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

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SILVICULTURE

ABSTRACT

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.

HIGHLIGHTS

The cytokinin type and its concentration in the multiplication medium affected the proliferation rate but did not improve the rooting in difficult-to-root clones.
The use of *m*T in the *in vitro* multiplication phase improved plant elongation and proliferation.
With a concentration of 0.41 µmol of mT, the rooting of the clones with ease of rooting was also improved.

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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 less 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* sp. 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.

MATERIAL AND METHODS

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 Murashige and Skoog (MS) base medium (Murashige and Skoog, 1962).

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).

Shoots 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 (AIB), 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.

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 repeats 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 ($p<0.05\%$).

RESULTS

Multiplication stage

In this study, the elongation, proliferation and rooting ability of selected genotypes of *E. globulus* were evaluated using different concentrations of mT 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).

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 significatively 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).

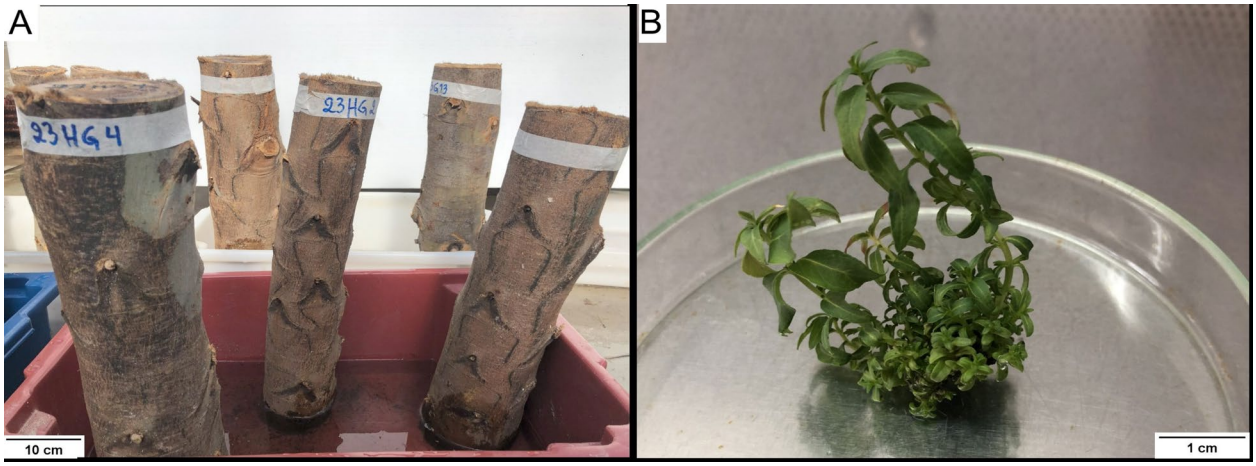

Figure 1: Micropropagation steps of *E. globulus*. (a) Rescue of individuals, trunk submerged in water to stimulate the sprouting of epicormic buds in INIA Las Brujas greenhouse; (b) 19G28 explant in multiplication stage with subcultures every four weeks after 90 days.

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.

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 (%)	Root length (cm)
Clone	3	32.030***	4.009**	10.453**	12.770**	3.376**	47.754**
Citoquinine	4	5.038***	10.038**	17.544**	11.039**	1.229***	18.654**
Clone:Citoquinine	12	1.607^{NS}	2.028^{NS}	4.030^{NS}	0.945^{NS}	0.432***	5.887**
Residue	380	0.748	0.743	0.87	0.862	0.126	1.895

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

In relation to the clones evaluated, 19G40 showed the lowest elongation, with the largest number of shoots less than 0.5 cm and between 0.5 and 1 cm in height and the lowest number of shoots over 1 cm. The rest of the clones did not show significant differences from each other for the elongation variable. Regarding proliferation, no significant differences were observed between clones (Table 3).

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).

DISCUSSION

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

Table 2: Number (and standard errors) of explants per interval of height and proliferation rate for each cytokinin treatment.

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.

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 clones. Tukey's test: the letters differ in $p<0.05$.

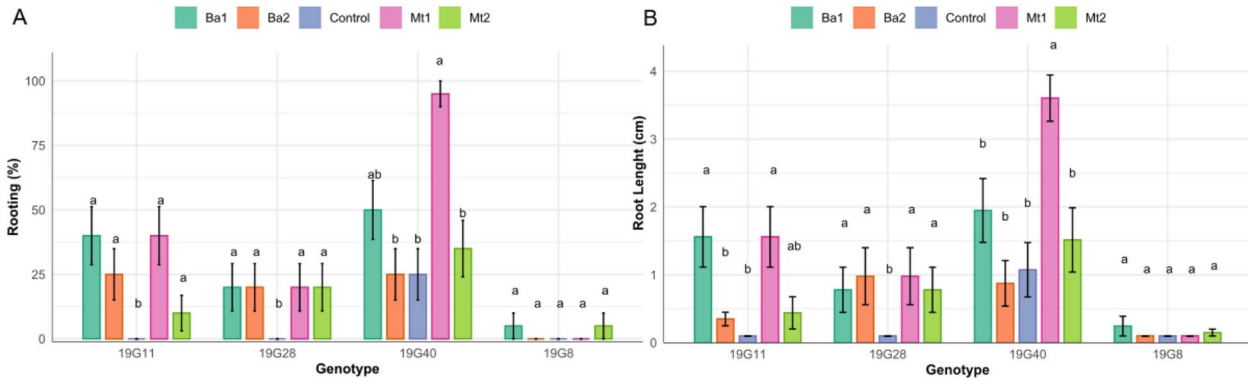

Figure 2: (a) Rooting percentage, and (b) root length as a function of genotype and concentration of benzyladenine (BA) and metatopoline (*m*T). Different letters indicate significant differences between treatments.

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 medium (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.

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

In vitro rooting

Eucalytpus 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 (AIA) (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 AIB 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.

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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