were cut and stained with safranin-fast green. Photographs were taken using an Olympus BX-41 microscope equipped with a digital camera (Olympus 12 MP). Thirty replicates were sectioned per treatment at 0, 7, 14, and 21 days after planting the propagules in the substrate.

Observations of the time course of root development were made, and digital photographs were taken. The anatomical events associated with the induction and formation of adventitious roots were then investigated, including the identification of the sites of root primordia initiation and the anatomical differences between IAA, IBA, and control treatments. Particular attention was given to whether these anatomical differences could act as barriers to the initiation and emergence of adventitious roots.

Statistical analysis

To identify pruning effects in a mini-clonal garden for mini-cutting production and propagation of *E. camaldulensis* clone selected in a semiarid condition, we examined the effects of propagulos collections on survival, number of sprouts per mini-stump and yield of sprouts at 5 successive collections. For statistical analysis, the assumptions of normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test) were calculated and tested, at a level of 5% significance and then submitted to ANOVA.

The quantitative data were checked for normality and then submitted to factorial ANOVA followed by estimated marginal means, Tukey's,

In sequence, to investigate whether mini-cuttings from mini-stumps of *E. camaldulensis* clone are responsive to adventitious rooting and the auxin can improve mini-

cutting rooting, we setup a experiment analyzing the effect of different doses of IAA and IBA auxins on mini-cutting rooting. Next, we investigated the optimum time for mini-cutting rooting inside the greenhouse, as well we identified different morphological events that result in adventitious rooting. To explain the rooting response of auxin-treated mini-cuttings to concentrations and days, and differentiate the morphological responses of mini-cuttings, we applied a generalized linear model (GLM) having a Poisson distribution and a log link function. In experiment II, we set auxin types and concentrations as fixed effects. For experiment III, we included both auxins and days as fixed effects. We ran all our analyses in R software version 4.3.3 (R Development Core Team, 2017) using packages dplyr, tibble and ggplot2.

A comprehensive overview of each step, from the clonal mini-garden to the experimental design sequence, is illustrated by Fig. 2. An overall picture of the mini-stumps is shown in Fig. 2B before the shoot collection events, while Figure 2C shows the mini-stumps after shoot collection events, and Fig. 2D with mature propagules on mini-stumps that were excluded from propagation.

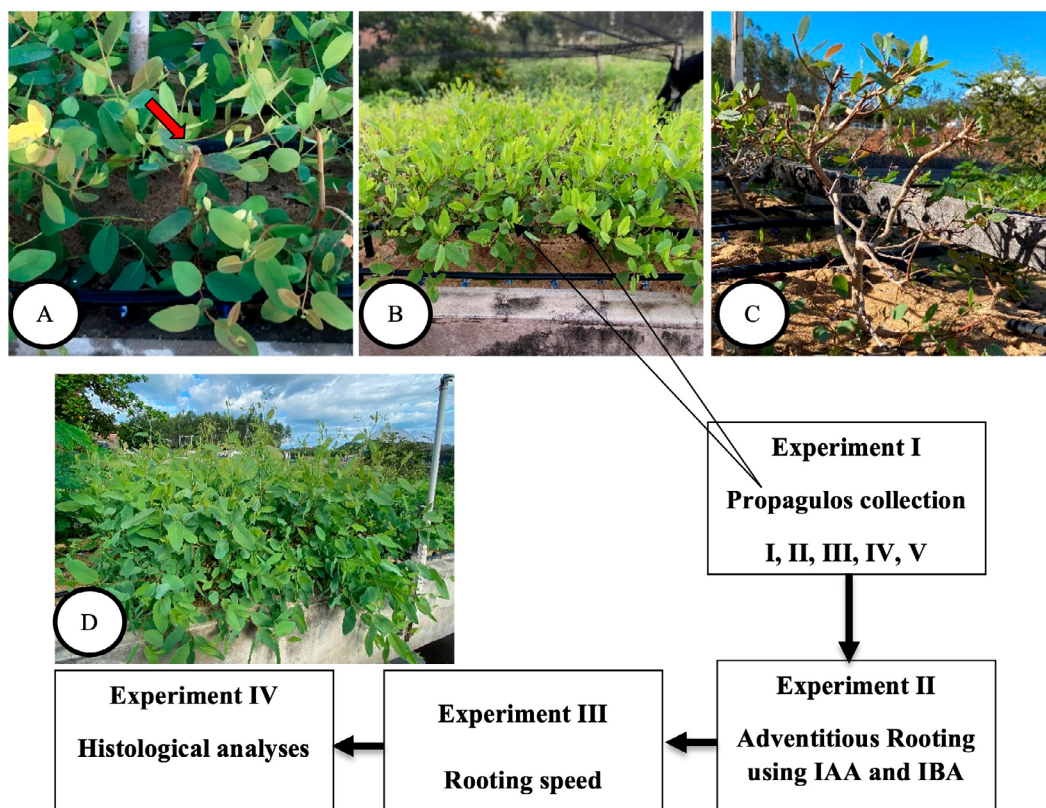


Figure 2. Clonal propagation of *Eucalyptus camaldulensis* through mini-cutting. (A) General view of the mini-stumps after breaking apical dominance. (B) Mini-stumps before the different shoot collection events with juvenile propagules. (C) the mini-stumps after shoot collection events. (D) Mini-stumps with mature propagules that were not utilized for propagation. Sequence of the experimental design: experiment I, II, III and IV.

Results

Mini-stump survival and production

The mini-stump prunings showed no significant differences across the five propagule collections in terms of survival, the number of sprouts per mini-stump, and sprout yield (sprouts/m²/month) (Fig. 3A, B, C). Survival was consistently high at 100% (Fig. 3A). The mini-stumps of the *E. camaldulensis* clone demonstrated efficient propagule production, yielding more than 13 propagules per mini-stump per month (Fig. 3B) and 900 sprouts per m² per month (Fig. 3C).

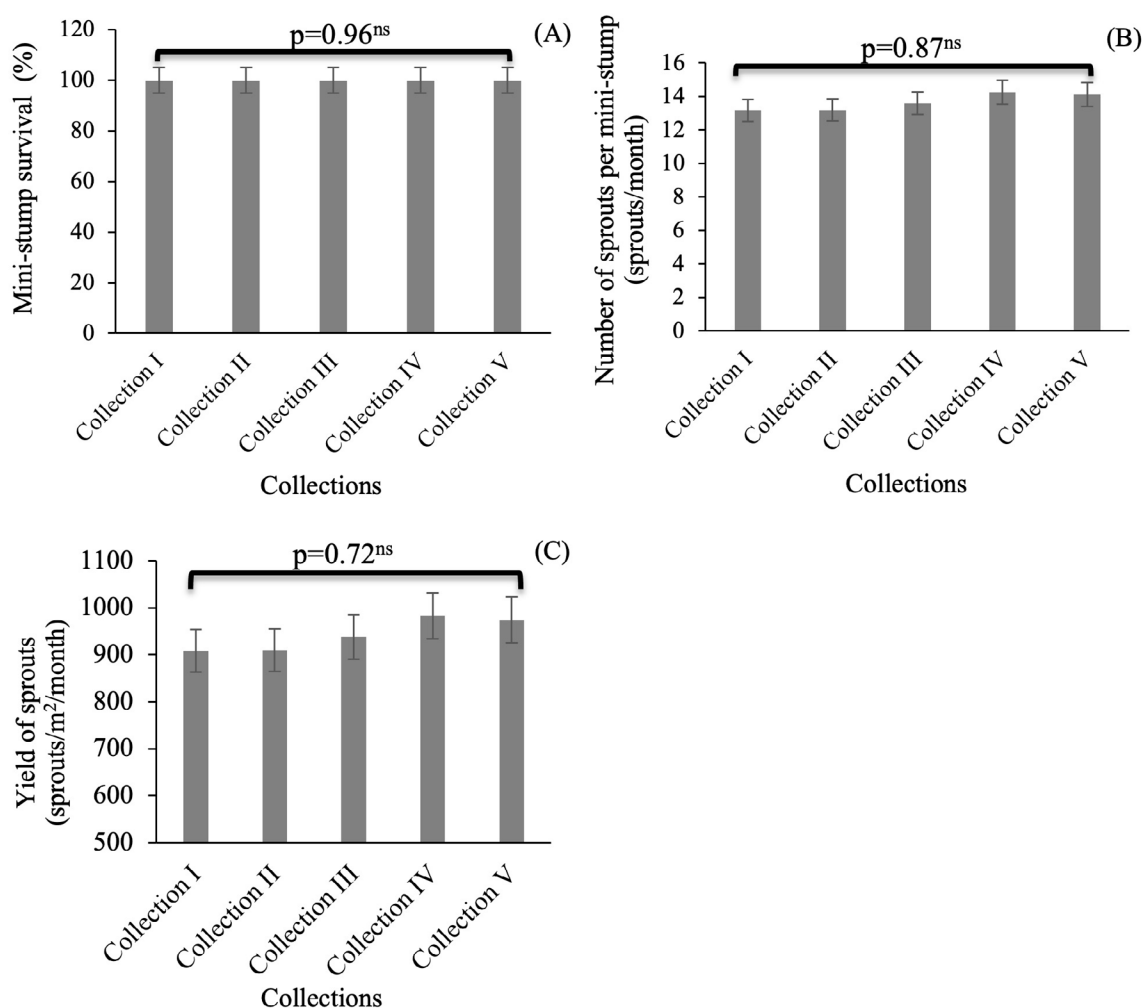


Figure 3. Impact of mini-stump pruning on propagule production in the *Eucalyptus camaldulensis* clone across successive sprout collections. (A) Mini-stump survival, (B) number of sprouts per mini-stump, and (C) the yield of sprouts (sprouts/m²/month). Bars indicate the standard error of the mean of four biological replicates (n=40). ns: not significant.

Influence of IAA and IBA

There was a significant interaction between auxins and their concentrations for most morphological variables, except for longest root length (cm per mini-cutting) and number of roots (units per mini-cutting), $p < 0.05$ (Table 1). Auxins significantly affected only base oxidation (%) and callus formation (%). Conversely, concentrations

significantly influenced rooting (%), survival (%), base oxidation (%), and callus formation (%).

The mini-cuttings of the *E. camaldulensis* clone showed responsiveness to adventitious rooting, with more than 55% rooting and survival in the control treatment (Figs. 4A, B). Both IAA and IBA improved rooting and survival equally, up to a concentration of 2,000 mg·L⁻¹. At a concentration of 4,000 mg·L⁻¹, the auxin IAA resulted in higher rooting rates (75%) compared to IBA (50%) (Fig. 4A, B). The highest percentages of rooting and survival were observed at 2,000 mg·L⁻¹, reaching 80% (Fig. 4A) and 85% (Fig. 4B), respectively.

Base oxidation was higher (14%) in mini-cuttings treated

with 1,000 mg·L⁻¹ of IAA (Fig. 4C), while callus formation was more prominent in the control and at 4,000 mg·L⁻¹ of IBA (Fig. 4D). However, different concentrations of IAA and IBA did not influence the number of roots (units per mini-cutting) or the longest root length (cm per mini-cutting) (Table 1 and Figs. 4E, F).

Collectively, these results suggest that the mini-cuttings of the *E. camaldulensis* clone are highly responsive to

Table 1. Effects of auxin types (IAA and IBA), concentrations (0 - control; 1,000; 2,000; and 4,000 mg·L⁻¹), and their interactions (Auxins types:Concentrations) on rooting (%), survival (%), base oxidation (%), callus formation (%), longest root length (cm mini-cutting⁻¹) and number of roots (unit mini-cutting⁻¹) in *Eucalyptus camaldulensis* mini-cuttings. ANOVA of generalized linear model (Poisson function) to rooting (%), survival (%), base oxidation (%), presence of callus (%), of *Eucalyptus camaldulensis* mini-cuttings subjected to auxins (IAA and IBA) and concentrations (0 - control; 1,000; 2,000; and 4,000 mg·L⁻¹).

Effect	Pr (>Chi)					
	Rooting	Survival	Base Oxidation	Presence of Callus	Longest Root Length	Number of Roots
Auxins types	0.0567	0.0566	0.0292	1.5 ⁻⁰⁹	0.1789	0.9364
Concentrations	4.5 ⁻¹⁴	1.6 ⁻¹⁴	< 2.2 ⁻¹⁶	< 2.2 ⁻¹⁶	0.5919	0.9462
Auxins types: Concentrations	0.0062	0.0179	1.8 ⁻¹²	1.6 ⁻¹²	0.7854	0.4426

Statistical analysis was performed using ANOVA based on a generalized linear model (Poisson function). The numbers in table indicate the p-value.

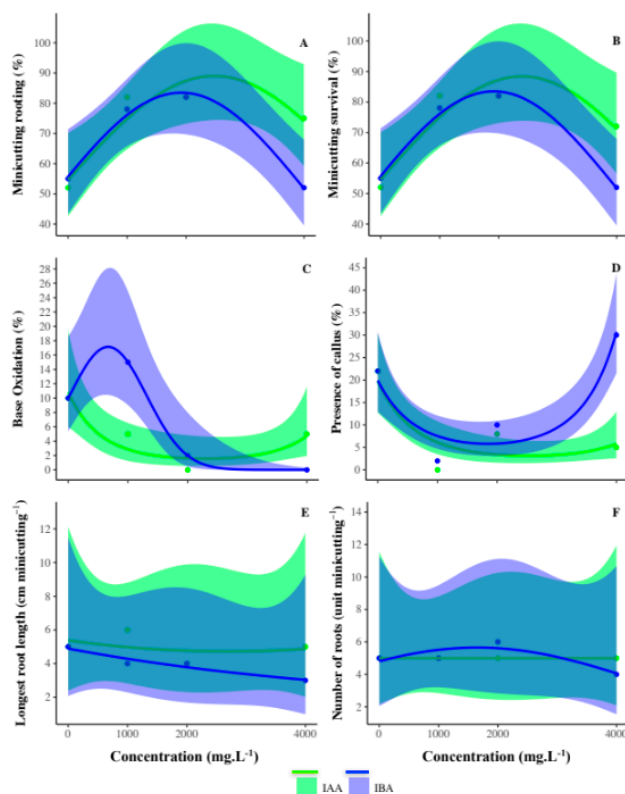


Figure 4. Effects of Indole-3-Acetic Acid (IAA) and Indole-3-Butyric Acid (IBA) on adventitious rooting of *Eucalyptus camaldulensis* clone propagated by mini-cuttings. (A) Mini-cutting rooting (%), (B) mini-cutting survival (%), (C) base oxidation (%), (D) presence of callus (%), (E) longest root length (cm mini-cutting⁻¹), and (F) number of roots (unit mini-cutting⁻¹). 95% confidence interval predicted by generalized linear model (Poisson function).

adventitious rooting and that the auxins IAA and IBA can significantly enhance rooting and survival.

Rotting speed

There was a significant interaction between auxin application and days for rooting (%), callus formation (%), and oxidation (%), $p < 0.05$. Auxin application significantly affected rooting (%) and callus formation (%). However, the number of days in the greenhouse produced significant differences ($p < 0.05$) across all morphological variables (Table 2).

As expected, initial morphological changes in the basal region of the mini-cuttings were observed seven days after their introduction to the greenhouse (Fig. 5A, C). Adventitious root formation began with swelling at the bases of the mini-cuttings, followed by callus formation. Over time, the swelling response was similar among treatments with IAA, IBA, and the control, with higher averages (70%) recorded between 7 and 14 days (Fig. 5A). In contrast, greater callus formation (60%) was observed in the control treatment at 14 days (Fig. 5C). The mini-cuttings of the *E. camaldulensis* clone also exhibited low base oxidation, with the highest percentage (6%) occurring in the IAA treatment at 28 days (Fig. 5D).

Rhizogenesis in the mini-cuttings began on Day 14 for IAA and IBA treatments, while it was delayed for the control treatment (Fig. 5B). Thus, IAA and IBA treatments increased the rooting speed of the mini-cuttings, resulting in the highest rooting percentages throughout the evaluation period. For both IAA and IBA treatments, up to 45% of the mini-cuttings had rooted by Day 21, with rooting

Table 2. Effects of auxin application (control, IAA, and IBA), evaluation days, and their interaction (Auxin application:Evaluation days) on swelling (%), rooting (%), callus (%), oxidation (%), number of roots (unit mini-cutting⁻¹) and longest root length (cm mini-cutting⁻¹) in *Eucalyptus camaldulensis* mini-cuttings. ANOVA of generalized linear model (Poisson function) to swelling (%), rooting (%), callus (%), oxidation (%), number of roots (unit mini-cutting⁻¹) and longest root length (cm mini-cutting⁻¹) of mini-cuttings of *Eucalyptus camaldulensis* clone subjected to auxin application (control, IAA and IBA) and days. The numbers in table indicate the p-value.

Effect	Pr (>Chi)					
	Swelling	Rooting	Callus	Oxidation	Number of Roots	Longest Root Length
Auxins application	0.9307	5.8 ⁻¹²	2.1 ⁻⁰⁵	1.0000	0.0647	0.2089
Evaluation days	<2 ⁻¹⁶	< 2.2 ⁻¹⁶	< 2.2 ⁻¹⁶	<2 ⁻¹⁶	< 2 ⁻¹⁶	<2 ⁻¹⁶
Auxins application: Evaluation days	0.9712	2.4 ⁻¹³	6.9 ⁻⁰⁵	0.0122	0.7560	0.9990

Statistical analysis was performed using ANOVA based on a generalized linear model (Poisson function). The numbers in table indicate the p-value.

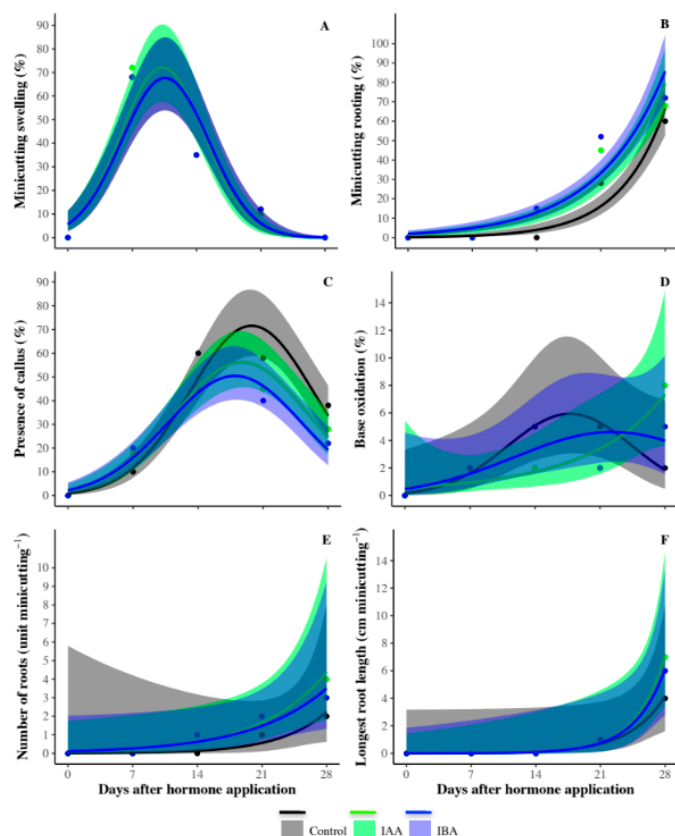


Figure 5. Indole-3-Acetic Acid (IAA) and Indole-3-Butyric Acid (IBA) increased the rooting speed of mini-cuttings from propagules of the *Eucalyptus camaldulensis* clone. (A) Mini-cutting swelling (%), (B) mini-cutting rooting (%), (C) presence of callus (%), (D) base oxidation (%), (E) number of roots (unit mini-cutting⁻¹), and (F) longest root length (cm mini-cutting⁻¹). 95% confidence interval predicted by generalized linear model (Poisson function).

stabilization occurring only after 28 days (Fig. 5B). Therefore, the time required for mini-cuttings to remain in the greenhouse to achieve adequate rooting percentages depends on auxin application, with a longer duration required in the absence of IAA or IBA (Fig. 5B).

Root length and the number of roots did not differ significantly among the control, IAA, and IBA treatments (Table 2) and remained relatively stable until Day 21. By Day 28, the longest root reached 4.8 cm, with an average of 2.6 roots per mini-cutting (Figs. 5E, F).

Histological analyses

The shoots of the *E. camaldulensis* clone contained a central pith surrounded by xylem, vascular cambium, phloem, cortex, and epidermis (Fig. 6A). The stems were rectangular in transverse section, with a prominent ring of sclerenchyma cells at the periphery of the vascular tissue. Additionally, the cortex, near the epidermis, contained cavities with oil (idioblasts) (Fig. 6A). Secondary growth was evident in all studied mini-cuttings, characterized by cambium activity and well-developed secondary phloem and xylem.

Adventitious roots originated from the xylem, phloem, or vascular cambium in mini-cuttings treated with IAA and IBA (Figs. 6B, C). However, in untreated mini-cuttings (without auxins), roots arose from the phloem or vascular cambium (Fig. 6B). Root formation was direct, with the same behavior observed in auxin-treated and untreated mini-cuttings; no simultaneous formation of callus and adventitious roots occurred. The sclerenchyma ring was ruptured by root primordia formation (Fig. 6C). After seven days, groups of dividing cells (meristemoids) became oval

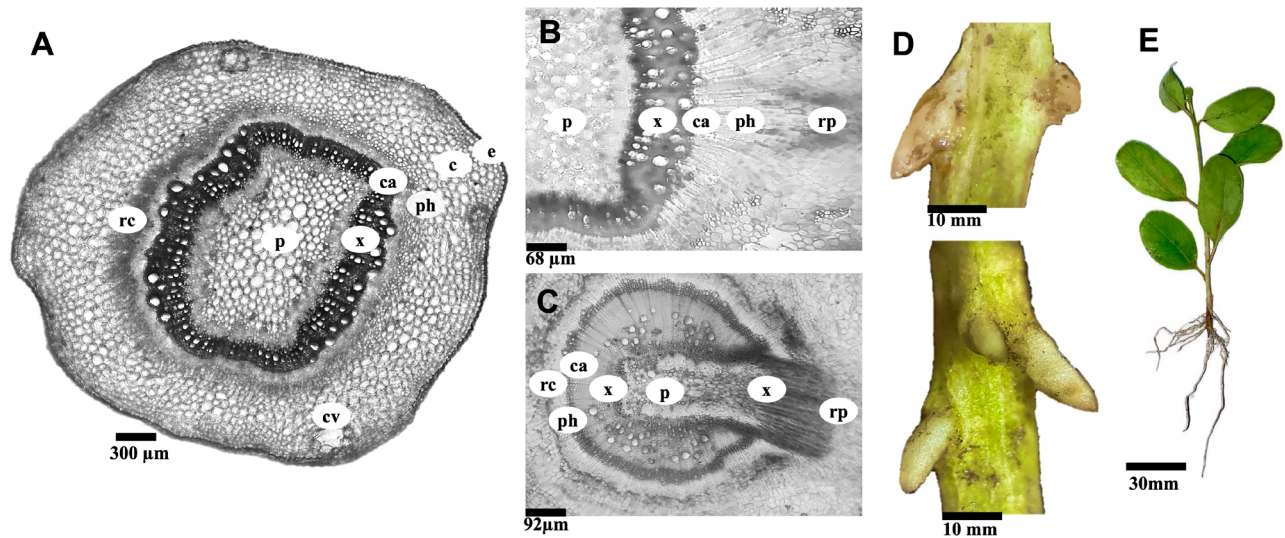


Figure 6. Transverse sections of the basal regions of *Eucalyptus camaldulensis* mini-cuttings and their role in adventitious root formation. (A) Mini-cutting base overview, (B) detail showing the formation of adventitious root primordium from vascular and phloem tissue, (C) developing adventitious root from pith tissue, (D) external adventitious root primordia, and (E) root elongation. **c:** cortex, **e:** epidermis, **ph:** phloem, **p:** pith, **rc:** sclerenchyma ring, **ca:** vascular cambium, **cv:** cavity containing oil (idioblasts), **x** = xylem.

in shape and differentiated into root primordia (Fig. 6C). These primordia extended perpendicularly to the main axis toward the epidermis.

At approximately 14 days, young white roots began to emerge from the epidermis and became visible outside the propagule (Fig. 6D). Adventitious roots developed from both corner and side positions, up to 2 cm above the base of the mini-cuttings (Fig. 6D). In the following weeks, the roots continued to grow and initiated the production of secondary lateral roots (Fig. 6E).

These results suggest that root primordia formation occurred within one to two weeks, and the sclerenchyma ring did not prevent adventitious rooting. In mini-cuttings treated with IAA and IBA, adventitious root primordia originated from vascular, phloem, and pith tissues. In contrast, in untreated mini-cuttings, roots developed from the vascular cambium and phloem.

Discussion

The productivity of the mini-stumps stands out as a key deciding factor for the success of mini-cuttings, considering the productivity of mini-stumps as an objective parameter of the mini-garden's performance. The productivity of the mini-stumps is influenced by variables such as the type of clonal mini-garden, the nutritional management employed, seasonality, light quality and irradiance, air humidity and temperature, mineral nutrition, and water availability (Wendling et al., 2014; Batista et al., 2015; Vilasboa et al., 2022; Lima et al., 2022).

Here, we present evidence that the mini-stumps of the *E. camaldulensis* clone, cultivated under semiarid conditions, exhibited consistent performance due to 100% survival and the production of 13 propagules per mini-stump per month

in establishing a clonal mini-garden. This performance was maintained even in collections III and IV, conducted in August and September—months characterized by low relative humidity (60%) and high temperatures (28°C). Our results suggest that the mini-stumps of this specific genotype have excellent regrowth potential after pruning. In this sense, both the genotype and environmental control optimized the survival and productivity of the mini-stumps.

Regarding environmental conditions in semiarid regions, temperature and water availability stand out as variables that can influence mini-garden performance. However, water limitations can be mitigated with an efficient irrigation system, such as the drip irrigation used in the present study. Temperature is another factor influencing the productivity of mini-stumps and the quality of mini-cuttings (Trueman et al., 2013). The significant productivity of the mini-garden in this study at a temperature close to 30°C can be attributed to the complete adaptation of the mini-stumps to the environment, which favors the process of cell division. The processes of cell proliferation, sprout emission, and root formation are favored by higher temperatures, up to a certain extent (Lima et al., 2022). In this context, the application of mini-tunnels, a recent forestry practice used to increase the temperature of the mini-stump propagation environment during colder seasons (Batista et al., 2015; Lima et al., 2022; Vilasboa et al., 2022), is unnecessary in semiarid conditions due to naturally high temperatures.

In *E. camaldulensis*, regrowth ability from stumps (Heth et al., 1986; Reuveni et al., 1990), mini-stumps (Bindumadhava et al., 2011), and micro-stumps (Shanthi et al., 2015) has been documented, supporting the results of the present study. These studies demonstrate the resistance of this species to successive pruning, with a favorable response in the emission of epicormic shoots. Furthermore, in our study, both mini-stumps and shoots were free

of pathogens, ensuring their suitability for subsequent collection of mini-cuttings.

Notably, the productivity of mini-stumps observed in the present study exceeds values reported for *Eucalyptus mini-stumps* in clonal mini-gardens (Bindumadhava et al., 2011; Brondani et al., 2012) and is comparable to productivity achieved using mini-tunnels in different seasons (Batista et al., 2015; Lima et al., 2022; Vilasboa et al., 2022). Moreover, our data closely align with productivity levels reported for mini-stumps of other species grown under semiarid conditions (Souza et al., 2023; Silva et al., 2022). This reaffirms that high temperatures and low relative humidity, typical of semiarid regions, are not barriers to vegetative propagation by mini-cutting when proper management practices are employed. However, it is critical that plants undergo acclimatization and hardening after the rooting phase before being exposed to field conditions.

In greenhouses, where planting material for forests and plantations is rooted, air humidity is typically higher than in field conditions. Consequently, transplanting to the field can stress the trees, potentially affecting growth and development (e.g., height and diameter), as well as physiological and biochemical processes, including survival. Therefore, acclimatization after the rooting phase is essential, and the plant's reaction in the field depends on the clone's phenotypic plasticity (Gudynaitė-Franckevičienė & Plūra, 2022). The maintenance of survival and sprout production potential observed during August and September (periods of high temperature and low humidity) for the mini-garden in full sun suggests high survival and vigor rates are expected under field conditions for these plants.

Additionally, shoots from mini-stumps exhibited adventitious rooting potential. The high rooting rate observed in this study reflects a complex interaction between various factors, including the genetic control of the propagule donor plant (genotype), the nutritional and water balance of the mini-stumps, and the genotype's high regrowth capacity after pruning. The production of juvenile propagules with high vigor, combined with appropriate control of rooting conditions, contributes to these results (Wendling et al., 2014; Gianguzzi et al., 2020). Together, these factors influence rooting capacity and determine the success of clonal propagation by mini-cuttings (Wendling et al., 2014; De Almeida et al., 2017).

Efforts to increase the rooting rate of vegetative cuttings often involve optimizing environmental conditions, hormone applications, and physiological aspects of the hedge stock (Trueman et al., 2013). In this study, we identified auxin as a key factor for improving rooting potential in the selected *E. camaldulensis* clone. Adventitious rooting development is regulated by a crosslinked network of hormone signaling, with auxin playing a central role. Auxin accumulation at rooting sites, mediated by polar transport, is a specific initial response of rooting-competent tissues (Pizarro and Díaz-Sala, 2019).

Procedures to improve adventitious root induction often involve auxin treatments and other factors, such as light or temperature, which interact with auxin activity (De Almeida

et al., 2017). For instance, Vilasboa et al. (2022) reported differentially expressed transcripts during adventitious root formation in response to auxin and other factors, highlighting the molecular regulation of this process, which involves auxin-, gibberellin-, jasmonic acid-, and ethylene-mediated responses, wounding, sugar signaling, and cell cycle regulation. Therefore, the application of exogenous auxin is necessary for stimulating adventitious root development in most tree species (Díaz-Sala, 2020).

In mini-cutting, IAA and IBA are commonly used to enhance rooting rates (Hartmann et al., 2011). However, the choice of auxin depends largely on the species or genotype (Steffens and Rasmussen, 2016; Ayala et al., 2022). Our work describes the application of both IAA and IBA at an optimal concentration of 2,000 mg·L⁻¹ to improve rooting.

At the onset of clonal propagation of *E. camaldulensis*, rooting rates ranged between 4% and 50%, depending on the cloned genotype (Heth et al., 1986; Reuveni et al., 1990). Using the mini-cutting technique, some genotypes achieved up to 97% adventitious rooting (Bindumadhava et al., 2011). However, for recalcitrant genotypes, micropropagation techniques inducing somatic embryos improved propagation capacity, enabling large-scale multiplication of elite trees (Girijashankar, 2012). The adventitious rooting recorded from 33 superior performing *E. camaldulensis* clones propagated by microcutting was between 24.8% to 100%, depending on the genotype (Shanthi et al., 2015). The adventitious rooting rates observed in this study (>80%) underscore the high propagation potential of this genotype under semiarid conditions.

As noted, the genetic background of the plant material is of great importance for the rooting of *E. camaldulensis*. The results of the present study show that the *E. camaldulensis* genotype selected in a semiarid conditions has a high potential for adventitious rooting, with rates exceeding 80% when applying 2,000 mg·L⁻¹ of IAA or IBA. In this sense, rooting is not a limiting factor for the large-scale multiplication of this genotype. A common practice in mass propagation systems is to select clones with rooting levels of 70% or higher (Xavier et al., 2013), while clones with poorer rooting performance are discarded. Furthermore, the application costs of IAA and IBA are offset by the benefits of these auxins, as their application can reduce the time required for rooting. Optimizing the rooting duration in the greenhouse can maximize the utilization of facilities, mitigate the adverse impacts of high humidity on the adventitious root system, prevent post-rooting mortality (Brondani et al., 2012), and consequently reduce the costs of genotype multiplication.

Regarding the origin of adventitious roots in stem cuttings, it can vary significantly according to the plant species and types of propagules (Bryant and Trueman, 2015). Anatomical analysis of mini-cutting stems showed similarities in structural development patterns across treatments with IBA, IAA, and the control. There are two patterns of root development in vegetative propagules: direct and indirect rhizogenesis. In the direct pattern, competent cells initiate cell division, and root formation occurs directly

from the original stem tissues (Xavier et al., 2013). On the other hand, the indirect pattern involves the formation of irregular masses of parenchyma cells (callus formation), which is then followed by rooting (Hartmann et al., 2011). As described for other *Eucalyptus* species (Goulart et al., 2014; Bryant and Trueman, 2015), the rooted mini-cuttings of the *E. camaldulensis* genotype presented a direct pattern of adventitious root formation.

However, as previously described, auxin accelerated anatomical changes at the bases of mini-cuttings, most notably at the root primordia formation stage. Unlike what was reported by Bryant and Trueman (2015), the precise location of adventitious root initiation was easy to identify in *E. camaldulensis* mini-cuttings. Anatomical analyses showed that roots were emitted close to the vascular cambium in both control and IAA- or IBA-treated mini-cuttings, a pattern also observed in other forest tree species, such as *Eucalyptus grandis* × *Eucalyptus urophylla* (Goulart et al., 2014), *Corymbia torelliana* F.Muell., and *E. camaldulensis* (Bryant and Trueman, 2015).

Only in IAA- and IBA-treated mini-cuttings was disorganization of the pith and xylem tissue observed, which could be related to the capacity of these phytohormones to promote cell proliferation (Sánchez et al., 2007; Wang et al., 2022). Auxin has been shown to induce xylem differentiation in *Arabidopsis* (Ohashi-Ito et al., 2013). The application of exogenous auxin is expected to stimulate the activation of cells in the vascular cambium, thus facilitating cell multiplication and rhizogenesis (Sá et al., 2022).

Some literature reports suggest that, in certain cases, the presence of a sclerenchyma ring may delay or completely obstruct root emission in vegetative propagules (Wendling et al., 2015). However, the continuous and discontinuous sclerenchyma layers observed in the mini-cuttings of the *E. camaldulensis* genotype exhibit different behavior and do not appear to inhibit the emission and development of adventitious roots. Taken together, our results demonstrate that the genotype selected in a semiarid conditions has a natural competence for adventitious rooting.

The mini-cutting technique applied has notable applications in propagating the selected genotype. For example, the productivity and absence of mortality in mini-stumps during successive pruning, combined with the rooting rate, ensure the genotype's potential to be multiplied via mini-cutting. Rooting rates can be further increased by applying IAA and IBA, as these auxins exhibit equivalent rooting performance. This provides substantial benefits for commercial production, including reduced time to root formation, potentially due to the activation of multiple tissues (pith and xylem) for the direct origin of the root system.

The findings from this study offer valuable insights for mini-garden management and adventitious rooting, facilitating the effective application of the mass mini-cutting technique in commercial nurseries to propagate the *E. camaldulensis* genotype selected in a semiarid conditions.

Conclusions

Altogether, the present data support the following conclusions: (I) The mini-cutting technique is applicable and viable for the propagation of the *E. camaldulensis* genotype under semiarid conditions. (II) The survival and productivity of mini-stumps were not influenced by low relative humidity (60%) and high temperature (28°C), as well as the response of propagules to adventitious rooting. (III) The pattern of root tissue initiation is directly from the vascular cambium and consequently the sclerenchyma ring surrounding the vascular system was not a mechanical barrier to root formation. However, IAA and IBA treatment can contribute to the disorganization of pith and xylem tissues, leading to the formation of adventitious roots. (IV) The application of rooting hormones, IAA and IBA, on mini-cuttings enhanced the rooting ability of the *E. camaldulensis* genotype. Additionally, auxin application at the base of the mini-cuttings, also induces faster rooting compared to the control. The accelerated rooting process shortened the duration required for mini-cuttings to root in the greenhouse, making it a viable option for adoption in commercial nurseries to enhance operational efficiency and optimize their facilities.

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