Optimización de las condiciones *in vitro*/ *ex vitro* para la multiplicación clonal de *Eucalyptus globulus* e híbridos interespecíficos

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Resumen

El programa de mejoramiento genético del Instituto Nacional de Investigación Agropecuaria (INIA, Uruguay) para *Eucalyptus globulus* y *E. maidenii* tiene como objetivo superar los problemas sanitarios y mejorar la baja capacidad de enraizamiento de estas especies. Esto mediante la selección de clones con un temprano cambio de follaje y cruzamientos interespecíficos entre *E. grandis* x *E. globulus* y *E. grandis* x *E. maidenii*, aprovechando la resistencia y la mayor capacidad de enraizamiento de *E. grandis*. Los clones del programa de mejoramiento se propagan mediante micropropagación, pero su limitada capacidad de enraizamiento y la baja sobrevivencia *ex vitro* dificultan su multiplicación. Con el objetivo de optimizar los protocolos de micropropagación y aclimatación de *E. globulus* e híbridos, se evaluó el suministro de diferentes tipos y concentraciones de citoquininas al medio de multiplicación. Además, se evaluó el uso de agentes microbianos durante el trasplante, a partir del desarrollo de celdas de crecimiento y condiciones óptimas de invernáculo. El suministro de metatopolina (mT) al medio de multiplicación mejoró la multiplicación y enraizamiento *in vitro*, con incrementos en las tasas de proliferación y elongación de los explantes, así como el porcentaje de enraizamiento y la calidad de raíz. Asimismo, la mejor calidad de planta *in vitro* obtenida con mT favoreció la aclimatación, con mayores porcentajes de supervivencia, altura y longitud de raíces. Por otro lado, la inoculación con dos cepas de *Azospirillum brasilense* y una cepa *Trichoderma asperellum* favoreció el crecimiento durante la aclimatación, lo que se reflejó en una mayor altura de planta y una mayor longitud de raíces principales y laterales. Estos resultados permitieron mejorar la eficiencia del protocolo de micropropagación, mediante el uso de mT, y el ajuste de las condiciones de aclimatación mediante la aplicación de agentes microbianos y el uso de celdas de crecimiento en condiciones de invernáculo.

Palabras clave: cultivo de tejidos, enraizamiento *in vitro*, citoquininas, benciladenina, agentes promotores del crecimiento vegetal

Optimization of *in vitro*/*ex vitro* conditions for clonal multiplication of *Eucalyptus globulus* and interspecific hybrids

Summary

The breeding program at the National Institute of Agricultural Research (INIA, Uruguay) for *Eucalyptus globulus* and its subspecies aims to overcome phytosanitary challenges and enhance their low rooting capacity. This is achieved through the selection of clones with early foliage change and interspecific crossings between *E. grandis* x *E. globulus* and *E. grandis* x *E. maidenii*, taking advantage of the resistance and better adventitious rooting capacity of *E. grandis*. Clones from the program are propagated through micropropagation; however, their limited rooting capacity and low *ex vitro* survival hinder their multiplication. With the aim of optimizing the micropropagation and acclimatization protocols for *E. globulus* and hybrids, the supply of different types and concentrations of cytokinins to the multiplication medium was evaluated. Additionally, the use of microbial agents during acclimatization was assessed, considering the development of growth units and optimal greenhouse conditions. The use of *Meta*-topolin (mT) improved *in vitro* multiplication and rooting, increasing the proliferation and elongation rates of the explants, as well as the rooting percentage and root quality. Furthermore, the enhanced *in vitro* plant quality obtained with mT enhanced acclimatization, resulting in higher survival rates, plant height and root length. On the other hand, inoculation with two strains of *Azospirillum brasilense* and a strain of *Trichoderma asperellum* promoted growth during acclimatization, resulting in greater plant height and longer main and lateral roots lengths. These results improved the efficiency of the micropropagation protocol through the use of mT, and by optimizing acclimatization conditions through the application of microbial agents and the use of growth cells under controlled greenhouse conditions.

Keywords: tissue culture, *in vitro* rooting, cytokinin, benzyladenine, plant growth promoting microorganism

1. Introducción

El complejo forestal de Uruguay está compuesto por dos grandes cadenas de producción, la industria de madera sólida y la industria de celulosa (Barros y Marisquirena, 2023; MGAP-DIEA, 2024). La industria de la celulosa está ampliamente dominada por empresas multinacionales con modelos de integración vertical que concentran su producción en plantaciones de *Eucalyptus grandis* y *E. dunnii* (Barros y Marisquirena, 2023; Morales et al., 2018). Estas dos especies ocupan el 55 % de la superficie forestal efectiva del país, la cual supera el millón de hectáreas (MGAP-DIEA, 2024). Además, en menor escala, existe un subsector compuesto por empresas medianas y pequeñas, en el cual la principal especie plantada es *E. globulus*, debido a su mayor demanda internacional y valor comercial (Balmelli et al., 2023; Morales et al., 2018). El área plantada de *E. globulus* y subespecies es del entorno al 12 % de la superficie forestal efectiva (MGAP-DIEA, 2024).

La reducción del área plantada de *E. globulus* es consecuencia de que la especie es altamente susceptible a la enfermedad foliar causada por *Teratosphaeria nubilosa*, la cual provoca manchas necróticas y una prematura defoliación del árbol (Balmelli et al., 2023; Morales et al., 2017; Pérez et al., 2009). Desde su aparición en 2007, esta enfermedad se ha expandido por todo el país, lo que ha afectado el crecimiento y sobrevivencia de las plantaciones jóvenes (Balmelli et al., 2014; Balmelli et al., 2016). Este problema sanitario ha provocado la sustitución de *E. globulus* por especies más tolerantes, como son *E. grandis*, *E. dunnii* y *E. smithii* (Balmelli et al., 2023; Morales et al., 2018). Como resultado, en los últimos años el área plantada de *E. globulus* se ha reducido, concentrándose en la región sur-este del país debido a una mayor adaptación a las condiciones marítimas (Morales et al., 2018).

Para superar estas limitantes, los programas de mejoramiento genético de *Eucalyptus* se enfocan en lograr avances genéticos en el corto tiempo y

basan sus estrategias en la selección de árboles superiores y la hibridación interespecífica (Assis y Mafia, 2007; Xavier y Silva, 2010). La selección de clones de *E. globulus* con un cambio precoz de follaje es un método eficaz de escape a *T. nubilosa*. Esto se debe a que el follaje adulto presenta una mayor resistencia a la enfermedad en comparación con el follaje juvenil. Además, el cambio de follaje en *E. globulus* presenta un fuerte control genético, con altos valores de heredabilidad (Balmelli et al., 2014; Balmelli et al., 2016; Quezada et al., 2023). Por otro lado, los cruzamientos interespecíficos con especies resistentes a enfermedades de interés permiten incorporar complementariedad, adaptabilidad y vigor híbrido entre parentales selectos (Assis y Mafia, 2007).

Los programas de mejoramiento genético recurren a la técnica de micropropagación para el rescate y la multiplicación de genotipos recalcitrantes de interés y materiales adultos con una disminuida capacidad de enraizamiento (De Almeida et al., 2017; Vilasboa et al., 2021). La micropropagación es una técnica de cultivo in vitro que consta de etapa principales, introducción de los materiales a condiciones *in vitro*, multiplicación, enraizamiento y posterior aclimatación. Esta técnica mejora el vigor y la capacidad de enraizamiento de los materiales mediante el proceso de rejuvenecimiento o revigoramiento (Wendling et al., 2014a, 2014b; Zhang et al., 2020). Además, la micropropagación permite producir plantas madres para estaquillado y plantas *in vitro* a escalas comerciales en corto tiempo y en áreas reducidas (Assis et al., 2004; De Almeida et al., 2017; Xavier y Silva, 2010). Sin embargo, la clonación de especies recalcitrantes como *E. globulus* presenta bajos porcentajes de enraizamiento, con raíces engrosadas, formación de callos y estructura quebradiza (Aumond et al., 2017; Brondani et al., 2012; Druege et al., 2019; Jofré et al., 2016). Además, durante la aclimatación se produce una alta mortalidad, ya que las microplántulas son altamente sensibles a las condiciones *ex vitro*, debido a la presencia de estomas no funcionales, baja capacidad fotosintética y una

débil conexión vascular entre el tallo y las raíces (Chaari-Rkhis et al., 2015; Grzelak et al., 2024).

Las líneas de investigación en micropropagación de *Eucalyptus* se enfocan en mejorar las condiciones de las etapas limitantes de enraizamento y aclimatación. En especies de fácil micropropagación, se busca optimizar las etapas del proceso con el objetivo de acortar los tiempos de producción y reducir los costos (Brondani et al., 2012; Trueman et al., 2023). Sin embargo, en especies de difícil micropropagación, el enfoque se centra en aumentar la capacidad de enraizamiento y facilitar la adaptación a condiciones *ex vitro* (Brondani et al., 2018; Faria et al., 2023; Trueman et al., 2018).

El ajuste de las condiciones de multiplicación *in vitro* es fundamental para mejorar la eficiencia de los protocolos, ya que en esta etapa se promueve la proliferación y elongación de los brotes y se define la calidad de las microplántulas a obtener. El desarrollo de microplántulas de mayor tamaño y vigor, con raíces funcionales, facilita su posterior adaptación a las condiciones *ex vitro* (Abiri et al., 2020; Trueman et al., 2018). El uso de citoquininas en el medio de multiplicación es necesario para estimular la proliferación *in vitro*; sin embargo, el tipo y la concentración pueden afectar el enraizamiento en etapas posteriores (Tahir et al., 2022). La benciladenina (BA), citoquinina comúnmente utilizada en los protocolos de micropropagación, ha presentado efectos inhibitorios sobre el enraizamiento. Para superar esta limitante y optimizar la multiplicación, se ha propuesto la sustitución de la BA por citoquininas similares, como la metatopolina (mT), una citoquinina de mayor actividad y rápida degradación (Aremu et al., 2017; Strnad et al., 1997).

Durante la aclimatación se busca aumentar la sobrevivencia de las microplántulas mediante la promoción del crecimiento radicular inicial y un mayor control de las condiciones de temperatura y humedad para reducir el estrés del trasplante (Brondani et al., 2018; Maravilha et al., 2023). En el estaquillado de *Eucalyptus*, la inoculación con microorganismos promotores del crecimiento vegetal es una práctica ampliamente utilizada, ya que

incrementa la capacidad de enraizamiento y mejora la calidad de las raíces formadas (Mafia et al., 2007). Sin embargo, son escasos los estudios de uso de microorganismos aplicados en la aclimatación de las microplántulas, lo que representa una oportunidad para optimizar esta etapa (Dwiyani et al., 2024). Por otro lado, las condiciones *in vitro* difieren grandemente de las condiciones del trasplante suponiendo un estrés para las plantas. Con el fin de facilitar la adaptación al trasplante y dar soporte a las raíces durante la aclimatación, se desarrolló una estructura que denominamos *celdas de crecimiento*, a partir de la modificación de rizotrones empleados para el estudio de las raíces (Reni et al., 2024). Complementariamente, las celdas de crecimiento se utilizaron en invernáculo, bajo sistema de humedad controlada mediante nebulizador ultrasónico.

Esta maestría se desarrolló en el marco del programa de mejoramiento genético de *E. globulus* y *E. maidenii* del Instituto Nacional de Investigación Agropecuaria (INIA, Uruguay). El programa tiene como objetivo obtener genotipos de buen comportamiento productivo y sanitario, especialmente frente a *T. nubilosa*. Para esto se utilizan dos estrategias en paralelo, la selección de individuos de *E. globulus* con cambio precoz de follaje y la selección de híbridos con *E. grandis* resistentes a *T. nubilosa*. En ambos casos el rescate de los clones y la obtención de plantas madres para técnicas de estaquillado se realiza por micropropagación, para lo cual es necesario levantar varias limitantes relacionadas a la dificultad de enraizamiento y aclimatación de las microplántulas.

El objetivo general de esta tesis fue evaluar las condiciones *in vitro* y *ex vitro* del protocolo de micropropagación de *E. globulus*, *E. grandis* x *E. globulus* y *E. grandis* x *E. maidenii*, con el fin de mejorar la capacidad de enraizamiento y la sobrevivencia durante la aclimatación.

En los objetivos específicos se enlistan:

— Evaluar el efecto del suministro de citoquininas en el medio de multiplicación sobre la multiplicación, enraizamiento *in vitro*, y aclimatación en clones de *E. globulus* e híbridos.

— Evaluar el efecto de la inoculación de plántulas al inicio de la aclimatación con microorganismos promotores de crecimiento vegetal sobre la supervivencia y crecimiento radicular y aéreo.

2. Potential of metatopoline in the *in vitro* multiplication and rooting of *Eucalyptus globulus* Labill. clones

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2.1. Resumen

Antecedentes: *Eucalyptus globulus* Labill. es una especie de gran interés para la industria de la celulosa. Sin embargo, tiene una baja capacidad de enraizamiento, lo que dificulta su clonación a escala comercial. En la micropropagación, el suministro de citoquininas es necesario para estimular la proliferación, pero el tipo y concentración de citoquinina pueden afectar negativamente el enraizamiento adventicio. Este estudio evaluó el efecto de la metatopolina (mT) y la benciladenina (BA) en la multiplicación *in vitro*, elongación y enraizamiento de clones de *E. globulus*. En el medio de multiplicación se suministraron concentraciones de 0,44 y 0,88 µmol BA, 0,41 y 0,83 µmol mT, y un medio control sin citoquininas.

Resultados: Durante la fase de multiplicación, mT aumentó la tasa de proliferación. Además, mT presentó menor producción de explantes pequeños, menores a 0,5 cm de altura. En la fase de enraizamiento, los clones de fácil enraizamiento tratados con 0,41 µmol de mT alcanzaron porcentajes de enraizamiento de 91 %, mientras que con el uso de BA fue de 50 %. Sin embargo, en los clones de difícil enraizamiento no se observaron diferencias en relación con el tipo de citoquinina. Los explantes tratados con mT desarrollaron raíces más largas que los provenientes de BA, que presentaron raíces similares a las del tratamiento control (T).

Conclusión: Con el uso de mT en el medio de multiplicación, se mejoró la elongación, la proliferación y el enraizamiento *in vitro* de *E. globulus*, lo

que resulto en una mejor calidad de explante *in vitro* previo a la etapa de aclimatación.

Palabras clave: benciladenina; citoquinina; tejido de cultivo vegetal; especies recalcitrantes.

2.2. Summary

Background: *Eucalyptus globulus* Labill. is a species of great interest to the pulp industry. However, it has a low rooting capacity, which makes it difficult to clone on a commercial scale. In micropropagation, the supply of cytokinins is necessary to stimulate proliferation, but the type and concentration of cytokinins can negatively affect adventitious rooting. This study evaluated the effect of metatopoline (*m*T) and benzyladenine (BA) on *in vitro* multiplication, elongation, and rooting of *E. globulus* clones. In the multiplication medium, concentrations of 0.44 and 0.88 µmol BA, 0.41 and 0.83 µmol *m*T, and a control medium without cytokinins were supplied.

Results: During the multiplication phase, *m*T increased the proliferation rate. In addition, *m*T presented lower production of small explants, less than 0.5 cm in height. In the rooting phase, easy rooting clones treated with 0.41 µmol of *m*T reached rooting percentages of 91%, while with the use of BA it was 50%. However, in clones with difficult rooting, no differences were observed in relation to the type of cytokinin. Explants treated with *m*T developed longer roots than those originated with BA, which presented roots similar to those of the control treatment (T).

Conclusion: With the use of *m*T in the multiplication medium, the *in vitro* elongation, proliferation and rooting of *E. globulus* was improved, therefore improving the quality of the *in vitro* explants for the acclimatization stage.

Keywords: benzyladenine; cytokinin; plant tissue culture; recalcitrant species.

2.3. Introduction

Eucalyptus globulus is outstanding for the pulp and paper industry due to its wood with high cellulose contents and low lignin, which ensure optimal yields (Assis and Mafia, 2007; Carrillo et al., 2017). However, the species has a low capacity for adventitious rooting, making it difficult to multiply by commercial cloning techniques (Assis and Mafia, 2007; de Almeida et al., 2015). According to Assis et al., (2004) the rooting capacity of stem cuttings of *Eucalyptus* decreases with ontogenetic aging, having a maximum rooting potential at the high juvenility level (mini-cotyledon cuttings). Moreover, according to these authors, part of the juvenility obtained by the *in vitro* rejuvenation process and/or in basal shoots of felled adult trees is gradually eroded during the growth of clones in clonal hedges.

Micropropagation has great potential in *E. globulus* due to the possibility of increasing the rooting capacity in adult materials (Trindade and Pais, 1997). Through successive *in vitro* subcultures, a progressive rejuvenation and reinvigoration of the materials occurs (Wendling et al., 2014; Faria et al., 2023; Isah, 2023). This stage of rejuvenation facilitates the passage from adult phase materials to juvenile phases, increasing the capacity for adventitious rooting (Trindade and Pais, 1997; Baccarin et al., 2015; Isah, 2023). However, the recalcitrant behavior of *E. globulus* is also manifested in micropropagation, which limits the efficiency of this technique in the species (Bennett et al., 1994; Trindade and Pais, 1997; Calderon-Baltierra et al., 2004).

The use of cytokinins in the micropropagation of *Eucalyptus* is fundamental to stimulate the proliferation of the shoots, although depending on the clone, the type and concentration of cytokinin can inhibit the rooting process (Neigishi et al., 2014; Druege et al., 2019; Vilasboa et al. 2019). Benzyladenine (BA) is the cytokinin commonly used by micropropagation laboratories for its easy availability and low cost (Bairu et al., 2007; Aremu et al., 2017). However, there are multiple reports against BA for causing physiological disorders and negative effects on rooting in a wide variety of

species (Aremu et al., 2017; Trueman, 2018; Naaz et al., 2019). In order to overcome the limitations associated with BA, analogues have been identified and developed as alternatives to this cytokinin (Aremu et al., 2017). Among them, metatopoline (*m*T) stands out, a highly active aromatic-type cytokinin similar to BA in chemical structure (Strnad et al., 1992; Strnad et al., 1997). The difference from the BA is the presence of a hydroxyl group on the benzyl ring in the *m*T structure (Aremu et al., 2012). *m*T has been shown to favor conditions in the *in vitro* multiplication phase, such as proliferation rate, and alleviating physiological disorders like hyperhidricity and shoot necrosis, with lower subsequent impact on the rooting process in species such as *Syzygium cumini* and *Harpagophytum* procumbens (Bairu et al., 2011; Aremu et al., 2012, 2017; Naaz et al., 2019).

The aim of the work was to reduce physiological alterations caused by excessive or prolonged use of BA, as well as the reduction of oxidative stress in *Eucalyptus globulus* micropropagated plants. For this purpose, the known positive effect of *m*T on multiplication rate and *in vitro* rooting efficiency was evaluated in *E. globulus* by different concentration of BA and *m*T supplied at the multiplication stage.

2.4. Material and methods

2.4.1. Plant material

Four clones of *E. globulus* (19G8, 19G11, 19G28 and 19G40) from the breeding program of the Instituto Nacional de Investigación Agropecuaria (INIA, Uruguay) were used for the test. The introduction of selected individuals was carried out by sprouting the basal portion of the stem in the INIA Las Brujas greenhouse. Considering the topophytic effect (Assis 2001), the stumps were taken from the basal part of 21-month-old field trees. They were placed in trays with the base of the trunk submerged in water to stimulate the budding of epicormic buds (Figure 1a). The epicormic shoots were extracted and surface disinfected with 10% sodium hypochlorite with two drops of Tween 20 under agitation for 10 minutes and three rinses with

sterile distilled water. Explants were introduced to a MS base culture medium (Murashige and Skoog, 1962).

2.4.2. Multiplication and rooting stage

The multiplication medium was composed of macronutrients from modified MS salts according to Castillo et al. (2020), micronutrients from Woody Plant Medium (WPM) salts (Lloyd and McCown, 1980), and vitamins (de Fossard et al., 1974). It was supplemented with 7.2 $g.L^{-1}$ agar, 10 $g.L^{-1}$ sucrose and 0.05 µM naphthalene acetic acid (ANA) as auxin; adjusted to pH 5.8. Different cytokinins and concentrations (evaluation treatments) were added to this basal medium: BA at 0.44 µM (0.1 $g.L^{-1}$) (BA1); BA at 0.88 µM (0.2 $g.L^{-1}$)(BA2); *m*T at 0.41 µM (0.1 $g.L^{-1}$) (*m*T1); *m*T at 0.83 µM (0.2 $g.L^{-1}$) (*m*T2); and a medium without cytokinins (T). The conditions of the growth chamber were 23 °C (+/-2 °C), 16 hours of photoperiod with fluorescent light and a light intensity of 30 $\mu mol.m^{-2}.s^{-1}$. Subcultures were performed every four weeks and at 90 days the proliferation rate was evaluated as the number of new shoots per explant, and the elongation of shoots was evaluated as the number of explants per height range: less than 0.5 cm, between 0.5 and 1 cm, and greater than 1 cm, measure with a graph paper (Figure 1b).

Figure 1: Micropropagation steps of *E. globulus*. (a) Rescue of individuals, trunk submerged in water to stimulate the sprouting of epicormic buds in INIA Las Brujas greenhouse; (b) 19G28 explant in multiplication stage with proliferating shoots that are subcultured every four weeks after 90 days.

Shoots that reached 1 cm in length were transferred to *in vitro* rooting medium, composed of one third MS salts, 5.5 $g.L^{-1}$ agar, 10 $g.L^{-1}$ sucrose, and 3.0 µM indole butyric acid (IBA), adjusted to a pH of 5.8. The *in vitro* rooting period was 21 days, and the first seven days were in total darkness. The conditions of the growth chamber were 23 °C (+/-2 °C), 16 hours of photoperiod with fluorescent light and a light intensity of 30 $\mu mol.m^{-2}.s^{-1}$. At the end of the rooting phase, the percentage of rooting and the root length were evaluated.

2.4.3. Experimental design and statistical analysis

A factorial experimental design was applied with four genotypes and five treatments (two concentrations of each cytokinin and the control without cytokinins), using 20 repetitions per genotype and treatment. The data obtained were processed using the R software, version 3.6.3 (R Core, 2018). Variables that did not show normal distribution after the Shapiro-Wilks test ($p>0.05$) were evaluated with Poisson distribution. Data were analyzed by analysis of variance (ANOVA, $p<0.05\%$) and comparison of means by Tukey's test ($p<0.05\%$).

2.5. Results

2.5.1. Multiplication stage

In this study, the elongation, proliferation and rooting ability of selected genotypes of *E. globulus* were evaluated using different concentrations of *m*T and BA. Analysis of variance (ANOVA) revealed significant effects of clone and treatment on the number of explants under 0.5 cm, explant between 0.5 and 1 cm, explant over 1 cm, proliferation rate, rooting percentage and root length. An interaction effect between treatments and clones was also detected for rooting percentage and root length (Table 1).

Table 1: Analysis of variance (ANOVA) for the average number of explants per interval of height, the average number of new shoots per plant, the percentage of rooting and the root length of *Eucalyptus* clones at 90 days in the multiplication stage and after 21 days in the rooting stage.

Treatment	GL	Mean Square					
		Explants under 0.5 cm	Explants between 0.5 and 1 cm	Explants over 1 cm	New shoots per plant	Rooting (%)	Rooth length (cm)
Clone	3	32.03***	4.00**	10.45**	12.77**	3.37**	47.75**
Citoquinine	4	5.03***	10.03**	17.54**	11.03**	1.22***	18.65**
Clon: Citoquinine	12	1.60^{NS}	2.02^{NS}	4.03^{NS}	0.94^{NS}	0.43***	5.88**
Residue	380	0.74	0.74	0.87	0.86	0.12	1.89

^{NS} no significance; *** significance at <0.001, ** significance at <0.01; *significance at <0.05.

Considering plant elongation in the different growing media, *m*T1, together with BA2 and *m*T2 were the treatments that differed significantly from the medium without cytokinin (T) ($p<0.05$) in the three height ranges (Table 2). BA1 showed significantly greater number of plants of less than 0.5 cm and fewer number of explants between 0.5 and 1 cm in height than *m*T1. For the height range of more than 1 cm, there were no significant differences between the media with cytokinins, however, with *m*T, there was a tendency for a greater number of shoots respect to BA. On the other hand, with both cytokinins at a higher concentration (BA2 and *m*T2), no significant differences were observed between them for the three height ranges (Table 2). Proliferation, assessed as the production of lateral shoots, showed that the medium without cytokinin had the lowest number of new shoots per explant. The highest number of new shoots per plant was observed with *m*T1, being the only medium with cytokinins that differed significantly from the medium without cytokinin (Table 2).

Table 2**:** Number (and standard errors) of explants per interval of height and proliferation rate for each cytokinin treatment of *Eucalyptus* clones at 90 days in the multiplication stage.

Cytokinin	Explants under 0.5 cm	Explants between 0.5 and 1 cm	Explants over 1 cm	New shoots per plant
BA1	6.92 (±0.56)ab	7.25 (±0.68)b	3.17 (±0.44)a	2.64 (±0.50)ab
BA2	6.08 (±0.56)bc	9.58 (±0.68)ab	3.67 (±0.44)a	2.45(±0.47)ab
*m*T1	4.17 (±0.56)c	10.42 (±0.68)a	4.58 (±0.44)a	3.11 (±0.53)a
*m*T2	5.75 (±0.56)bc	8.83 (±0.68)ab	4.00 (±0.44)a	2.46 (±0.50)ab
T	8.58 (±0.56)a	4.17 (±0.68)c	0.17 (±0.44)b	1.00 (±0.29)b

Different letters indicate significant differences among treatments. Tukey's test: the letters differ in $p<0.05$.

Table 3: Number of explants per interval of height and proliferation rate for each genotype of *Eucalyptus* clones at 90 days in multiplication stage.

Genotype	Explants under 0.5 cm	Explants between 0.5 and 1 cm	Explants over 1 cm	New shoots per plant
19G40	12.07 (±0.50) a	5.93 (±0.61) b	1.20(±0.50) b	1.92(±0.31) ab
19G8	5.27 (±0.50) b	8.53 (±0.61) a	3.27 (±0.5) a	3.60(±0.37) a
19G28	4.13 (±0.50) b	8.87 (±0.61) a	4.40(±0.5) a	2.36 (±0.43) ab
19G11	3.73 (±0.50) b	8.87 (±0.61) a	3.60(±0.5) a	1.39(±0.56) a

Different letters indicate significant differences among treatments. Tukey's test: the letters differ in $p<0.05$.

2.5.2. Rooting stage

The rooting capacity of the explants presented a strong dependence of the genotype, with different behaviors in the four clones evaluated. Clone 19G40 had the highest rooting capacity, reaching the maximum rooting percentage (91%) and the maximum average root length (3.6 cm) in the *m*T1

treatment (Figure 2 and 3). In addition, the explants of clone 19G40 were the only ones that managed to root from the multiplication medium without cytokinin (T), with a rooting percentage of 25% and an average length of 1.08 cm. The rooting percentages for 19G11 - BA1 and 19G11 - *m*T1 were 40% and 38.9%, respectively. While by increasing the concentration of cytokinins in 19G11 - *m*T2 and 19G11 - BA2, the rooting decreased. Regarding genotype 19G28, a constant rooting with both cytokinin used was observed, with a rooting percentage of 20% and an average root length between 0.78 and 1 cm. Finally, genotype 19G8 had a very low rooting capacity regardless of the treatment used, with percentages less than 5% (Figure 2).

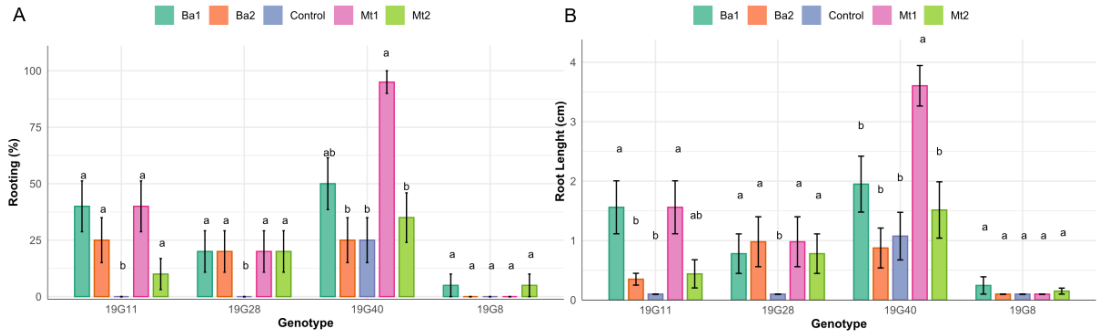

Figure 2: (a) Rooting percentage, and (b) root length as a function of genotype and concentration of benzyladenine (BA) and metatopoline (*m*T) of *Eucalyptus* clones at 21 days in the rooting stage. Different letters indicate significant differences between treatments.

Figure 3: *In vitro* rooting stage of 19G40 at 21 days in the rooting stage, (a) explants treated in multiplication medium with 0.41 µM *m*T; (b) explants treated with 0.44 µM BA.

2.6. Discussion

2.6.1. *In vitro* multiplication

While the problem of rooting of *E. globulus* clones is a well-known issue, a genotype-dependent response also occurs in the *in vitro* multiplication stages that allows a promising development of few clones (Trindade and Pais 1997; de Almeida et al., 2015). It is also known that the concentration and type of cytokinin used in the multiplication medium has an effect on the response of the explants during the multiplication stage, as well as in the subsequent stages of rooting and acclimatization (Druege et al., 2019; Vilasboa et al., 2019). In order to help manage hormonal alternatives for *in vitro* multiplication and avoid the loss of rooting capacity, the effect of different cytokinins on the multiplication and quality of *in vitro* explants was analyzed.

Explants in media with *m*T at a concentration of 0.41 µM showed increased proliferation and elongation compared to explants in media with BA. Similar responses were reported in several species, such as *Syzygium cumini* and *Saccharum* spp., with the use of *m*T (Aremu et al., 2012; Souza et al., 2019; Naaz et al., 2019). The use of *m*T, in addition to obtaining greater proliferation and elongation, increased the amount of photosynthetic pigments and the biomass of *Pterocarpus marsupium* Roxb. compared to BA (Ahmad and Anis, 2019). BA culture media stimulated the production of antioxidant compounds in plants, which may be associated with oxidative stress generated by cytokinin added to the medium (López-Orenes et al., 2013; Souza et al., 2019). In contrast, in a medium with *m*T, the activity of antioxidant enzymes was lower due to a possible lower oxidative stress (Souza et al., 2019). In addition to the type of cytokinin applied, proliferation and elongation in *Eucalyptus* is strongly influenced by the concentration of cytokinin and its balance with respect to the auxins of the medium (Brondani et al., 2012; de Oliveira et al., 2015). In our results, it was observed that both concentrations of the same cytokinin did not present significant differences in the elongation and proliferation of the explants. However, in easily rooted clones in multiplication media supplied with *m*T or BA at lower concentration, higher rooting percentages were observed.

Corymbia and *Eucalyptus* micropropagation protocols usually have an initial phase of multiplication and a subsequent phase of elongation. In the multiplication phase, high concentrations of cytokinins are used to stimulate the proliferation of the shoots (Faria et al., 2022). In the multiplication medium BA is supplied in concentrations of 0.5 to 1.0 mg L-1 (2.2 to 4.4 µM) combined with ANA as auxin in concentrations of 0.05 to 0.1 mg L-1. While in the elongation phase, to promote elongation, cytokinin concentrations are reduced, supplying BA at concentrations of 0.05 to 0.1 mg L^{-1} (0.22 to 0.44 µM) and ANA and/or IBA as auxins in concentrations of 0.5 to 1.0 mg L^{-1}. These protocols were reported in *E. benthamii* (Brondani et al., 2012), *E. cloeziana* (de Oliveira et al., 2015), *E. grandis* × *E. globulus*, and *E. urophylla*

× *E. globulus* (De Oliveira et al., 2016), *E. microcorys* (Faria et al., 2022); and *C. maculata* (Molinari et al., 2023). In the elongation phase, better elongation results were also reported when cytokinins were removed from the culture médium (Gómes et al., 2007; Brondani et al., 2012). The removal of cytokinins from the multiplication medium seeks to solve habituation problems (Gómes et al., 2007; De Oliveira et al., 2015), or to counteract situations of oxidative stress, which can arise due to a high concentration of cytokinins in the culture medium (López-Orenes et al., 2013; Souza et al., 2019). In our work, the multiplication medium without cytokinins impaired the elongation, proliferation and subsequent rooting of the materials, as was also observed by Neigishi et al., 2014.

2.6.2. *In vitro* rooting

Eucalyptus globulus is a recalcitrant species in which the genetic factor exerts a great influence on the *in vitro* response, on the period of rejuvenation and on the rooting capacity (Trindade and Pais, 1997; Fett-neto et al., 2001; Aumond et al., 2017). In this sense, *E. globulus* clones with easy and difficult rooting are differ in that the easy-to-root clones have higher endogenous levels of indole-butyric acid (IAA) (Neigishi et al., 2014). In *E. globulus* clones, *in vitro* rooting percentages ranging from 75% to 95% have been achieved by modifications of the growth regulators of the medium (Bennett et al., 1994; Trindade and Pais, 1997). In these works, the best results were obtained by combining two cytokinins, interspersing the use of BA and kinetin in the multiplication medium (Bennett et al., 1994), and by the use of IBA in rooting (Trindade and Pais, 1997). In our results, it was observed that it was possible to achieve rooting percentages similar to those reported when using *m*T in easy to root clones. However, in these same clones, lower rooting percentages were obtained with the use of BA. Additionally, in easy to root clones such as 19G40, lower shoot elongation was observed. On the other hand, in clones of difficult rooting, such as clones 19G8 and 19G28, the type and concentration of cytokinin had no effect on the percentage of rooting. The improved rooting performance of *m*T

compared to BA is consistent with several reports indicating higher percentages of rooting and survival in acclimatization when providing *m*T (Bairu et al., 2007; 2011; Naaz et al., 2019). These results have also been reported in *Eucalyptus*, were the use of *m*T increased the rooting and acclimatization of *E. grandis* × *E. urophylla* (van der Westhuizen, 2014). The main metabolite of topolines (meta-Topolin and meta-Topolin riboside) is *O-glucoside*, an easily degradable compound, which disappears during rooting and acclimatization (Bairu et al., 2011; Grira et al., 2023). On the other hand, the main metabolite of BA is N6-benzyladenine-9-glucoside, which is more stable but has a negative effect on rooting and acclimatization (Werbrouck et al., 1996; Bairu et al., 2011; Aremu et al., 2017).

From this work, it is concluded that, for the studied genotypes, the use of *m*T (in concentrations of 0.4 µM) in the multiplication medium improved elongation, while in the rooting stage it enhanced rooting percentage and root growth in easily-rooted clones. In turn, a negative effect trend was also observed when the cytokinin concentration in the multiplication medium was increased to 0.8 µM for BA and *m*T.

2.7. Authorship contribution

Project Idea: AC; GB

Processing: FE; AC; MB; MBB; MC; LR

Analysis: FE; AC; MDR

Writing: FE; MDR

Review: FE; AC; MB; MBB; MC; LR; GB; MDR

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3. Use of growth cells and rhizosphere microorganisms in the acclimatization of *Eucalyptus globulus* and hybrids

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3.1. Resumen

Eucalyptus globulus es una especie de interés para el sector forestal debido a su calidad de madera. Sin embargo, presenta un comportamiento recalcitrante que dificulta su clonación a escala comercial. La principal limitante en la micropropagación de *E. globulus* es su baja capacidad de enraizamiento y adaptación a condiciones *ex vitro*. Este trabajo busca mejorar el enraizamiento y la aclimatación de las microplántulas. Se evaluaron el tipo y la concentración de citoquinina *in vitro* y el uso de microorganismos rizosféricos en un sistema de enraizamiento que se denominó *células de crecimiento*. Para reducir el efecto negativo de las citoquininas durante el enraizamiento, se evaluó el efecto del suministro al medio de multiplicación de benciladenina (BA) a 0,1 mg L^{-1} y metatopolina (mT) a 0,1 y 1,0 mg L^{-1}. Usando celdas de crecimiento en la aclimatación, se evaluó el efecto de la inoculación con *Azospirillum brasilense* y *Trichoderma asperellum* sobre el desarrollo de radicular y altura de planta. El uso de mT incrementó el área y proliferación de explantes, así como la elongación de los brotes en la multiplicación. El tratamiento con 0,1 mg L^{-1} incrementó en un 20 % el porcentaje de enraizamiento en comparación con la BA, y en la aclimatación aumentó en un 20 % el porcentaje de sobrevivencia, presentando mayor altura y longitud de raíces. La inoculación con

microorganismos durante la aclimatación favoreció el crecimiento de las microplántulas, con una mayor altura y longitud de raíces primarias y secundarias.

3.2. Summary

Eucalyptus globulus is a species of interest to the forestry sector due to the quality of the wood. However, it presents recalcitrant behavior that makes it difficult to clone it on a commercial scale. The main limitations in the micropropagation of *E. globulus* are the low rooting percentages and its limited adaptation to *ex vitro* conditions. This work sought to improve the rooting and acclimatization performance of micropropagated plants. We evaluated the type and concentration of *in vitro* cytokinins and the use of rhizospheric microorganisms in a rooting system that we called growth cells. To avoid the negative effect of cytokinins during rooting, the effect of supplying benzyladenine (BA) delivery at 0.1 $mg.L^{-1}$ and *meta*-Topolin (mT) at 0.1 and 1.0 $mg.L^{-1}$ in the multiplication medium was evaluated. Using growth cells in acclimatization, the effect of inoculation with *Azospirillum brasilense* and *Trichoderma asperellum* on root development and plant height was evaluated. The use of mT increased the area and proliferation of the clusters, as well as the elongation of shoots during multiplication. Treatment with 0.1 $mg.L^{-1}$ of mT increased *in vitro* rooting by 20% compared to BA; in acclimatization it favored the percentage of plant survival by 20%, presenting greater height and root length. Inoculation with microorganisms during acclimatization favored micro-plantlet growth, promoting greater height with longer primary and secondary roots.

Keywords: Rooting, Cytokinin, *meta*-Topolin, Micropropagation, *Azospirillum*, *Trichoderma*

3.3. Introduction

Eucalyptus globulus is of great interest to the pulp industry due to the quality of its wood, characterized by high cellulose content and low lignin levels (Carrillo et al. 2017). As a result, it presents high international demand and significant commercial value (Morales et al. 2018). Nonetheless, in spite of its advantages, *E. globulus* has a low rooting capacity, which hinders its rescue and multiplication in breeding programs, as well as its cloning on a commercial scale (Trindade and Pais 1997; de Almeida et al. 2017).

Micropropagation is a widely used technique in forestry improvement programs for the propagation of recalcitrant species and clones, as well as for the recovery of high-value materials (Assis et al. 2004; Trueman et al. 2018). Micropropagation improves vigor and rooting capacity of explants through the process of rejuvenation and/or reinvigoration through successive *in vitro* subcultures (Wendling et al. 2014 a, b; Isah 2023). In addition, it allows for uniform large-scale production in confined spaces, under controlled and pollution-free nutritional and environmental conditions (Assis et al. 2004; Abiri et al. 2020).

However, in recalcitrant species such as *E. globulus*, micropropagation shows low rooting capacity and a strong genotype-dependent response (Trindade and Pais 1997; Fett-Neto et al. 2001; Negishi et al. 2014), as well as the formation of poorly functional and fragile roots (Brondani et al. 2012). On the other hand, adequate control of the *ex vitro* environmental conditions is crucial to avoid micro-plantlet transplant stress and low survival rates (Brondani et al. 2018; Abiri et al. 2020; Faria et al. 2022). In *E. globulus* there are also reports of good *in vitro* multiplication behavior of genotypes with greater difficulties in rooting and acclimatization (Esquivel et al. 2024).

To overcome the low *in vitro* response of *E. globulus* and improve rooting and acclimatization, easily propagated materials are preferred. One strategy is the early selection of genotypes easy-to-root (Trindade and Pais 1997; Negishi et al. 2014). Another strategy is to carry out controlled crosses with easy-to-root species, such as *E. grandis* (de Oliveira et al. 2016; De

Almeida et al. 2015; Vilasboa et al. 2021). Adjusting the micropropagation protocols, optimizing the rooting and acclimatization, offers the opportunity to utilize agronomically remarkable recalcitrant genotypes, improve process efficiency, increase productivity, and reduce associated costs (Faria et al. 2023).

The use of cytokinins in the *in vitro* multiplication medium is essential to stimulate proliferation and delay senescence. However, the type and concentration can after on the rooting and acclimatization stages (Kieber and Schaller 2018; Druege et al. 2019; Vilasboa et al. 2019; Esquivel et al. 2024). Benzyladenine (BA) is the cytokinin commonly used in tissue culture protocols, but it is not exempt from causing physiological problems and inhibiting rooting (Aremu et al. 2017; Trueman et al. 2018). In order to overcome this limitation, analogues to benzyladenine have been developed, such as *meta*-Topolin (*m*T), an aromatic rapidly-degrading cytokinin (Strnad et al. 1992; Strnad et al. 1997). *Meta*-Topolin has been reported to increase proliferation and enhance rooting in diverse species (Aremu et al. 2014; Naaz et al. 2019; Krakhmaleva et al. 2023) as well as in *Eucalyptus* spp. (van der Westhuizen 2014; Esquivel et al. 2024).

On the other hand, the use of microorganisms that promote plant growth has great potential during the acclimatization of different woody species (Ortas et al. 2017; Dwiyani et al. 2024). However, its effect on *Eucalyptus* has been poorly studied. Nevertheless, it has been reported that these microorganisms enhance rooting and growth of cuttings (Mafia et al. 2007; Texeira et al. 2007). Plant growth-promoting microorganisms have two mechanisms of action. The direct mechanisms produce and secrete assimilable compounds for plants, such as macro and micronutrients, hormones, among others. They also carry out an indirect mechanism, playing the role of biological controllers (Fibach-Paldi et al. 2011; Gupta et al. 2015). *Azospirillum* spp. is a rhizobacterium reported to promote the growth, rooting, and germination of *Eucalyptus* (Puente et al. 2010; Angulo et al. 2014). In the rhizosphere, it produces and secretes indole acetic acid (IAA)'s inducers, that

is absorbed by the roots (Puente et al. 2018; González-Díaz et al. 2019). *Trichoderma* spp. is a fungus widely used as a biocontrol agent in mini-cuttings against *Oidium* spp., *Botrytis cinerea*, and *Cylindrocladium* spp., increasing rooting and survival in *Eucalyptus* species (Fortes et al. 2007). The combined application of *Azospirillum brasilense* with *Trichoderma* spp. biostimulates wheat and corn crops, without compromising the production of hormones or other compounds (Janzen et al. 1992; Öğüt et al. 2005; Moreira et al. 2022; Fernandez et al. 2024).

An active root system uses its energy for root growth and exploration of the soil to allow absorption of water and nutrients, providing support to the aerial part. But root initiation is a very delicate process, being very sensitive to plant movements during acclimatization. To facilitate the initiation and development of root primordia, a “growth cell” structure adaptable to use in moisture-saturated environment was developed. Although there is reported experience on the use of rhizotrons in *Eucalyptus* cuttings (M'Bou et al. 2008), it has been focused on the root study in a non-invasive way (Hamer et al. 2016; Reni et al. 2024).

Considering the background of *in vitro* behavior, the objective of this work was to study multiplication and acclimatization methodologies in clones of *E. globulus, E. grandis* × *E. globulus*, and *E. grandis* × *E. maidenii*. We evaluated the effect of supplying of type and concentration of cytokinins on the multiplication and rooting *in vitro* stages, and the use of growth cells as a support structure in the acclimatization. In addition, using growth cells in acclimatization, the effect of inoculation with *Azospirillum brasilense* and *Trichoderma asperellum* on root development was evaluated.

3.4. Material and methods

3.4.1. Plant material

Materials from the tree breeding program of the National Institute of Agricultural Research (INIA, Uruguay) were used for the study. Twenty-one-month-old trees growing in the field were cut down and the basal 50 cm of

their stems were transferred to INIA Las Brujas' greenhouse. The budding of the epicormic buds was stimulated by immersing the trunks in water (Fig. 1a). The shoots were extracted and surface disinfected with sodium hypochlorite (10 $g.L^{-1}$) and Tween 20 (1 $ml.L^{-1}$) for 10 minutes and then rinsed three times with sterile distilled MS base medium (Murashige and Skoog, 1962) (Fig. 1b). From the materials introduced, five clones were selected for the tests that did not show any incidence of callogenesis, hyperhydricity or oxidation: two *E. globulus* clones (21G8 and 18G13), two *E. grandis* × *E. globulus* clones (17HG6 and 17HG28), and one *E. grandis* × *E. maidenii* clone (17HM51).

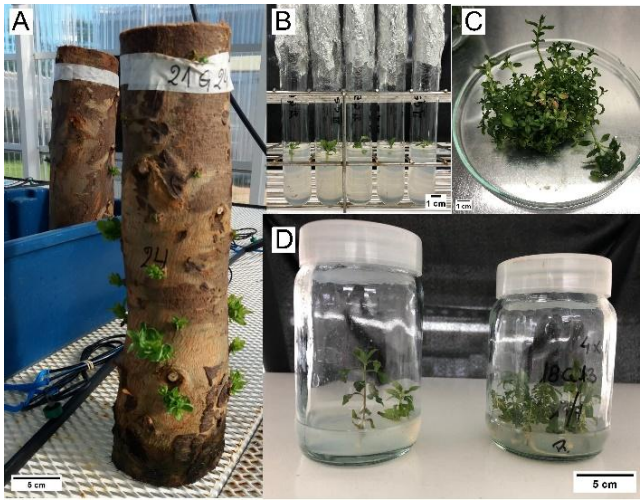

Fig. 1. Micropropagation of *E. globulus* and hybrids. A) Proliferation of epicormic buds of basal trunks. B) *In vitro* introduction. C) *In vitro* multiplication and formation of cluster structures. D) *In vitro* rooting stage of shoots over 1 cm in height.

3.4.2. Evaluation of cytokinin

3.4.2.1 *In vitro* multiplication

Clusters, defined as explants with more than 10 shoots according to Brondani et al. (2012), were subcultured in a multiplication medium composed of macronutrients from modified MS according to Castillo et al. (2020), micronutrients from Woody Plant Medium (WPM) salts (Lloyd and McCown, 1980), and vitamins (de Fossard et al. 1974). It was supplemented with 7.2 $g.L^{-1}$ agar (Sigma), 10 $g.L^{-1}$ sucrose, and 0.05 µM naphthaleneacetic

acid (NAA); adjusted to pH 5.8 (Fig. 1c). The cytokinins assessed were BA a concentration of 0.4 µM (0.1 mg.L^{-1}), and *m*T at 0.4 µM (0.1 mg.L^{-1}) and 0.9 µM (1.0 mg.L^{-1}). The conditions of the growth chamber were 23 °C (+/-2 °C), 16 daylight hours of photoperiod, with cold white LED light and a light intensity of 30 µmol.m^{-2}.s^{-1}. Subcultures were performed every three weeks and at 90 days the size of the clusters, the proliferation rate (such as the number of shoots greater than 1 cm in height per cluster), explant height and photosynthetic pigments (chlorophyll a, chlorophyll b, chlorophyll a + b, and carotenoids) were evaluated.

3.4.2.2 *In vitro* rooting

Shoots over 1 cm in height were transferred to total darkness for one week in a medium composed of one third MS salts, 5.5 g.L^{-1} agar, 10 g.L^{-1} sucrose, and 3.0 µM indole butyric acid (IBA), adjusted to pH 5.8 (Fig. 1d). After one-week, cold white LED lighting was applied at a light intensity of 30 µmol.m^{-2}.s^{-1} and a photoperiod of 16 hours. At three weeks, the percentage of rooting and root length were evaluated.

3.4.2.3 Acclimatization

Rooted shoots were transplanted into growth cells (see details below). Five of each clone explants were placed per cell, with a distance of 5 cm from each other, and using sand and perlite (3:1) (Fig. 2). The cells were placed with an inclination of 45° in the greenhouse, inside a humidity chamber of between 80 and 90% humidity, controlled by an ultrasonic nebulizer (capacity 30 m^3 and 8 liters). At four weeks, percent survival, explant height, and length of primary and secondary roots were assessed.

3.4.3. Evaluation of microbial agents in acclimatization

3.4.3.1 *In vitro* multiplication and rooting

Rooted explants of clones 17HM51 and 17HG28 were used for the assay. Explants came from the multiplication and rooting conditions described above, using 0.1 mg L^{-1} of BA in the multiplication medium.

3.4.3.2 Acclimatization

Four rooted shoots were transplanted per growth cells and under greenhouse conditions described above. The effect of the combined inoculation of a solution of *Azospirillum brasilense* (strains Az39 and CFN535) at a concentration of 1×10^5 CFU.ml^{-1} (colony forming units) and a solution of *Trichoderma asperellum* (strain M2) at a concentration of 1×10^6 CFU.ml^{-1} and a control without inoculation was evaluated. The inoculation was carried out by discharging 1 ml of each solution at the base of each plant transplanted into growth cells. At four weeks, the percent survival, plant height, root dry weight, primary and secondary root length, and leaf area of the second pair of leaves from the apex were evaluated.

3.4.4. Use of growth cells

The growth cells were designed based on rhizotrons built with two plates of transparent acrylic and polyurethane foam joined by a PVC U profile. The dimensions of the growth cell were 27 cm high, 40 cm wide, and 2 cm thick, and were stacked on a wooden support to place them at a 45° position inclination. The growth cells were filled with substrate at 80% capacity so that the transplanted plants remain within the two acrylics, immobilizing the plant and generating a microclimate similar to *in vitro* conditions, with the aim of reducing their exposure to unfavorable conditions (Fig. 2).

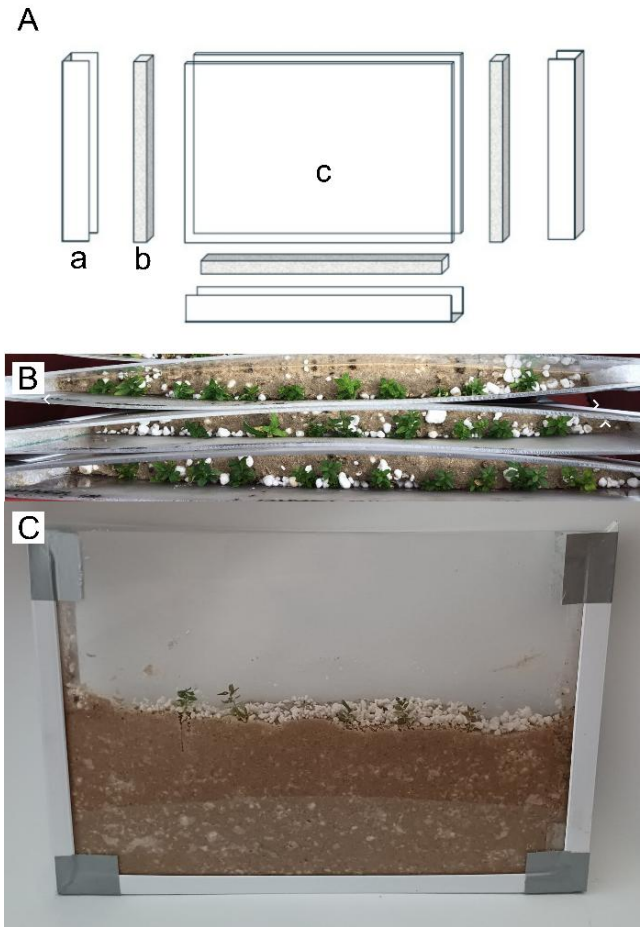

Fig. 2. A) Schematic of the growth cell, a) PVC U profile 1 cm wide, b) polyurethane foam 1 cm wide, c) transparent acrylic plates 40 cm long, 27 cm wide, and 0.2 cm thick. B) and C) Growth cell from vertical and horizontal views.

3.4.5. Image processing

To measure the clusters area and leaf area, as well as the height of the shoots and the length of the roots, the samples were photographed from a height of 50 cm, with a camera (ES65, Samsung) in a vertical position and perpendicular to the surface. Images were processed with the FIJI program (Schindelin et al. 2012), using the Trainable Weka Segmentation (Arganda-Carreras et al. 2017) and Smart-Root (Lobet et al. 2011) plugins. Trainable Weka Segmentation was used for the segmentation of the clusters and

leaves, while Smart-Root was used to analyze the height of the explant and the length of the primary and lateral roots.

3.4.6. Photosynthetic pigment analysis

25 mg of fresh leaf tissue suspended in 5 ml of DMSO in the dark for 48 hours were used. Absorbance was determined in spectrophotometer (Jenway 6300) at visible wavelengths of 480, 649, 665 nm. The equations for determination of carotenoids, chlorophyll a, b, and a + b were based on the method described by Welbrun (1994).

3.4.7. Experimental design and statical analysis

For the cytokinin assay, a randomized design in factorial arrangement (5×3) was used, in which five genotypes and three cytokinins were evaluated. In the multiplication, rooting and acclimatization stages, 5 explants per experimental unit (flask and growth cell) and 6 replicates were used.

In the trial of plant growth promoters during acclimatization, the experiment was carried out in a completely randomized design in a factorial arrangement (2×2). Two clones and the effect of inoculation with *Azospirillum brasilense* and *Trichoderma asperellum* and a control without inoculation were evaluated. For this stage, 4 explants per growth cell and 6 replicates were used.

The data obtained were processed using the R software, version 3.6.3 (R Core, 2023). Variables that did not show normal distribution after the Shapiro-Wilks test ($p > 0.05$) were normalized by Box Cox. Data were analyzed by analysis of variance (ANOVA, $p < 0.05$) and comparison of means by Tukey's test ($p < 0.05$).

3.5. Results

3.5.1. Evaluation of cytokinin

3.5.1.1 *In vitro* multiplication

An increase in the final cluster area was observed in the *m*T multiplication media, while shoot elongation with *m*T depends on the clone (Fig. 3). An effect of the clone and cytokinin interaction was observed (Annex 1), for clone 21G8, which presented the lowest cluster area among the clones. For the same cluster area, between 1 and 3 additional shoots were generated with *m*T compared to BA (Fig. 4). Regarding the analysis of photosynthetic pigments, no significant differences were observed between BA and *m*T at concentrations of 0.1 $mg.L^{-1}$. However, as the concentration of *m*T increased, there was a decrease in the content of chlorophyll a, a + b, and carotenoids (Fig. 5).

In relation to the behavior of the materials evaluated, the *E. grandis* × *E. maidenii* clone (17HM51) developed greater cluster area and a tendency of a greater proliferation, and higher shoots. In contrast, *E. globulus* clones (18G13 and 21G8) showed a low cluster area, which was also reflected in low proliferation (Fig. 3a and 4). In addition, clone 21G8 exhibited the lowest elongation of the shoots. On the other hand, clone 17HM51 had high levels of chlorophyll a, a + b, and carotenoids, differing significantly from *E. globulus* clone 21G8, which had the lowest values.

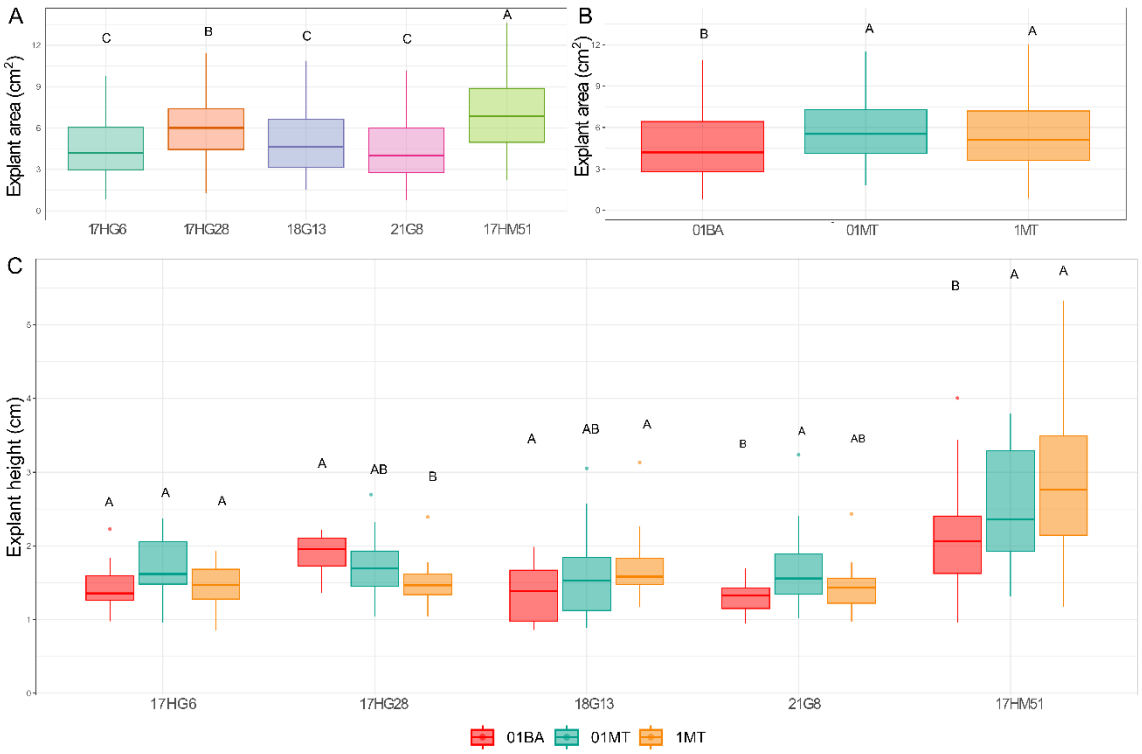

Fig. 3. Explant area and height length as a function of genotype and concentration and type of cytokinins of *Eucalyptus* clones at 90 days in the multiplication stage A) and B) Explant area based on genotypes and cytokinins, respectively. C) Explant height on genotypes depending on the cytokinins supplied. 01BA - 0.1 mg L^{-1} BA, 01MT - 0.1 mg L^{-1} of *m*T, and 1MT - 1.0 mg L^{-1} of *m*T.

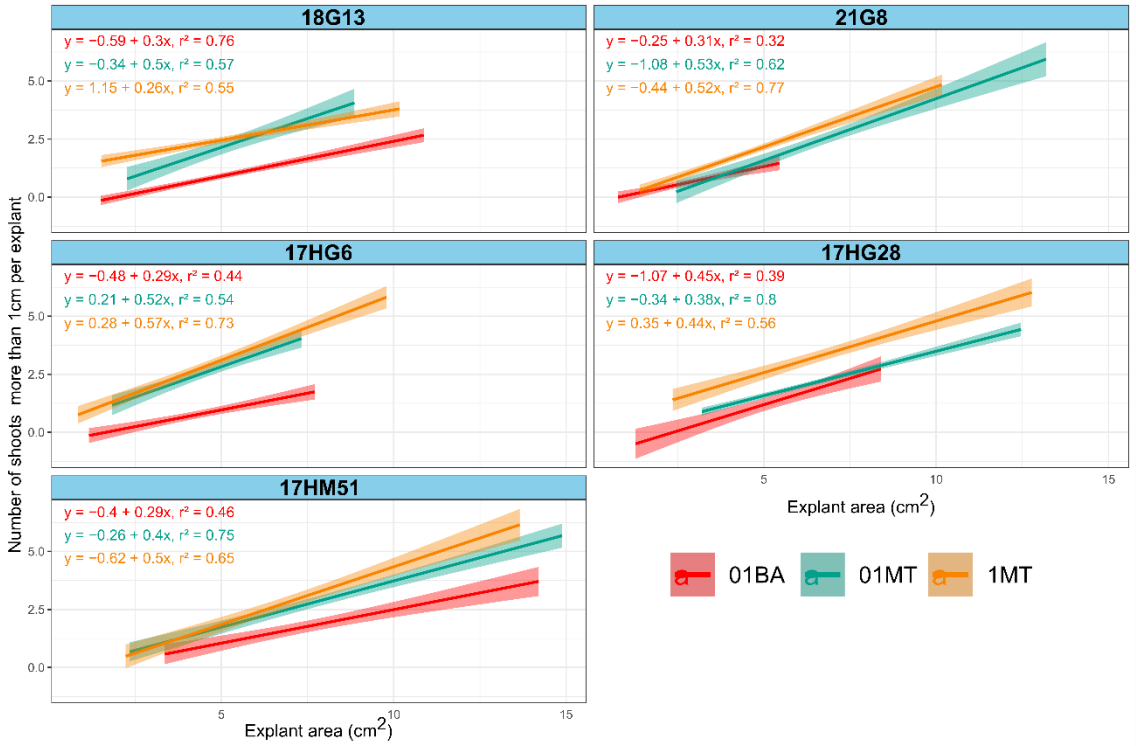

Fig. 4. Proliferation (as the number of shoots of more than 1 cm height per explant) and cluster area for the different clones of *Eucalyptus* at 90 days based on the cytokinin supplied to the multiplication medium.

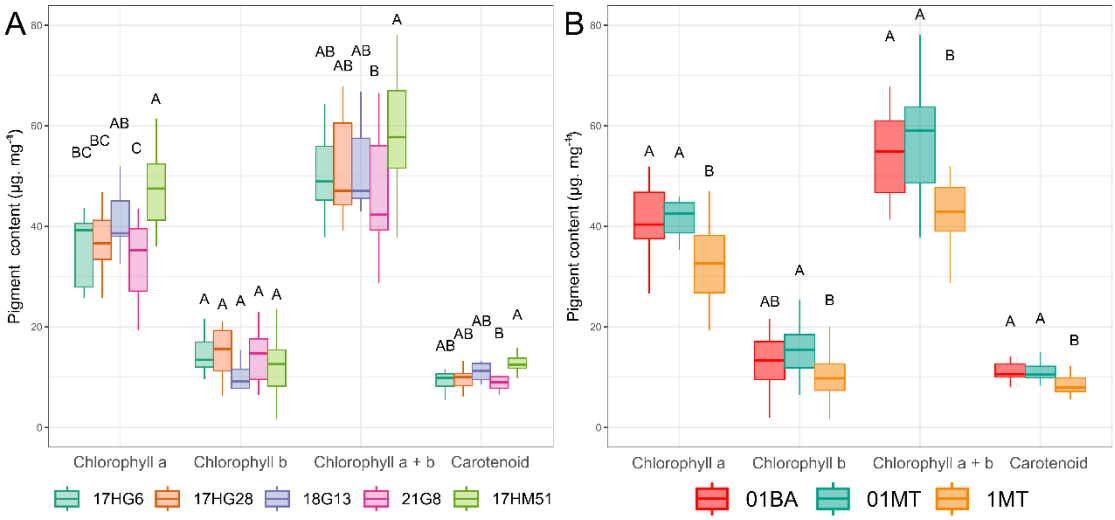

Fig. 5. Analysis of photosynthetic pigments as a function of genotype and cytokinin at 90 days in the multiplication stage. Chlorophyll a, b, a + b, and carotenoid content depending on the clones (a) and the cytokinins supplied to the multiplication medium (b).

3.5.1.2 *In vitro* rooting

Explants pretreated with 0.1 mg.L^{-1} of *m*T presented higher rooting percentages, differing significantly from those treated with BA and with *m*T at 1.0 mg.L^{-1}. In addition, the use of 0.1 mg.L^{-1} of *m*T in multiplication favored the development of longer roots during the rooting stage (Fig. 6). The *E. grandis* × *E. maidenii* clone (17HM51) had the highest rooting percentages, differing significantly from *E. globulus* (18G13 and 21G8) and *E. grandis* × *E. globulus* 17HG28 clones. In addition, clone 17HM51 developed the longest roots, while clones 21G8 and 17HG6 showed the shortest roots lengths (Fig. 6).

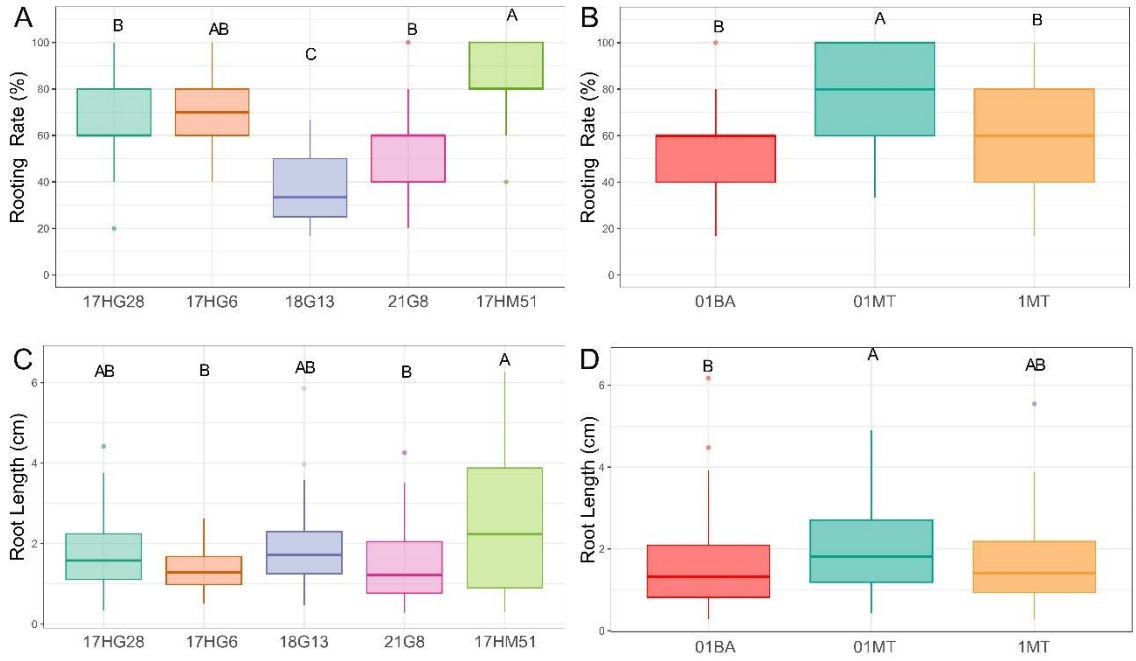

Fig. 6. Rooting percentage and root length as a function of genotype and cytokinin at 21 days in the rooting stage. Rooting percentage for the different clones (a) and cytokinins (b), and primary root length for the different clones (c) and cytokinins (d).

3.5.1.3 Acclimatization

An effect of clone and cytokinin on survival percentage, height, and primary root length was observed. For the clone an effect was observed for lateral roots, but not so for cytokinins. An interaction effect between clone and cytokinin was also recorded for primary root length (Annex 1).

Explants from *m*T multiplication media had higher survival percentages and height, differing significantly from those treated with BA. *E. globulus* clone 18G13 showed low survival relative to the rest. Although clone 21G8 did not show significant differences, a trend towards lower survival was observed. On the other hand, the *E. grandis* × *E. maidenii* clone (17HM51) was the clone that showed the highest growth in height, differing from the rest of the materials (Fig. 7).

Clone 21G8 exhibited limited root growth, with the shortest primary and secondary root lengths. An interaction between genotype and cytokinin was observed for the length of primary roots (Annex 1); in clones 21G8 and 17HG28, the use of *m*T increased the length of the primary roots, while with BA they practically did not present root growth (Fig. 8).

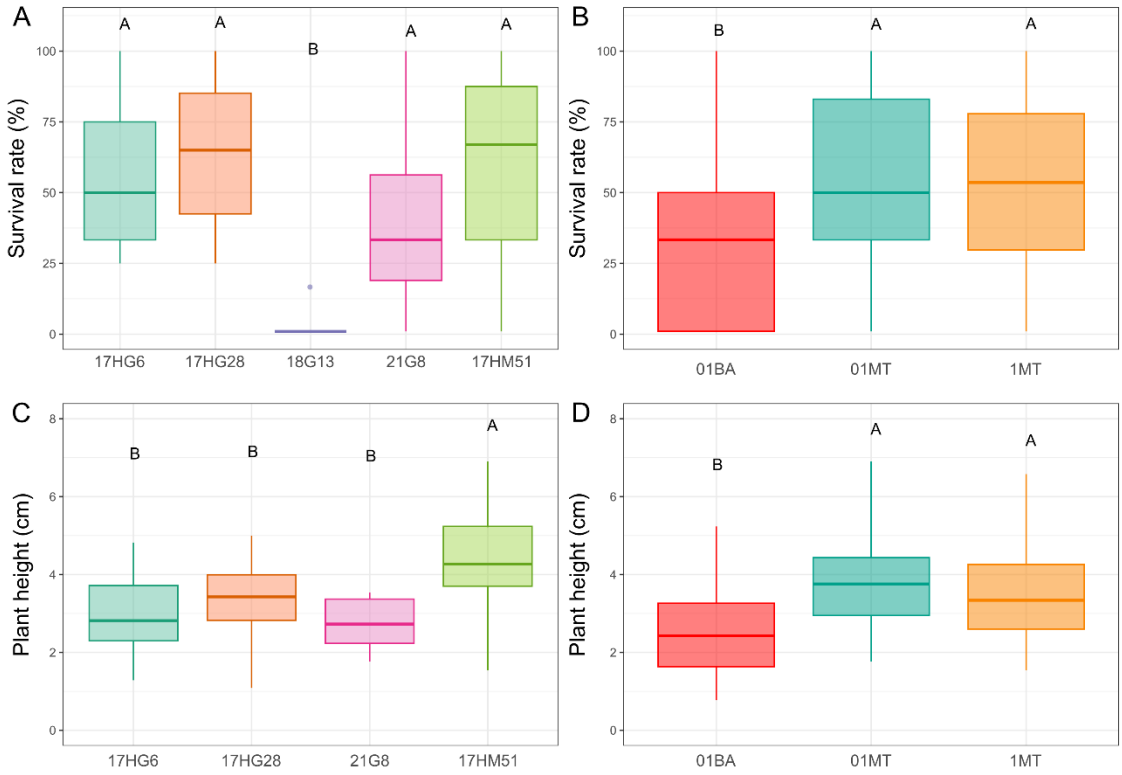

Fig. 7. Percentage of survival at the end of acclimatization for the different clones (A) and cytokinins (B). Acclimated plants height for clones (C) and cytokinins (D).

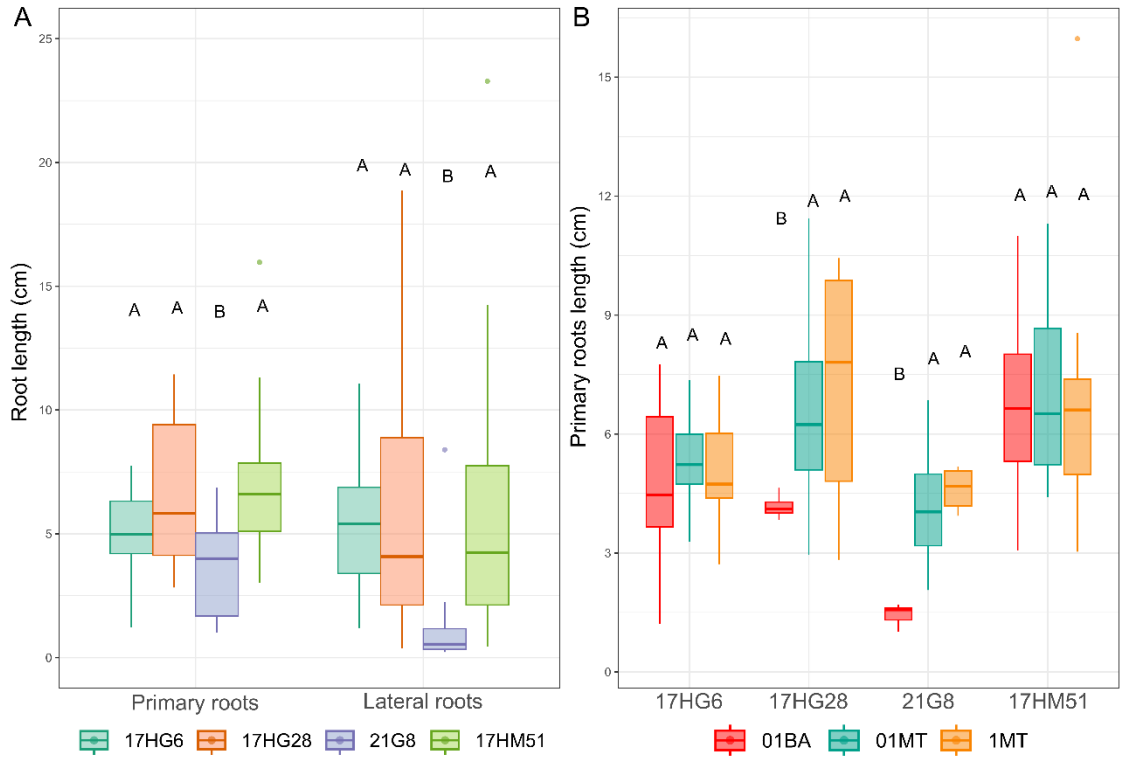

Fig. 8. Length of primary and secondary roots as a function of genotype and cytokinin at 28 days in the acclimatization. (A) Length of primary and lateral roots for clones. (B) Length of primary roots as a function of clones and cytokinin.

3.5.2. Evaluation of microbial agents in acclimatization

Inoculation with *A. brasilense* and *T. asperellum* increased the length of the primary and lateral roots during acclimatization, and root weight compared to those not inoculated (Fig. 9D, E and F) (Fig. 10). In addition, inoculation favored aerial growth of clones 17HM51 and 17HG28 during acclimatization (Fig. 9B).

A clone effect was observed for survival and leaf area; however, no significant effect of inoculation was recorded in these variables. Likewise, an effect of inoculation on explant height, root dry weight, and primary root length was observed (Annex 2).

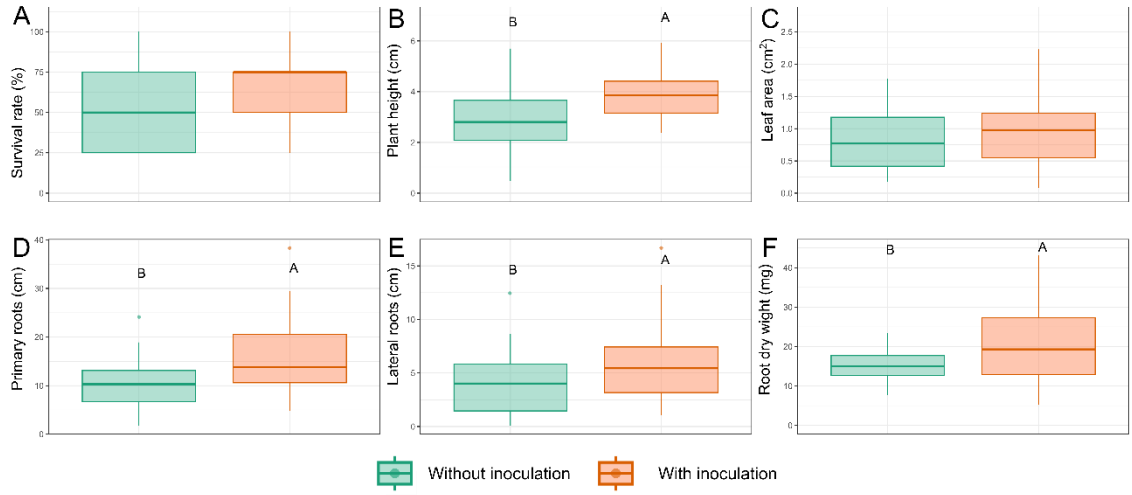

Fig. 9 Acclimatization variables of *Eucalyptus* clones 17HM51 and 17HG28 as a function of genotype and inoculation, evaluated at 28 days. (A) survival rate, (B) plant height, (C) leaf area, (D) primary roots length, (E) lateral roots length, and (F) root dry weight of the first pair of leaves developed.

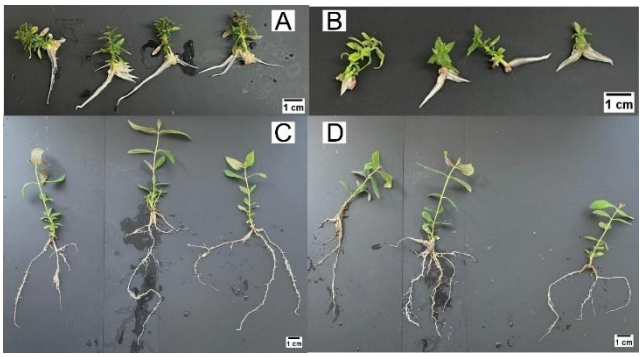

Fig. 10. Rooting (21 days) and acclimatization (28 days) stages of *E. grandis* × *E. maidenii* clone 17HM51. (A) and (B) *In vitro* rooted explants from treatments with a) 0.1 mg-L^{-1} of *m*T, and b) -0.1 mg-L^{-1} of BA. (C) and (D) Acclimated micro-seedlings c) inoculated with *Azospirillum brasilense* and *Trichoderma asperellum*, and d) without inoculation (control).

3.6. Discussion

3.6.1. Plant material

The *E. globulus* clones evaluated presented difficulties in micropropagation, with limited response to *in vitro* multiplication, due to low rooting percentages, and high mortality in acclimatization. *E. globulus* is a recalcitrant *in vitro* species, reported for its difficulty in establishing itself in the *in vitro* introduction and multiplication medium (Trindade and Pais et al. 1997). It exhibits low rooting capacity *in vitro* and limited adaptation during acclimatization, resulting in high mortality (Fett-Neto et al. 2001; Esquivel et al. 2024). On the other hand, hybrids of *E. grandis* × *E. maidenii* and *E. grandis* × *E. globulus* proved to be relatively easier materials to micropropagate. *Eucalyptus grandis* has been reported as an easily rooted species (De Almeida et al. 2015; Vilasboa et al. 2021). Interspecific hybridization between *E. globulus* and its subspecies, with easily rooted materials, tends to increase rooting capacity and facilitate propagation (Assis et al. 2004; de Oliveira et al. 2012-2016). The genotypes used in this work have been selected for their response to *T. nubilosa* without considering their rooting capacity, which has proven to be very low.

3.6.2. *In vitro* multiplication

The cytokinins supplied to the multiplication medium have a strong effect on the behavior of *in vitro* explants at this stage, as well as in the response during rooting and acclimatization (Aremu et al. 2017; Druege et al. 2019; Vilasboa et al. 2019). In our results, the use of *m*T favored the proliferation of the clusters in all clones and the elongation of the shoots from *E. globulus* clones compared to a medium supplied with BA. These same effects have been reported for *E. globulus* and *E. grandis* × *E. urophylla*, in which *m*T acts as a promoter of proliferation rate and elongation in *in vitro* multiplication (van der Westhuizen 2014; Esquivel et al. 2024).

Plant tissues metabolize exogenous cytokinins into free base forms, such as ribosides, N-glycosides, O-glycosides, and nucleotides, depending

on the type of cytokinin delivered (Hošek et al. 2020; Grira et al. 2023). BA is deactivated mainly by N-glycosylation, forming N6-benzyladenine-9-glucoside, an irreversible process (Strand et al. 1997; Bairu et al. 2011; Aremu et al. 2012). On the other hand, *m*T, due to the presence of a hydroxyl group at the N9 position of the adenine ring, is deactivated by O-glycosylation, forming ß-D-glucopyranosyl-benzyladenine-9-riboside, in a reversible process, since the action of β-glucosidase can easily convert it into active forms of cytokinins (Werbrouck et al. 1996; Strand et al. 1997; Bairu et al. 2011; Aremu et al. 2012; Grira et al. 2024). This gives *m*T greater biological activity over longer periods, which could explain the higher proliferation compared to BA (Strand et al. 1997; Aremu et al. 2017; Grira et al. 2024).

The supply of cytokinins to the *in vitro* multiplication medium delay the senescence of plant tissues by regulating oxidative stress and antioxidant capacity (Cortleven and Schmülling 2015; Honig et al. 2018; Vylicilova et al. 2020). The effect of cytokinins on senescence is strongly related to tissue age and light conditions (Cortleven and Schmülling 2015; Honig et al. 2018). During senescence, the supply of cytokinins increases the activity of the catalase (CAT) and ascorbate peroxidase (APX) enzymes, and reduces H_2O_2 levels, protecting the photosynthetic system and cell membranes, which prevents the degradation of chlorophylls against oxidative damage (Zavaleta-Mancera et al. 2007; Mik et al. 2011; Honig et al. 2018). Associated with this, it has been reported that *m*T has greater anti-senescent activity compared to BA (Holub et al. 1998; Honig et al. 2018). Also, *in vitro* explants treated with *m*T have lower levels of H_2O_2 and anti-oxidative enzymes compared to the use of BA (Souza et al. 2019), as well as higher levels of photosynthetic pigments in *m*T-treated shoots compared to BA (Ahmad and Anis 2019). In our results, no significant differences for photosynthetic pigments were observed between *m*T and BA at the same concentration. However, a decrease in photosynthetic pigments was evidenced with the increase in the concentration of *m*T. This could be associated with the fact that high

concentrations of cytokinins can induce an increase in H_2O_2 levels and an overexpression of antioxidant enzymes (Synkova et al. 1999; Mlejnek et al. 2003; López-Orenes et al. 2013; Honig et al. 2018). This suggests the search for a balance between the material that continues in micropropagation determined by the size of the cluster and the explants that go on to rooting. Understanding this balance where the genotype, hormonal levels of the medium and growth conditions interact can be very relevant in genotypes that are difficult to propagate in a vegetative way.

3.6.3. *In vitro* rooting

Cytokinins are auxin antagonists and exert an inhibitory effect on the adventitious rooting process. However, in the initial stages, low concentrations of cytokinins can have a positive influence, stimulating the cell cycle (de Klerk 2002; da Costa et al. 2013; Druege et al. 2019; Tahir et al. 2022). For this reason, rooting media generally exclude cytokines or deliver them at very low concentrations (Trueman et al. 2018). Likewise, in *Eucalyptus* the cytokinins supplied to the multiplication medium can have a negative effect on subsequent rooting, which will depend on the type and concentration supplied, as well as on the response of the genotype (Trueman et al. 2018; Vilasboa et al. 2019; Esquivel et al. 2024).

In our results, explants from *m*T multiplication media had higher rooting percentages and longer roots compared to those treated with BA. This could be due to the metabolization of each cytokinin and the behavior of its metabolites during rooting (Aremu et al. 2017). BA is metabolized to N6-benzyladenine-9-glucoside, a relatively stable compound that is concentrated in the basal zone of explants (Strand et al. 1997; Bairu et al. 2011; Aremu et al. 2012). On the other hand, *m*T is deactivated into ß-D-glucopyranosyl-benzyladenine-9-riboside, which is a reactivatable compound that degrades faster (Werbrouck et al. 1996; Aremu et al. 2017). With *m*T, there is less accumulation of N-glycosides in plants, and more accumulation of O-

glycosides, which would explain the better rooting compared to BA (Bairu et al. 2011; Grira et al. 2024).

3.6.4. Acclimatization

Acclimatization is a limiting stage in the micropropagation of *Eucalyptus*. During the transplant to *ex vitro* conditions micro-plantlets suffered stress due to low photosynthetic activity and high stomatal conductance (Kumar and Rao 2012; Chaari-Rkhis et al. 2015; Grzelak et al. 2024). It also increases stress due to changes in humidity, temperature, and light conditions, which reduces survival rates (Brondani et al. 2018; Shiwani et al. 2022; Maravilha et al. 2023). Therefore, generating higher quality *in vitro* explants improves their ability to adapt to *ex vitro* conditions. A weak vascular connection between the stem and the root generates micro-plantlets more susceptible to transplant shock (Aremu et al. 2012; Brondani et al. 2012). In our results, the use of growth cells as a structure that fixes the plant in synergy with the ultrasonic nebulizer and the use of *m*T generated a greater survival in the acclimatization and a development of micro-plantlet with greater height and greater primary roots length. This is because the *in vitro* explants cultured with *m*T had more elongated shoots and superior quality roots, which facilitated acclimatization. Similar reports indicate that *in vitro* plants of higher aerial and root biomass are generated with *m*T, which ensure greater survival during acclimatization compared to BA (Aremu et al. 2012; Naaz et al. 2019).

During acclimatization, promoting micro-plantlets growth and development allows for a reduction in stress caused by transplantation, as well as shortening the acclimatization stage (Shiwani et al. 2022). The use of plant growth promoters has great potential during acclimatization, as it enables the adaptation of micro-plantlets by promoting rooting through certain microorganisms (Ortas et al. 2017; Shiwani et al. 2022; Dwiyani et al. 2024). Improvements in survival have been reported with combined inoculation of *Bacillus subtilis* and *Pseudomonas corrugate* in acclimatization in *E. tereticornis* (Pandey et al. 2000; Aggarwal et al. 2012).

Our results showed, that inoculation with *A. brasilense* and *T. asperellum*, promoted root growth and plant height, favoring adaptation and better rustication. This could be because *Azospirillum* is a bacterium reported to induce rooting in *Eucalyptus* cuttings since it releases auxin precursors into the rhizosphere (Puente et al. 2018; González-Díaz et al. 2019). While *Trichoderma* is reported as a biological control agent, it also plays a role as a promoter of *Eucalyptus* rooting (Fortes et al. 2007). Although the inoculation with both microorganisms did not allow determining the individual effects and/or if there was a synergistic effect, it did show an improvement in the acclimatization of the micro-plantlets compared to the control treatment.

3.7. Conclusions

This work has allowed the preliminary identification of two components that improve the micropropagation process. This system considers the predisposing conditions of *in vitro* culture (use of cytokinin in the multiplication stage) and the use of microbial agents in a cell structure that promotes the interaction between the plants and the microorganism facilitating hardening. It is undoubtedly an open field of study and more research is needed that explores the interaction and identification of efficient microorganisms that facilitate obtaining acclimatized plants.

3.8. Acknowledgements

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3.10. Annex

Annex 1. Analysis of Variance (ANOVA) for multiplication, rooting and acclimatization variables of *E. globulus*, *E. grandis* × *E. globulus*, and *E. grandis* × *E. maidenii* in relation to the cytokinin supplied.

Source of variation	Df	Mean Square							
		Explant area (*in vitro*)	Height explant (*in vitro*)	% rooting	Root length (*in vitro*)	Height explant (*ex vitro*)	Primary root (*ex vitro*)	Lateral root (*ex vitro*)
Cytokinins	2	1.470 ***	0.717 ***	4.321 ***	4.445 **	1.046***	4.127 ***	1.335 ^{NS}
Genotype	4	4.293 ***	6.2106 ***	4.831* **	11.84 ***	1.044***	9.5831 ***	1.748 ***
Interaction	8	1.867 ***	1.195 **	0.202$^{N}_{S}$	3.443 ^{NS}	1.054***	4.108 **	0.7837 ^{NS}
Residuals	406	2.141	1.619	0.249	1.423	0.844	2.115	0.936

NS no significance; *** significance at <0.001, **significance at <0.01; *significance at <0.05.

Annex 2. Analysis of Variance (ANOVA) for height, percentage of survivance, primary and lateral root length, weight dry root, and leaf area of acclimated explants of *E. grandis* × *E. globulus* and *E. grandis* × *E. maidenii* in relation to the inoculation.

Source of variation	Df	Mean Square					
		Height	% survivance	Primary root	Lateral root	Weight dry root	Leaf area
Inoculation	1	1.716 ***	0.522 NS	1.887 **	1.604 **	1.868 ***	0.473
Genotype	1	0.921 **	3.292 **	0.051 **	0.734 *	0.0594 NS	3.166 **
Interaction	2	0.010 NS	0.322 NS	0.0346 NS	0.097 NS	0.0285 NS	0.667
Residuals	86	1.793	0.427	1.294	1.306	1.313	2.558

NS no significance; *** significance at <0.001, **significance at <0.01; *significance at <0.05.

4. Conclusiones

De este trabajo de evaluaron componentes *in vitro* y *ex vitro* para mejorar la eficiencia de los protocolos de micropropagación clonal de *E. globulus*, *E. grandis* x *E. globulus* y *E. grandis* y *E. maidenii*.

El uso de mT mejoró el vigor y tamaño de las plantas obtenidas *in vitro*. La mT mejoró la proliferación en los clones de *E. globulus* e híbridos, y la elongación en los clones de *E. globulus* y *E. grandis* x *E. maidenii*. Durante la etapa de enraizamiento, el uso de mT incrementó el porcentaje de enraizamiento y la longitud de las raíces para los clones evaluados. La obtención de una planta *in vitro* con mayor vigor permitió una mayor adaptación a las condiciones *ex vitro*. En cuanto a las concentraciones evaluadas, los mejores resultados se observaron a concentraciones mT de 0,1 mg. L.

La inoculación con dos cepas de *Azospirillum brasilense* (Az39 y CFN535) y una cepa de *Trichoderma asperellum* (M2) en el trasplante mejoró el desarrollo radicular y aéreo, lo que favoreció la adaptación de las microplántulas.

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