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Microplants production of *Eucalyptus cloeziana* from indirect organogenesis

Producción de microplantas de *Eucalyptus cloeziana* por organogénesis indirecta

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SUMMARY

Few studies have focused on the *in vitro* multiplication methods for *Eucalyptus cloeziana*. In this work we developed an indirect organogenesis protocol to obtain micropropagated plants. The interactions of plant growth regulators in juvenile tissues were evaluated. Hypocotyl, cotyledon and root tissues obtained from *in vitro* germinated seedlings were subjected to combinations of α-naphthaleneacetic acid (NAA), thidiazuron (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D). *In vitro* callogenesis, adventitious bud induction, shoot elongation, and *ex vitro* survival and rooting were evaluated. Callogenic structures were observed in all evaluated tissues. Morphogenic characteristics related to the meristematic center was observed. The tissues that presented the callus mass were subcultured in a regeneration culture medium supplemented with 1.0 mg L^{-1} of 6-benzylaminopurine (BAP). Only hypocotyl and cotyledon induced adventitious bud and shoot elongation. Hypocotyl subjected to 2.0 mg L^{-1} TDZ had the highest number of elongated shoots. The two tissues (*i.e.*, hypocotyl and cotyledon) combined with thidiazuron were characterized by the *ex vitro* survival of microcuttings and by adventitious rooting. Results confirmed tissue culture efficiency for microplants production by indirect organogenesis from hypocotyl and cotyledon cultivated with TDZ, and its implementation can be an alternative for forest tree breeding programs of *E. cloeziana*.

Key words: adventitious rooting, callogenesis, indirect regeneration, adventitious bud.

RESUMEN

Pocos estudios se han centrado en los métodos de multiplicación *in vitro* para *Eucalyptus cloeziana*. En este trabajo se desarrolló un protocolo de organogénesis indirecta para obtener plantas micropropagadas. Se evaluaron las interacciones de los reguladores del crecimiento en tejidos juveniles. Los tejidos del hipocótilo, cotiledón y raíces obtenidos de plántulas germinadas *in vitro* se sometieron a combinaciones de ácido α-naftaleneacético (ANA), tidiazurón (TDZ) y ácido 2,4-diclorofenoxiacético (2,4-D). Se evaluaron la callogénesis *in vitro*, la inducción adventicia de yemas, el alargamiento de brotes, y la sobrevivencia y enraizamiento *ex vitro*. Se observaron estructuras calogénicas en todos los tejidos evaluados. Se observaron características morfogénicas relacionadas con la formación de centros meristemáticos. Los tejidos que presentaron la masa del callo fueron subcultivados en medio de cultivo de regeneración suplementado con 1,0 mg L^{-1} de 6-bencilaminopurina (BAP), y solo los tejidos del hipocótilo y cotiledón formaron yemas adventicias y brotes alargados. Los tejidos del hipocótilo sometidos a 2,0 mg L^{-1} de tidiazuron obtuvieron mayor número de brotes alargados. Los dos tejidos (hipocótilo y cotiledón) combinados con TDZ se caracterizaron por sobrevivencia y el enraizamiento adventicio *ex vitro* de las microestacas. Los resultados confirmaron la eficiencia del cultivo de tejidos para la producción de microplantas por organogénesis indirecta a partir de tejidos del hipocótilo y cotiledón cultivados con TDZ y su implementación puede ser una alternativa para los programas de mejoramiento forestal de *E. cloeziana*.

Palabras clave: enraizamiento adventicio, callogénesis, regeneración indirecta, brote adventicio.

INTRODUCTION

The genera *Eucalyptus* and *Corymbia* are the most cultivated exotic species of the forest sector. They are well recognized for their silvicultural characteristics, fast growth, great ecological plasticity and wood properties, making them attractive for numerous industrial applications (Brondani *et al*. 2018, Arriel *et al*. 2019). Among these genera, several species still have been evaluated for commercial plantation establishment, because of the growing demand for forest products. Such species include *Eucalyptus cloeziana* F. Muell., which stands out for its wide applications, including the production of raw materials for furniture, construction, energy and pulp and paper industries, and has established itself in the industrial sector in Brazil (Alves *et al*. 2017).

In vitro plant regeneration techniques have been used as complementary tools to forest tree breeding, where the development of a tissue culture protocol is essential to obtain whole plants on a large scale. The phases of tissue regeneration, bud multiplication, shoot elongation and subsequent rooting and acclimatization can only be performed after the establishment of aseptic cultures with good vegetative vigor (Silva *et al*. 2019). One of the current methodologies adopted as an alternative to obtain complete plants is indirect organogenesis by the induction of callus, and it has several applications, such as the production of transgenic plants (Silva *et al*. 2019).

The use of indirect organogenesis allows the formation of adventitious bud and shoot elongation for producing large quantities of microplants in a short period and enables the study of different morphogenetic and physiological phenomena (Hesami and Daneshvar 2018), production of secondary metabolites (Mutawil *et al*. 2016), transgenic plants, polyploidy, and somaclonal variations (Hesami and Daneshvar 2018) and callus culture for growing cells in suspension (Silva *et al*. 2019).

Callogenesis and bud induction occur from the redifferentiation of the cells forming new tissue, as these processes are directly controlled by morphophysiological events that regulate endogenous mechanisms, proteins and DNA methylation (Pan *et al*. 2010). Thus, the influence of the incubation conditions has been studied in several *Eucalyptus* species (Fernando *et al*. 2016) during organogenesis. These changes may occur due to the composition of the culture medium, nutrition, balance between the plant growth regulators, type and ontogenetic age of the explant and determination of the tissues (Brondani *et al*. 2012, 2018, Oliveira *et al*. 2015, Mittal and Sharma 2017, Souza *et al*. 2019).

Eucalyptus cloeziana is important in the forest sector, therefore obtaining an efficient and reproducible protocol for indirect tissue regeneration is essential to propagate improved genotypes. Furthermore, the knowledge generated may contribute to plants production by specialized forest companies, producers of improved plants and research institutions in different world regions. In this context, the present study aimed at establishing an indirect organogenesis protocol for *E. cloeziana* from hypocotyl, cotyledon and root tissues, regarding: (i) tissue competence and plant growth regulator concentrations for the induction of callus structures; (ii) presence of meristematic center; (iii) *in vitro* adventitious bud induction and shoot elongation; (iv) *ex vitro* survival of microcuttings and adventitious rooting.

METHODS

Source of tissues. Hypocotyl, cotyledon and root tissues were collected from *E. cloeziana* F. Muell seedlings germinated *in vitro*. The seeds originated from a Seed Producing Area (SPA) in Anhembi, state of São Paulo, Brazil, from cultivar LCFA026 of the Forest Science and Research Institute (IPEF).

Seed disinfection and in vitro germination. Seeds were pre-treated by imbibition in distilled water for 24 hours. Subsequently, seeds were washed under tap water for 5 minutes and sterilized first in a 70 % ethanol solution for 90 seconds and then in sodium hypochlorite (NaOCl, 2.0-2.5 % active chlorine) containing one drop (0.05 mL) of tween-20 for 25 minutes. After the disinfection process, seeds were washed three times with autoclaved distilled water. The seeds selected for cultivation were those that showed a state of turgidity and white color (Oliveira *et al*. 2015). Seeds were *in vitro* inoculated in glass test tubes (20 × 100 mm) containing 6 mL of the culture medium composed only of distilled water and agar. Seeds were kept under ambient growth room conditions until tissue was collected.

Preparation of the culture medium. The culture medium was prepared with deionized water, 6 g L^{-1} of agar and 30 g L^{-1} of sucrose. The pH of the medium was adjusted to 5.8 with 0.1 N - HCl and 0.1 N - NaOH before the agar was added, and afterwards, the mixture was autoclaved at 121 °C (~1.0 kgf cm^{-2}) for 20 minutes. Before the culture medium was autoclaved, a plant growth regulator was added.

Culture conditions under growth room. The growth room had a temperature of 25 °C (± 2 °C), photoperiod of 16 hours of light and irradiance from cool white fluorescent lamps of 32 µmol m^{-2} s^{-1}.

Callus culture. Seedlings obtained *in vitro* after 20 days of germination were used to collect the tissues (*i.e.*, explants). Fragments of root (middle portion measuring 0.5 cm in length), hypocotyl (middle portion measuring 0.5 cm in length) and cotyledons (sectioned at 50 % of the area) were individually inoculated in glass test tubes (20 × 100 mm) containing 6 mL of Woody Plant Medium

(WPM) (Lloyd and McCown 1980). The culture medium was supplemented with different combinations and concentrations of plant growth regulators, namely, 0.0, 2.0 and 4.0 mg L^{-1} of α-naphthaleneacetic acid (NAA); 0.0, 1.0 and 2.0 mg L^{-1} of thidiazuron (TDZ); and 0.0, 1.0 and 2.0 mg L^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D). Each of the treatments consisted of 10 replicates (the experimental unit was test tubes containing one explant). The experiment was conducted in a completely randomized design in a factorial arrangement (3 × 27), where the factors were three types of explants (*i.e.*, hypocotyl, cotyledon and root) and 27 combinations of plant growth regulators (*i.e.*, NAA × TDZ × 2,4-D). After inoculation, explants were kept in a growth room in the dark for 30 days. After treatments had been applied for 30 days, the percentage of callogenesis was evaluated.

Adventitious bud induction. Callus were transferred to the adventitious bud induction medium, constituted by WPM culture medium and supplemented with 1.0 mg L^{-1} of 6-benzylaminopurine (BAP). Explants were inoculated in glass containers (6 × 7 cm) containing 40 mL of a culture medium. Subcultures were performed every 30 days under growth room conditions. The experiment was conducted in a completely randomized design in a factorial arrangement (3 × 27), where the factors were three types of explants (*i.e.*, hypocotyl, cotyledon, and root) and 27 combinations of plant growth regulators (NAA × TDZ × 2,4-D). The percentage of adventitious buds induced in 90 days was evaluated.

Shoot elongation. Only the explants that regenerated adventitious buds were used for the shoot elongation phase. Explants with initiation of 3 to 5 shoots for culture were inoculated in WPM culture medium supplemented with 0.5 mg L^{-1} BAP and 1.0 mg L^{-1} of indole-3-butyric acid (IBA). Explants were inoculated in glass containers (6 × 7 cm) containing 40 mL of culture medium. Subcultures were performed every 30 days under growth room conditions. The experiment was conducted in a completely randomized design with a factorial arrangement (3 × 27), where the factors were three types of explants (*i.e.*, hypocotyl, cotyledon, and root) and 27 combinations of plant growth regulators (NAA × TDZ × 2,4-D). At the end of the experiment, the number of elongated shoots (≥ 1 cm) in 90 days was evaluated.

Ex vitro survival, rooting and acclimatization. In vitro elongated shoots (*i.e.*, microcuttings with a length equal to or larger than 1 cm) were collected and transplanted *ex vitro* to a mini-incubator system (Brondani *et al*. 2018). The substrate used to promote the rooting of the microcuttings was a mixture of decomposed *Pinus* bark and medium-sized vermiculite, at a 1:1 (v v^{-1}) ratio. The mini-incubator system was kept under growth room conditions. The survival percentage and the adventitious roots of the microcuttings were evaluated at 20 days of *ex vitro* incubation. For acclimatization, the rooted plants were transplanted to individual containers (250 mL) containing the same proportion of the substrate used for rooting. In this phase, a nutrient solution composed of MS half-strength (Murashige and Skoog 1962) culture medium was applied every 7 days. The acclimatization period lasted 20 days. After acclimatization, the plants were transplanted to larger plastic cups (500 mL) and transferred to a greenhouse (50 % natural light). The growth phase in the greenhouse lasted 80 days, and plant survival was evaluated. The experiment was conducted in a completely randomized design with a factorial arrangement (3 × 27), where the factors were three types of explants (*i.e.*, hypocotyl, cotyledon and root) and 27 combinations of plant growth regulators (*i.e.*, NAA × TDZ × 2,4-D). The steps outlined in the methods section are briefly illustrated in figure 1.

Histological analyses. Samples of the callus were fixed in a modified formaldehyde and glutaraldehyde solution (glutaraldehyde 1 %, paraformaldehyde 4 % in sodium phosphate buffer, $NaH_2PO_4.H_2O$; 0.1 M; pH 7.2) (Karnovsky 1965) and were submitted to six vacuum series (-600 mmHg) for 30 minutes each. Samples were subsequently stored for 30 days at 4 °C and dehydrated by ethanol-alcohol series in increasing concentrations (*i.e*., 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 %, v v^{-1}), remaining in each solution for 15 minutes. Samples were placed in an infiltration medium (Historesin®, Leica) for 24 hours and prepared according to the manufacturer's instructions, remaining 28 days at 24 °C. The blocks containing the samples were sectioned longitudinally or transversally to 5 µm thickness using a Microm HM 355S automatic rotary microtome (Thermo Scientific®). Each section was stained with toluidine blue (0.05 %, v v^{-1}) in sodium phosphate buffer and citric acid (Sakai 1973) for 30 minutes and mounted on histological slides with synthetic resin (Entellan®). The histological sections were analyzed and photographed under an optic microscope (Opton®), and the images were captured at a micrometric scale. A descriptive analysis was performed on each sample, aimed at identifying the disposition of the tissues connecting the axillary buds and nodes, the region of the adventitious root emergence and evident meristematic zones.

Statistical analyses. The data measured in all experiments were analyzed using Hartley's test ($P > 0.05$) and the Shapiro-Wilk's test ($P > 0.05$) to assess the homogeneity of the variances and the normal distribution of data, respectively. Data were transformed as needed by the Box-Cox test. Next, an analysis of variance (ANOVA, $P < 0.05$) was performed. According to the significance of ANOVA, the mean values of the treatments were compared by the Duncan's test ($P < 0.05$). The steps outlined in the methods section are briefly illustrated in figure 1.

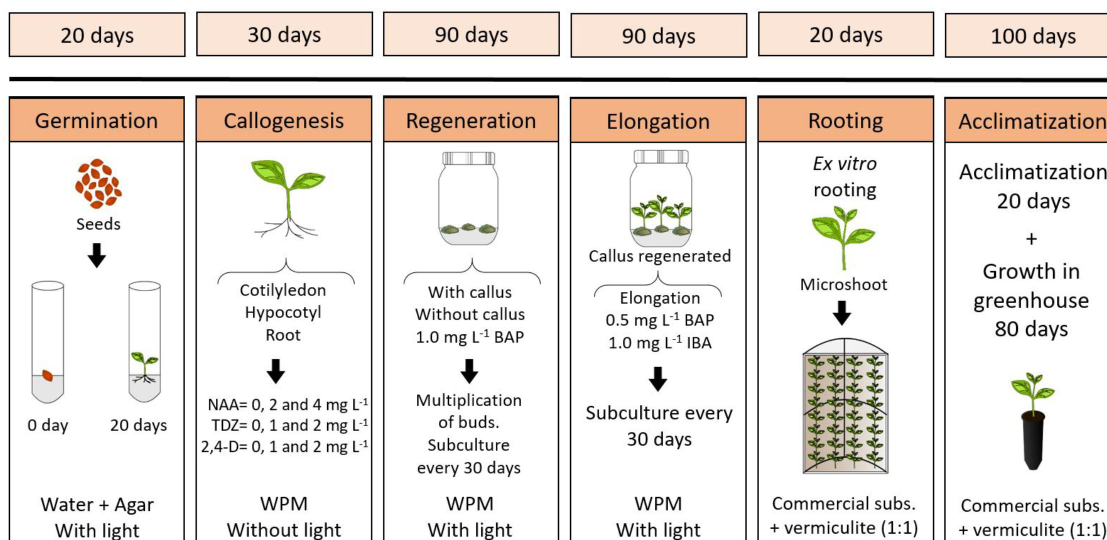

Figure 1. Flowchart of the experiment with detail of the *in vitro* seed inoculation phase until the acclimatization of microplants of *E. cloeziana* obtained by indirect regeneration in 350 days.

Diagrama de flujo del experimento con detalles de la fase de inoculación de semillas *in vitro* hasta la aclimatización de microplantas de *E. cloeziana* obtenidas por regeneración indirecta en 350 días.

RESULTS

Callus culture. Seeds of *E. cloeziana* were inoculated *in vitro* to obtain seedlings as a source of explants, and cultures with no visible microbial infection (bacteria and/or fungi) were selected. After 30 days of the callogenesis process with different tissues and combinations of plant growth regulators, induction to callogenesis was observed in all treatments, with an overall mean callogenesis of 78.4 %. Percentages of callogenesis of 30-100 % were observed for the hypocotyl, 40-100 % for the cotyledon and 20-100 % for the root (table 1).

Intense meristematic activities related to the organogenic competence of the tissues induced by combinations of plant growth regulators were observed. Cellular areas of meristematic competence were identified in the cells of the superficial tissues of callus structures, characterized by thin cell walls, reduced cell size, isodiametric cells and a high nuclear/cytoplasmatic ratio (figure 2A). In addition, the formation of a vascular bundle and the presence of a region with intense meristematic activity denotes cell competence for the formation of new tissues (figure 2A). High accumulation of ergastic substances in cells, intense meristematic activity (figure 2B) and the presence of meristemoids (figure 2C) were observed in several anatomical sections, indicating morphogenic potential.

Applications of 1.0 mg L^{-1} 2.4-D and 4.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2,4-D provided an effective hormonal balance for the initiation of the adventitious root formation from the callus in the hypocotyl and cotyledon, respectively (figure 2D).

Adventitious root formation was observed in the hypocotyl and cotyledon (figure 3A), showing the formation of a meristematic center with a connection to the callus mass (figure 3B). High nuclear/cytoplasmic ratio was observed in isolated cells for the combination of NAA and 2,4-D in all tissues tested (figure 3C). In addition, meristematic centers were also observed in the upper ends of the callus structures (figure 3D), indicating intense morphogenic activity.

Adventitious bud induction. Tissues that formed a callus were transferred to the culture medium supplemented with 1.0 mg L^{-1} BAP. Only 1.5 % of the callus demonstrated organogenic competence for adventitious bud induction, where the best responses occurred for the combinations of 1.0 mg L^{-1} TDZ, 2.0 mg L^{-1} TDZ and 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} TDZ. Hypocotyl and cotyledon tissues were able to form adventitious buds (table 2).

Callus formed from hypocotyl did not present a significant difference between applications of 2.0 mg L^{-1} TDZ and 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} TDZ, which resulted in mean shoot values of 22.2 % and 20.0 %, respectively (table 2). On the other hand, among the callus obtained from the cotyledon, a significant difference was observed between the treatments that were composed by the combinations of 1.0 mg L^{-1} TDZ and 2.0 mg L^{-1} TDZ, with the latter being more responsive to the regeneration medium, resulting in 55.5 % of the callus regenerating buds (table 2). The other tissues that did not show adventitious bud regeneration in the 90-day period were discarded.

The histological sections confirmed the regeneration of adventitious buds, which originated from the callus that presented organogenic competence, thereby indicating meristematic regions (figure 4A) and the vascular connection of shoots with the cell mass of origin (figure 4B).

Table 1. Percentage of callogenesis in *E. cloeziana* explants according to the combinations of plant growth regulators and explants at 30 days of *in vitro* cultivation.

Porcentaje de calogénesis en explantes de *E. cloeziana* de acuerdo con las combinaciones de reguladores del crecimiento de las plantas y explantes a los 30 días de cultivo *in vitro*.

NAA (mg L^{-1})	TDZ (mg L^{-1})	2,4-D (mg L^{-1})	Explant		
			Hypocotyl	Cotyledon	Root
0.0	0.0	0.0	MP	66.7^{Ba} (±16.7)	25.0^{DEb} (±16.4)
0.0	0.0	1.0	40.0^{CDb} (±16.3)	88.9^{Aa} (±11.1)	37.5^{CDb} (±18.3)
0.0	0.0	2.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
0.0	1.0	0.0	50.0^{Cc} (±16.7)	85.7^{Aa} (±14.3)	70.0^{Bb} (±15.3)
0.0	1.0	1.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
0.0	1.0	2.0	50.0^{Cc} (±16.7)	55.6^{Bb} (±17.6)	66.7^{Ba} (±16.7)
0.0	2.0	0.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
0.0	2.0	1.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
0.0	2.0	2.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	0.0	0.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	0.0	1.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	0.0	2.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	1.0	0.0	80.0^{Bb} (±13.3)	100.0^{Aa} (±0.0)	MP
2.0	1.0	1.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	1.0	2.0	90.0^{ABb} (±10.0)	100.0^{Aa} (±0.0)	20.0^{Ec} (±13.3)
2.0	2.0	0.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	2.0	1.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	2.0	2.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
4.0	0.0	0.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
4.0	0.0	1.0	50.0^{Cb} (±16.7)	100.0^{Aa} (±0.0)	MP
4.0	0.0	2.0	90.0^{ABa} (±10.0)	90.0^{Aa} (±10.0)	20.0^{Eb} (±13.3)
4.0	1.0	0.0	90.0^{ABb} (±10.0)	100.0^{Aa} (±0.0)	40.0^{Cc} (±16.3)
4.0	1.0	1.0	90.0^{ABb} (±10.0)	100.0^{Aa} (±0.0)	50.0^{Cc} (±16.7)
4.0	1.0	2.0	90.0^{ABb} (±10.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
4.0	2.0	0.0	40.0^{CDb} (±16.3)	100.0^{Aa} (±0.0)	MP
4.0	2.0	1.0	MP	90.0^{Aa} (±10.0)	MP
4.0	2.0	2.0	30.0^{Db} (±15.3)	40.0^{Ca} (±16.3)	MP

Means followed by the same uppercase letter in the columns and means followed by the same lowercase letter in the rows do not differ significantly by the Duncan's test at the 5 % probability level. Data are expressed as the mean (±standard error). MP = missing parcel due to bacterial manifestation.

Despite the high callus formation intensity of the tissues by the application of plant growth regulators, the root did not present competence for the induction of adventitious buds in the regeneration medium.

Shoot elongation. Only the callus showing adventitious bud regeneration (table 2) were subdivided into standard explants with initiation of 3 to 5 shoots for culture (figure 4A). The elongation medium was supplemented with 0.5 mg L^{-1} BAP and 1.0 mg L^{-1} IBA to establish microstumps *in vitro*. Hypocotyl originating from the application of 2.0 mg L^{-1} TDZ showed the highest number of elongated shoots at 90 days of culture (table 3), resulting in a mean of 41.5 shoots per explant. All callus showed the formation of shoots (*i.e.*, microcuttings), which were collected for the rooting phase.

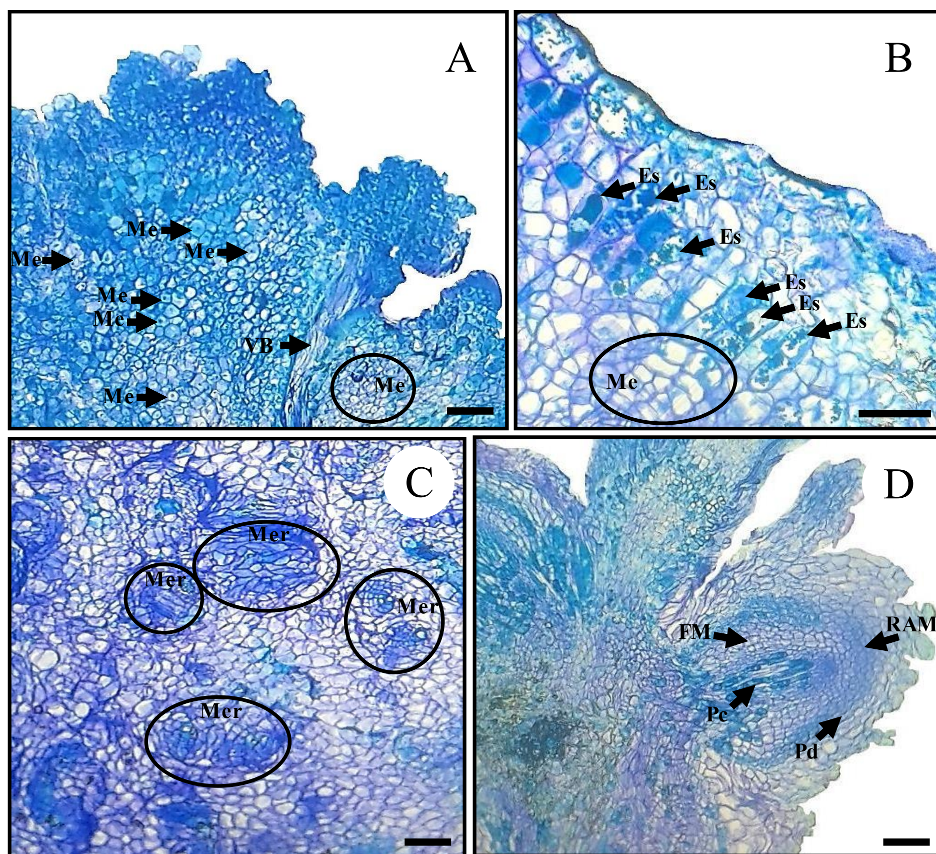

Figure 2. Histology of callus of *E. cloeziana*. A) Callus showing formation of a vascular bundle (arrow) and area with meristematic activity (arrow and region) supplemented with 2.0 mg L^{-1} TDZ and cotyledon. B) Detail of cells showing high accumulation of ergastic substances (arrows) and a region with meristematic activity in the presence of 2.0 mg L^{-1} TDZ and cotyledon. C) Region of callus showing formation of meristemoids with 1.0 mg L^{-1} TDZ and cotyledon. D) Detail of the formation of adventitious roots from the callus, with the root apical meristem with 1.0 mg L^{-1} 2,4-D and hypocotyl. Vascular bundle (VB), meristematic activity (Me), ergastic substances (Es), meristemoids (Mer), root apical meristem (RAM), fundamental meristem (FM), procambium (Pc), and protoderm (Pd). Bar = 100 µm.

Histología de callos de *E. cloeziana*. A) Callo que muestra la formación de un haz vascular (flecha) y área con actividad meristemática (flecha) con suplementación de 2,0 mg L^{-1} de TDZ y cotiledón. B) Detalle de las células que muestran una alta acumulación de sustancias ergásticas (flechas) y una región con actividad meristemática en presencia de 2,0 mg L^{-1} de TDZ y cotiledón. C) Región del callo que muestra la formación de meristemoides con 1,0 mg L^{-1} de TDZ y cotiledón. D) Detalle de la formación de raíces adventicias del callo, con el meristemo de la raíz apical con 1,0 mg L^{-1} de 2,4-D e hipocotilo. Haz vascular (VB), actividad meristemática (Me), sustancias ergásticas (Es), meristemoides (Mer), meristemo de la raíz apical (RAM), meristemo fundamental (FM), procambio (Pc) y protodermo (Pd). Barra = 100 µm.

Ex vitro survival, rooting and acclimatization. Microcuttings were collected (figure 5B) from the microstumps rooted in the shoot elongation phase (figure 5A), and these microcuttings were placed in a mini-incubator system for rooting. The mini-incubator system was maintained in a growing room under ambient conditions.

Survival and *ex vitro* rooting of the microcuttings (figure 5C) were evaluated at 20 days. Cotyledon subjected to 1.0 mg L^{-1} TDZ and hypocotyl subjected to 2.0 mg L^{-1} TDZ showed satisfactory survival and rooting, and complete plants were obtained (table 4). The remaining combinations of plant growth regulators tested in this phase did not result in rooting, and tissue mortality was observed (table 4).

Rooted microcuttings were acclimatized for 20 days and afterwards grown in a greenhouse, where they showed normal development and growth (figure 5D), enabling completion of the adventitious regeneration protocol of plants in 350 days.

DISCUSSION

Several studies have reported on the efficiency of callus induction from hypocotyl and cotyledon with *Eucalyptus* species, such as those developed for *E. camaldulensis* Dehnh. (Dibax *et al*. 2010), *E. saligna* Smith. (Silva *et al*. 2015) and *E. globulus* Labill. (Salla *et al*. 2018). In the present study with *E. cloeziana*, callus formation occurred

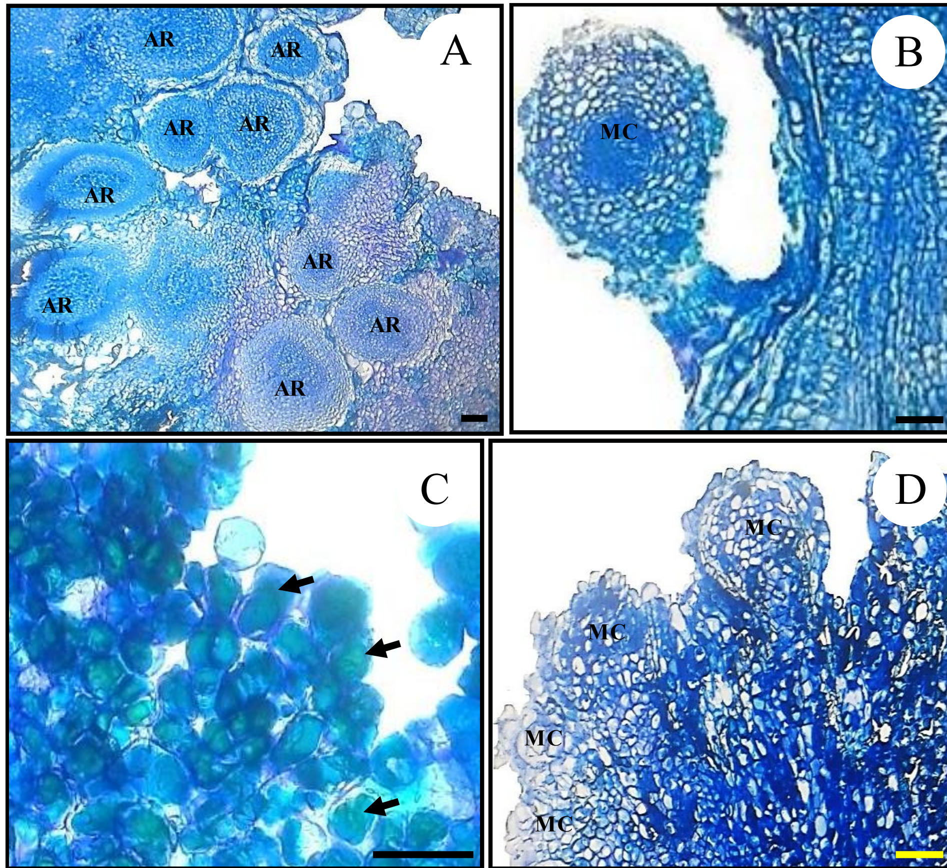

Figure 3. Histology of callus of *E. cloeziana*. A) Callus structure showing meristematic activities and initiation of adventitious root formation with 4.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2,4-D and cotyledon. B) Evidence of meristematic center showing connection with the callus mass with 1.0 mg L^{-1} TDZ and cotyledon. C) Presence of isolated cells with high nuclear/cytoplasmic ratio (arrows) with 4.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2,4-D and cotyledon. D) Evidence of meristematic center at the upper ends of the callus mass with 2.0 mg L^{-1} TDZ and cotyledon. Adventitious root (AR), meristematic center (MC). Bar = 100 µm.

Histología de callos de *E. cloeziana*. A) Estructura del callo que muestra actividades meristemáticas e iniciación de la formación de raíces adventícias con 4,0 mg L^{-1} de ANA + 2,0 mg L^{-1} de 2,4-D y cotiledón. B) Evidencia de centro meristemático que muestra conexión con la masa de callos con 1,0 mg L^{-1} de TDZ y cotiledón. C) Presencia de células aisladas con alta relación nuclear/citoplasmática (flechas) con 4,0 mg L^{-1} de ANA + 2,0 mg L^{-1} de 2,4-D y cotiledón. D) Evidencia de centro meristemático en los extremos superiores de la masa del callo con 2,0 mg L^{-1} de TDZ y cotiledón. Raíces adventicias (AR), centro meristemático (MC). Barra = 100 µm.

in all tissues tested, independent from the plant growth regulator combinations; however, the highest percentages of callogenesis were observed when cotyledon tissue was used (table 1).

Hypocotyl and cotyledon extracted from *E. cloeziana* seedlings were the sources of explants most suitable for callus formation, including evidence of adventitious root induction and buds at this organogenic phase. Vascular bundle formation and an area with meristematic activity in the callus were observed with the combination of 2.0 mg L^{-1} TDZ and the cotyledon (figure 2A). Aggarwal *et al*. (2010) found intense meristematic activity in cells of the superficial layer in tissues of *E. tereticornis* Smith., and these cells were later organized into buds. This observation was also reported in *E. cloeziana*, both for root and bud formation (figures 2A-D). In addition, a high accumulation of ergastic substances in callus from cotyledon combined with 2.0 mg L^{-1} TDZ (figure 2B) and callus showing the formation of meristems in the combination of 1.0 mg L^{-1} TDZ and cotyledon (figure 2C) were observed. In a study on the induction of callus with BAP and NAA and/or IAA (indole-3-acetic acid) in *E. dunnii* Maiden., Oberschelp *et al*. (2015) observed the presence of subepidermal meristemoids and meristematic areas developing at the end of the hypocotyl and evidence of a high number of druses in buds or ergastic substance, observations similar to those made in the present study with *E. cloeziana*. Initiation of adventitious root occurred from the callus in the hypocotyl and cotyledon (figures 2D and 3A).

The presence of a meristematic center with a vascular connection to the callus mass was observed in cotyledon treated with 1.0 mg L^{-1} TDZ (figure 3B) and 2.0 mg L^{-1}

Table 2. Percentage of induction of adventitious buds in *E. cloeziana* explants according to the combinations of plant growth regulators and explants at 90 days of *in vitro* cultivation.

Porcentaje de inducción de yemas adventicias en explantes de *E. cloeziana* de acuerdo con las combinaciones de reguladores del crecimiento de las plantas y explantes a los 90 días de cultivo *in vitro*.

NAA ($mg\ L^{-1}$)	TDZ ($mg\ L^{-1}$)	2,4-D ($mg\ L^{-1}$)	Explant		
			Hypocotyl	Cotyledon	Root
0.0	0.0	0.0	MP	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
0.0	0.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
0.0	0.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
0.0	1.0	0.0	0.0^{Bb} (±0.0)	42.8^{Ba} (±20.2)	0.0^{Ab} (±0.0)
0.0	1.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
0.0	1.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
0.0	2.0	0.0	22.2^{Ab} (±14.7)	55.5^{Aa} (±17.5)	0.0^{Ac} (±0.0)
0.0	2.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
0.0	2.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	0.0	0.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	0.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	0.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	1.0	0.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	MP
2.0	1.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	1.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	2.0	0.0	20.0^{Aa} (±13.3)	0.0^{Cb} (±0.0)	0.0^{Ab} (±0.0)
2.0	2.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	2.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
4.0	0.0	0.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
4.0	0.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	MP
4.0	0.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
4.0	1.0	0.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
4.0	1.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
4.0	1.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
4.0	2.0	0.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	MP
4.0	2.0	1.0	MP	0.0^{Ca} (±0.0)	MP
4.0	2.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	MP

Means followed by the same uppercase letter in the columns and means followed by the same lowercase letter in the rows do not differ significantly by the Duncan's test at the 5 % probability level. Data are expressed as the mean (±standard error). MP = missing parcel due to bacterial manifestation.

Table 3. Number of elongated shoots per *E. cloeziana* explant according to combination of plant growth regulators and explants at 90 days of *in vitro* culture.

Número de brotes alargados por explante de *E. cloeziana* de acuerdo con la combinación de reguladores del crecimiento de las plantas y explantes a los 90 días de cultivo *in vitro*.

NAA ($mg\ L^{-1}$)	TDZ ($mg\ L^{-1}$)	Explant	Shoot number (shoot $explant^{-1}$)
0.0	1.0	Cotyledon	20.0^{B} (±3.5)
0.0	2.0	Hypocotyl	41.5^{A} (±16.5)
0.0	2.0	Cotyledon	12.0^{B} (±2.9)
2.0	2.0	Hypocotyl	9.5^{C} (±3.5)

Means followed by the same uppercase letter do not differ significantly by the Duncan's test at the 5 % probability level. Data are expressed as the mean (±standard error).

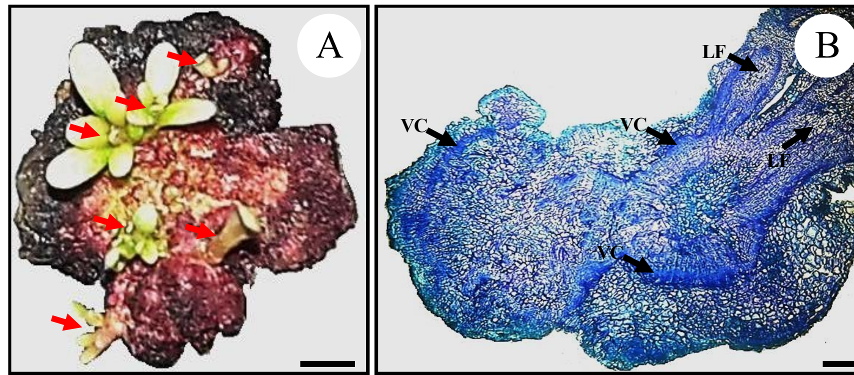

Figure 4. Histology of organogenic callus of *E. cloeziana* obtained from the combination of 2.0 mg L^{-1} TDZ and hypocotyl. A) Details on the induction of adventitious buds with shoot initiation, leaves and apical meristem (arrows). Bar = 1 cm. B) Adventitious leaf primordia showing vascular connection with the callus mass. Adventitious leaf primordia (LF), vascular connection (VC). Bar = 100 µm.

Histología de callos organogénicos de *E. cloeziana* obtenidos de la combinación de 2,0 mg L-1 de TDZ y hipocotílico. A) Detalles sobre la inducción de yemas adventicias con iniciación de brotes, hojas y el meristemo apical (flechas). Barra = 1 cm. B) Primordios de hojas adventicias que muestran conexión vascular con la masa del callos. Primordios de hojas adventicias (LF), conexión vascular (VC). Barra = 100 µm.

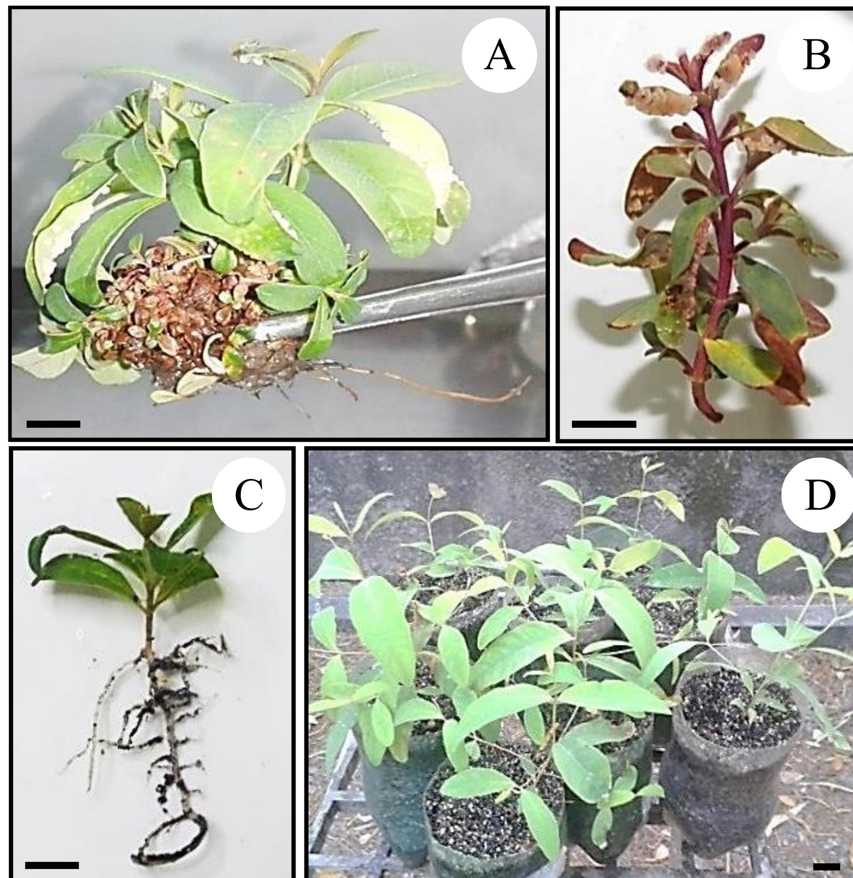

Figure 5. Stages of shoot elongation and *ex vitro* rooting of *E. cloeziana*. A) Details of *in vitro* rooted microstump, with visible formations of shoots. Bar = 1 cm. B) Details of a shoot collected (*i.e.*, microcutting) from a microstump where callus structures were present on the leaves. Bar = 1 cm. C) Details of microcutting rooted under *ex vitro* conditions at 20 days. Bar = 1 cm. D) Acclimatized plants grown in a greenhouse at 100 days. Bar = 2 cm.

Etapas de alargamiento de brotes y enraizamiento *ex vitro* de *E. cloeziana*. A) Detalles del microcepa enraizado *in vitro*, con formaciones visibles de brotes alargados. Barra = 1 cm. B) Detalles de un brote recogido (microestaca) de una microcepa donde las estructuras de callos estaban presentes en las hojas. Barra = 1 cm. C) Detalles de microestaca enraizado en condiciones *ex vitro* a los 20 días. Barra = 1 cm. D) Plantas climatizadas cultivadas en un invernadero por 100 días. Barra = 2 cm.

Table 4. Percentage of survival and *ex vitro* rooting of *E. cloeziana* microcuttings according to the concentration of plant growth regulators and explants at 20 days of cultivation.

Porcentaje de supervivencia y enraizamiento *ex vitro* de microestaca de *E. cloeziana* de acuerdo con la concentración de reguladores del crecimiento de las plantas y explantes a los 20 días de cultivo.

NAA (mg L^{-1})	TDZ (mg L^{-1})	Explant	Survival (%)	Rooting (%)
0.0	1.0	Cotyledon	66.7^B (±12.6)	53.3^A (±13.3)
0.0	2.0	Hypocotyl	100.0^A (±0.0)	55.6^A (±17.6)
0.0	2.0	Cotyledon	0.0^C (±0.0)	0.0^B (±0.0)
2.0	2.0	Hypocotyl	0.0^C (±0.0)	0.0^B (±0.0)

Means followed by the same uppercase letter in the columns do not differ significantly by the Duncan's test at the 5 % probability level. Data are expressed as the mean (±standard error).

TDZ (figure 3D), which can regenerate buds. This characteristic is important for the plant regeneration, such as reported by Mycock and Watt (2012), when evaluating the callus anatomy in *E. grandis* W. Hill ex Maiden × *E. urophylla* S. T. Blake observed that the roots developed from the stem region immediately above the callus or from the callus itself and that of the primordial meristems of the root appear to have been derived from the pericycle of the stem, *i.e.*, from the parenchyma layer between the endodermis and the phloem.

The efficiency of *in vitro* organogenesis depends primarily on the source of the explant, on the components of the culture medium and on the environmental conditions (Hesami and Daneshvar 2018, Silva *et al*. 2019). Another factor influencing the organogenic responses in *Eucalyptus* is the endogenous and exogenous hormonal balance between the cytokinin and auxin present in the plant tissue, which serve as inducing agents for *in vitro* morphogenesis (Silva *et al*. 2019, Souza *et al*. 2019). Many studies report a favorable effect of TDZ at different stages of callus formation (Jafari *et al*. 2017), with TDZ concentrations below 2.5 mg L^{-1}, being recommended for effective applications to the tissue of woody species, since high concentrations of this plant growth regulator may limit the indirect organogenesis by reducing the induction of buds and increasing hyperhydricity (Hesami and Daneshvar 2018). At 90 days after the start of the experiment, the combination of plant growth regulators and type of explants had an effect on the indirect organogenesis in *E. cloeziana*. In this context, the use of 1.0 and 2.0 mg L^{-1} TDZ, considering cotyledon and hypocotyl, respectively, produced the best responses due to the higher percentage of adventitious buds compared to the other evaluated treatments (table 2, figures 4A-B).

As for the number of elongated shoots arising from bud regeneration from callus, the best results were observed with the use of 2.0 mg L^{-1} TDZ, using hypocotyl (table 3). Data reported in literature corroborate those found in this study, since TDZ promotes cell division (Fernando *et al*. 2016, Jafari *et al*. 2017). Some studies have identified TDZ as one of the most effective cytokinins for bud regeneration in *Eucalyptus*. The efficiency of TDZ was demonstrated in other species of the genus *Eucalyptus*, such as in *E. camaldulensis* (Dibax *et al*. 2010). This ability of the callogenic tissues to redifferentiate into buds is essential for the shoot elongation phase (Salla *et al*. 2018).

Hypocotyl and cotyledon combined with TDZ (*i.e.*, 1.0 and 2.0 mg L^{-1} TDZ) favored the *ex vitro* microcuttings survival (66.7-100.0 %) and adventitious rooting (53.3-55.6 %) (table 4). These results confirm the need for an adequate choice of plant growth regulator and tissue for the development of a protocol aiming at whole plant regeneration. The successful regeneration of plants through indirect organogenesis has been reported for a limited number of commercially important eucalypt species.

Hypocotyl and cotyledon of *in vitro* cultured seedlings were the most responsive tissues as explants for the regeneration of new *E. cloeziana* plants via indirect organogenesis, showing a response similar to that of other studies (Mittal and Sharma 2017). A possible cause for this tissue differentiation, considering that these events may vary according to the plant genotype (Salla *et al*. 2018), is related to the regulation of physiological processes that favor the formation of new tissues because they contain cells with high juvenility and cellular competence (Wendling *et al*. 2015), leading to increased organ production and development (Gupta and Karmakar 2017).

Ex vitro survival and rooting of *E. cloeziana* microcuttings obtained by indirect organogenesis is difficult to achieve; however, the efficiency of this protocol was observed in 350 days (figures 5A-D). Rooting is one of the most difficult phases of micropropagation of woody species and is usually conducted under *ex vitro* conditions (Brondani *et al*. 2012, 2018). However, modifications in the rooting procedures have allowed results to be obtained at adequate levels (Brondani *et al*. 2012), whereby various combinations of plant growth regulators can be effective in promoting survival and plant rooting through

indirect organogenesis (Aggarwal *et al*. 2010, Oliveira *et al*. 2015).

The use of the mini-incubator system was adequate for the survival, rooting and initial acclimatization of *E. cloeziana* plants (table 4). These results corroborate other studies using the same type of system, where higher survival and rooting in microcuttings of *Corymbia citriodora*, *E. urophylla*, *E. benthamii* (Brondani *et al*. 2012, 2018) and *E. cloeziana* (Oliveira *et al*. 2015) were obtained.

Regarding the *in vitro* culture time, considering the phases of germination (20 days), callogenesis (30 days), bud regeneration (90 days), shoot elongation (90 days), *ex vitro* survival and rooting (20 days), and acclimatization (20 days) and hardening (80 days), the protocol proposed (350 days) can be considered an alternative for the propagation of the species for numerous applications within forest tree breeding.

The developed methodology can be tested in other *Eucalyptus* and *Corymbia* species that present rooting difficulties, considering that, in the current processes of *in vitro* rejuvenation, between 12 and 19 successive subcultures (360 to 523 days) are commonly used to obtain juvenility/reinvigoration of the mature tissues and, consequently, to increase the rhizogenic competence of the propagules. Notably, the protocol was developed for explants collected from seedlings obtained by *in vitro* germination, and thus, the feasibility of applying the technique to selected adult plants needs to be tested.

In conclusion for *E. cloeziana*, (*i*) tissue competence and plant growth regulator concentrations were effective for the induction of callus structures; (*ii*) meristematic center and high nuclear/cytoplasmic ratio were observed in the combination of NAA and 2,4-D; (*iii*) *in vitro* adventitious bud induction and shoot elongation occurred with 1.0 mg L^{-1} TDZ combined with cotyledon, 2.0 mg L^{-1} TDZ combined with hypocotyl and cotyledon, and 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} TDZ combined with hypocotyl; (*iv*) cotyledon combined with 1.0 mg L^{-1} TDZ and hypocotyl combined with 2.0 mg L^{-1} TDZ were characterized by the *ex vitro* survival of microcuttings (66.7-100.0 %) and by adventitious rooting (53.3-55.6 %).

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