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Use of biodegradable polyester-based microvessels for micropropagation of mature *Eucalyptus microcorys*

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Abstract

Background: Micropropagation, an *in vitro* vegetative propagation technique using small propagules is one of the main applications of plant tissue culture. It can be used to clone specific plants with desired traits and reduce the cost of plant propagation. In this study, we developed a protocol for micropropagation of *Eucalyptus microcorys* F.Muell using a selected mature tree, in which we tested various combinations of different culture media and evaluated the use of biodegradable polyester-based microvessels during the adventitious rooting and acclimatisation phases.

Methods: Epicormic shoots were used as an explant source. After the *in vitro* explant establishment and multiplication, we tested 8 combinations of BAP, NAA and IBA in the elongation phase. Three types of microvessels were tested in the adventitious rooting phase and acclimatisation of the microcuttings.

Results: Epicormic shoots had an establishment percentage of 40.6% and a total of 820 explants were generated by the 11th subculture, with an average of 12 buds per explant. Best shoot elongation results were achieved with BAP (0.05 mg L^{-1}) + NAA (1 mg L^{-1}) and BAP (0.05 mg L^{-1}) + NAA (1 mg L^{-1}) + IBA (1 mg L^{-1}) combinations, whereas microvessel types M2 and M3 provided higher rooting and acclimatisation. According to the results of ISSR markers, at the end of 535 days of *in vitro* cultivation, cloning was successful between acclimatised micro-plantlets and the parent plant.

Conclusions: The micropropagation protocol using microvessels was efficient in producing *E. microcorys* clonal micro-plantlets and is recommended for further studies with this species, and for testing in the micropropagation of other species.

Keywords: *In vitro* culture; plant cloning; plant growth regulator; rooting container; vegetative propagation.

Introduction

Eucalypt plantations represent over 70% of the artificial forests in Brazil, with most forest products coming from plantations of *Eucalyptus* and *Corymbia* (IBÁ 2019). The establishment of eucalypt plantations has been growing over the years and is supported by advancements in breeding programmes and in cloning (Xavier & Silva 2010; IBÁ 2019). Even though eucalypt breeding in Brazil initially focused on commercial use due to the need for short-term genetic improvement, there were other breeding programmes where progeny strategies were used (Dutra et al. 2009; Xavier & Silva 2010). Progeny and provenance trials are important sources from which to select donor plants with desirable characteristics, yet long-term established trials are still rare.

The process for selecting and cloning trees via vegetative propagation (e.g., cutting, mini-cutting, and micro-cutting techniques) has advanced greatly due to the growing demand for fast-growing tree varieties, from forest companies to small landholders (Xavier & Silva 2010). Micropropagation is one of the main applications of plant tissue culture, defined as a technique of *in vitro* vegetative propagation using small propagules (Paiva & Gomes 2011; Trueman et al. 2018). This technique aims to clone species or hybrids that have high growth rates, tolerance to low temperatures and salinity, and resistance to pests and diseases (Dutra et al. 2009; Xavier & Silva 2010; Wendling et al. 2014; Brondani et al. 2018). The possibility of obtaining many clonal micro-plants in a short time and in a reduced area, led to an increase in the commercial use of micropropagation (Davey & Anthony 2010; Hartmann et al. 2011). Another advantage of micropropagation is the lower genetic variation from the original material, resulting in an authentic copy of the parent plant (Neumann et al. 2009).

Eucalypts comprise woody plants species on which the largest number of micropropagation studies have been conducted. However, there is still the need to improve micropropagation techniques, especially the collection of plant material and its subsequent tissue rejuvenation and/or reinvigoration. Developing genotype-independent protocols would also enable cost reductions (Dutra et al. 2009; Brondani et al. 2012). Improving rooting and acclimatisation phases of micropropagation is an opportunity to increase plant yields and the cost effectiveness of the process. To achieve that, this study tested the incorporation of microvessels in the rooting phase and acclimatisation of the micro-plantlets. A microvessel is a polyester-based 3D-printed vessel designed to be the final container for micro-plantlets due to its biodegradability when in contact with a substrate. This is due to the use of an unpigmented thermoplastic polyester, polylactic acid (PLA), made with lactic acid from materials of natural origin (e.g., corn starch, cassava, beetroot, sugar cane). In addition, the use of an unpigmented PLA filament is less toxic to plants compared with pigmented filaments, while also having an affordable production cost; currently 1 kg of PLA filament, enough to produce 1,000 microvessels, costs approximately $US19.00.

Eucalyptus microcorys F. Muell, known as tallowwood, is a native Australian species commonly found in northern New South Wales and southern Queensland at elevations of up to 800 m a.s.l. It belongs to the Myrtaceae family, *Eucalyptus* genus, Symphyomyrtus subgenus and Sebaria section (Pryor 1971). In addition to its good sprouting, tallowwood is known to be moderately resistant to frost, drought and fire, as well as to *Gloeophyllum trabeum* (Pers.) Murrill, a wood-decay fungus affecting many *Eucalyptus* species (Ferreira 1979; REMADE 2003; Calonego et al. 2013). Despite remarkable adaptive traits and high-quality timber (Oliveira et al. 2014; Souza et al. 1979), tallowwood plantations have not been as widely established in Brazil, as those containing other *Eucalyptus* species. To encourage the commercial use of tallowwood, this study tested a micropropagation protocol for one mature individual of *E. microcorys* using different microvessels in the adventitious rooting phase and acclimatisation of the micro-plantlets. The specific objectives were: i) to evaluate combinations of plant growth regulators at different phases in the protocol; ii) to identify the site of differentiated cells in the adventitious rooting through histological analysis; and iii) to confirm the genetic fidelity of the collected plant materials.

Methods

Plant material

Epicormic shoots for clonal micropropagation were collected from a selected mature tree of *E. microcorys* (Figure 1A) derived from an experimental eucalypt plantation (i.e., *Eucalyptus* spp. and *Corymbia* spp.) established in 1974 in the forest nursery of the Universidade Federal de Lavras, in Lavras, Minas Gerais state, Brazil (21°22'75" S, 44°96'98" W) (IPEF 1984).

Collection and induction of epicormic shoots

The selected mature tree was pathogen free and had a rectilinear stem with branches located in the lower portion of the crown to help collection and minimise ontogenetic age effects. We collected 45 branches of 50 cm length and diameter ranging between 1 to 7 cm. The leaves were removed prior to acclimatisation of branches in a greenhouse under controlled conditions (above 80% relative humidity (RH); 20-35°C) and intermittent irrigation with high pressure and low flow nozzles, automatically controlled by a humidistat. Individual branches were kept vertically upright in 5 L polyethylene pots filled with washed sand. The number of available shoots and buds were assessed after 45 days. Pots were arranged in a randomised factorial design according to diametric class (T1 – 1 to 3 cm diameter branches; T2 – 3 to 5 cm diameter branches; T3 – 5 to 7 cm diameter branches) and each group included 15 replications.

Preparation of culture medium and *in vitro* conditions

The culture medium was prepared with deionised water (with pH 5.8 adjusted with HCl or NaOH), agar (6 g L^{-1}) and sucrose (20 or 30 g L^{-1}). Plant growth regulators, namely 6-benzylaminopurine (BAP; $C_{12}H_{11}N_5$; 225.25 g), α-naphthaleneaceticacid (NAA; $C_{12}H_{10}O_2$; 186.20 g) and indole-3-butyric acid (IBA; $C_{12}H_{13}NO_2$; 203.24 g) were added to the medium before autoclaving at 121°C (~1.0 kgf cm^{-2}) for 20 min. Cultures were placed in a growth cabinet at 24±1°C with a 16 h photoperiod (40 μmol m^{-2} s^{-1}, using a cold white tube lamp).

In vitro establishment

Explants were derived from nodal segments of the middle portion of the epicormic shoots with axiliary buds (1.0 to 1.5 cm length) and free of leaves (Figures 1B and C). Explants were washed in running water for 10 min and immersed in 50% sodium hypochlorite solution (2.0 – 2.5% active chlorine v/v, H_2O/NaClO)

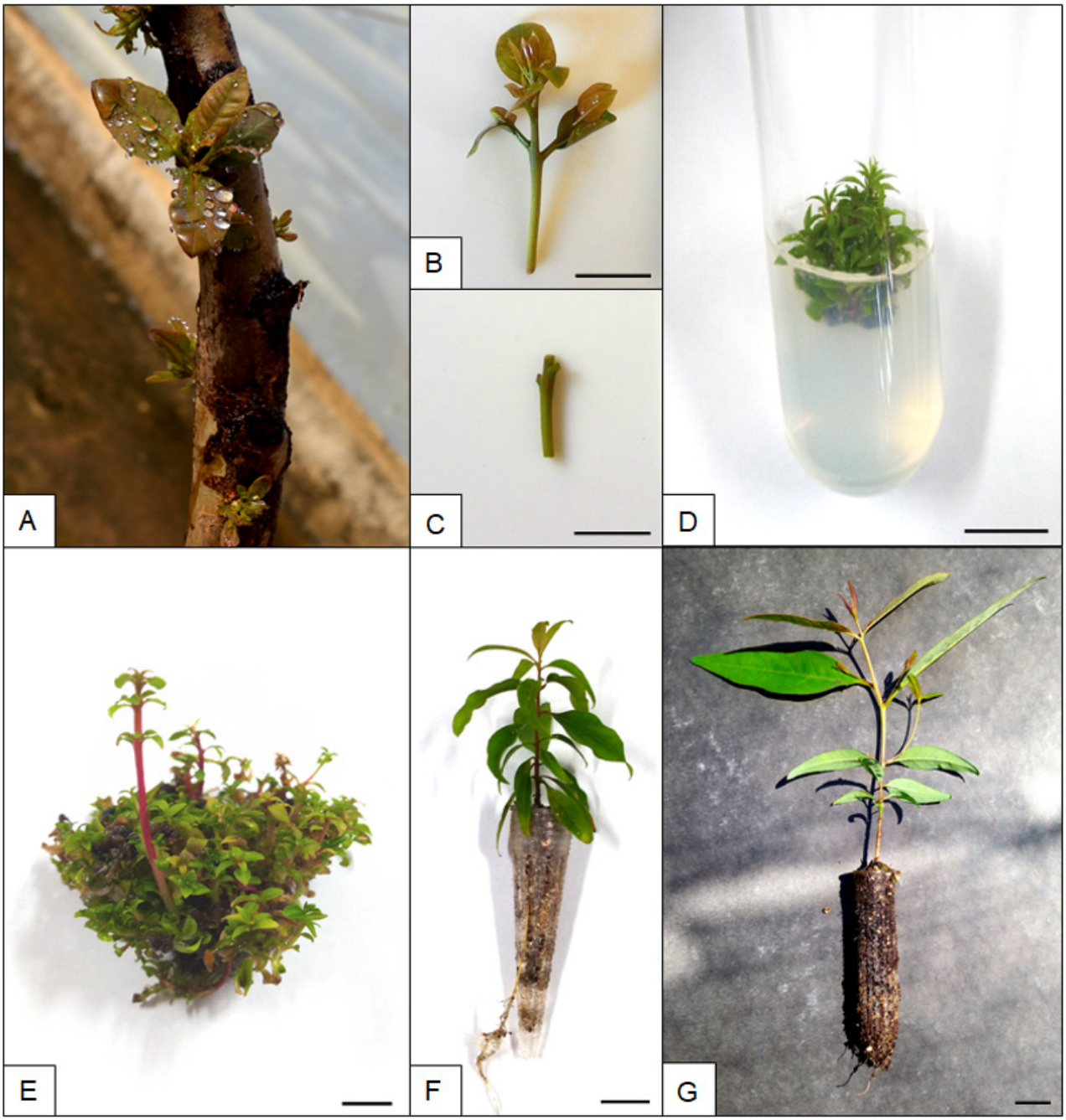

FIGURE 1: Micropropagation phases of a selected tree of *Eucalyptus microcorys*. (A) Epicormic shoots at 45 days in greenhouse; (B) Nodal segments from the middle portion of the epicormic shoots (C) used as explants; (D) Explants with axillary buds in WPM culture medium (0.5 mg L^{-1} BAP + 0.05 mg L^{-1} NAA) at 275 days; (E) Explant axillary buds during shoot elongation in WPM culture medium (0.05 mg L^{-1} BAP + 1 mg L^{-1} NAA + 1 mg L^{-1} IBA) at 90 days; (F) *In vitro* rooting of microcutting in microvessel (M2) at 45 days (root protruding from vessel); (G) Acclimatised microcutting (at 30 days) showing microvessel (M3) degradation. Bars = 1 cm.

for 5 min. At the end of the asepsis process, the explants were rinsed three times with autoclaved deionised water and inoculated in test tubes containing 12 mL of Murashige & Skoog (MS) medium (Murashige & Skoog 1962) without plant growth regulators. Tubes were kept in a growth room for 30 days, after which explants free from browning, fungal and bacterial contamination were considered established.

Multiplication

This phase was performed by adapting the multiplication phase procedure of the micropropagation protocol for *Eucalyptus benthamii* used by Brondani et al. (2012). Established explants including one axillary bud were inoculated in test tubes (2 × 15 cm) containing 12 mL of Woody Plant Medium – WPM (Lloyd & McCown 1981) supplemented with distilled water, agar (6 g L^{-1}), sucrose

(20 g L^{-1}), BAP (0.5 mg L^{-1}) and NAA (0.05 mg L^{-1}). Explants were subcultured to fresh multiplication medium every 25 days. After 275 days (11 subcultures), the number of explants and buds per explant were counted.

Elongation

Explants with 5 to 10 axillary buds, resulting from the 11th subculture, were inoculated in glass flasks (6 × 7 cm) containing WPM (40 mL) supplemented with NAA (0, 1 or 2 mg L^{-1}) and IBA (0, 1 or 2 mg L^{-1}). All culture media were supplemented with BAP (0.05 mg L^{-1}). Explants were subcultured to multiplication medium every 25 days and the number of shoots and elongated shoots (> 1 cm) was assessed after 120 days. The experiment was conducted as a completely randomised factorial design, comprised of eight combinations of two plant growth regulators (NAA and IBA), using eight replicates with four explants per replication.

Microvessels used for the adventitious rooting and acclimatisation

Microcuttings longer than 1 cm were collected and *in vitro* transplanted into perforated microvessels in a mini-incubator (Brondani et al. 2018). The three types of microvessels (M1, M2 and M3) used in the adventitious rooting phase were developed from PLA and 3D printed (Orion Delta) (Figure 2). The microvessels were either cylindrical- or cone-shaped with a 1 cm diameter and varying in height, volume, weight, number of elongated slits, and printing time (Table 1).

The mini-incubator is a sealed system consisting of a tray of rectangular cells (47 × 17 × 3 cm) filled with autoclaved sand to support the *in vitro* microvessels. The substrate used to promote rooting of the microcuttings was formulated using a commercial substrate composed of decomposed pine bark and vermiculite (1:1, v/v). Both components were autoclaved. The mini-incubator was kept at 24±1°C with a 16 h photoperiod (40 µmol m^{-2} s^{-1}). The moisture content of the substrate and RH of the mini-incubator were controlled by weekly addition of distilled water (20 – 50 mL) and covering with a polyethylene bag tightened around the edges of the tray (Brondani et al. 2012; Brondani et al. 2018). After 45 days, percentage survival and adventitious rooting of the microcuttings was evaluated. The experiment followed a completely randomised design, including three microvessel types with 15 replications, resulting in 45 microcuttings.

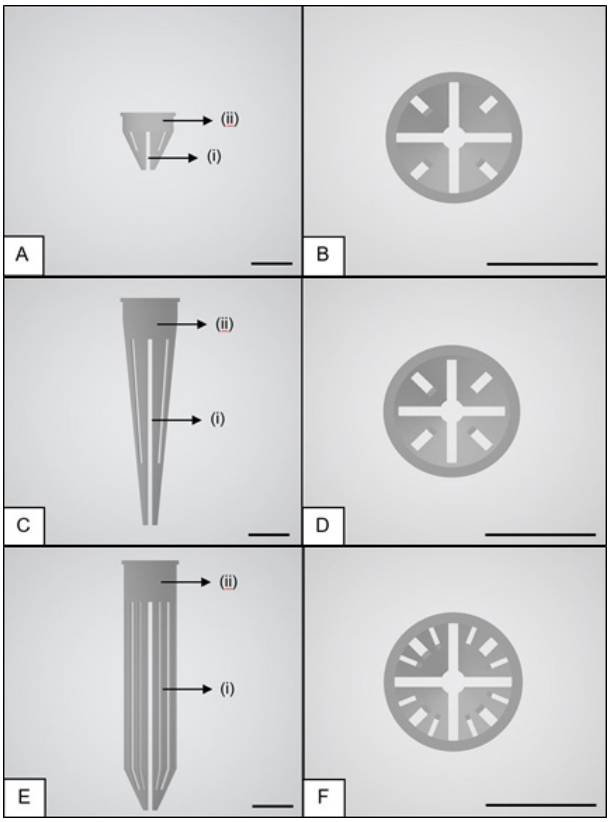

FIGURE 2: Microvessel type developed for the microcutting adventitious rooting and acclimatisation phases of a mature individual of *Eucalyptus microcorys*. (A) M1 (front); (B) M1 (from above); (C) M2 (front); (D) M2 (from above); (E) M3 (front); (F) M3 (from above). Notes: (i) Slits; (ii) Region for support and fitting of the microvessel. Bars = 1 cm. Using 3D printer Orion Delta. Patent registered on 13.03.2020, Brazilian Patent Trademark Office (INPI), BR2020200055130.

Microcuttings that had rooted in the microvessels (Figure 1F) were transferred to cylindric glass flasks sealed with polyvinyl chloride (PVC Boreda, Upside), which were opened every two days for 10 days. The microcuttings were then transferred to polyethylene tubes of 110 cm^3, containing a similar substrate used during the rooting phase (but not autoclaved) and

TABLE 1: Shape, number of elongated slits, dimensions (height, diameter), weight and printing time of microvessel types used during *in vitro* adventitious rooting and acclimatisation phases of *Eucalyptus microcorys* microcuttings.

		Dimensions					
Types[1]	**Shape**	**Number of elongated slits**	**Height (cm)**	**Diameter (cm)**	**Volume (cm^3)**	**Weight (g)**	**Printing time* (min)**
M1	Conical	8	1.5	1.0	0.75	0.53	6
M2	Conical	8	5.5	1.0	2.60	1.00	19
M3	Cylindrical	16	6.0	1.0	5.30	1.00	23

* Using 3D printer Orion Delta.

[1] Patent registered on 13.03.2020, Brazilian Patent Trademark Office (INPI), BR2020200055130.

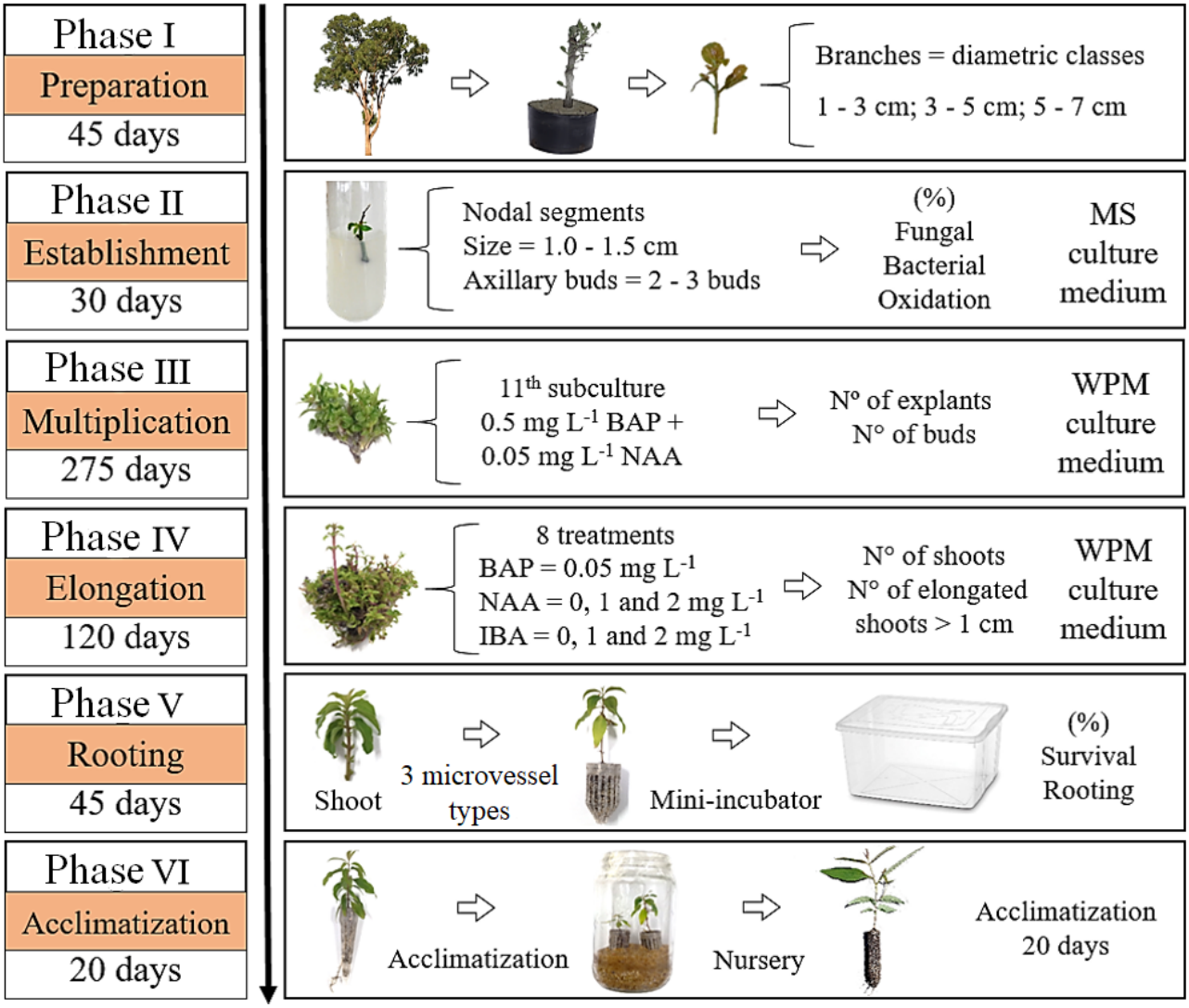

FIGURE 3: Phases of the protocol for the micropropagation of an *Eucalyptus microcorys* selected mature tree, from collection and induction of epicormic shoots (phase I) through to acclimatisation of micro-plantlets (phase VI), with total lasting 535 days.

moved to a greenhouse with 50% shade and sprinkler irrigation four times a day for a 10-day acclimatisation period. Survival of micro-plantlets was evaluated at the end of the acclimatisation period. The phases of micropropagation are represented in Figure 3.

Histological analysis

Anatomical studies were conducted to show the cell differentiation site in the adventitious roots of *E. microcorys*. Samples of stem segments with adventitious roots were fixed in formalin–acetic acid–alcohol (FAA) consisting of 37% formaldehyde, 70% ethyl alcohol and glacial acetic acid (Johansen 1940) for 48 h, and then dehydrated using an ethyl-alcohol series in increasing concentrations (70, 80 and 95%) at 24 h intervals. The samples were embedded in historesin (Historesin®, Leica) (1:1, resin:alcohol) for 7 days, then embedded in pure resin for another 7 days before being blocked in historesin. Blocks were sectioned transversely to a thickness of 8 µm using a microtome. The sections were dehydrated in ethyl alcohol and stained with toluidine blue (pH 4.7) and mounted on slides with permanent glue (Acrilex, São Paulo, Brazil) mixed with glycerin (50%) (Paiva et al. 2006).

Genetic fidelity

Leaf subsamples of acclimatised micro-plantlets (150 mg) and the adult donor plant (150 mg) were used for the extraction of genomic DNA by CTAB method (Doyle & Doyle 1987). Due to the lack of scientific studies on *E. microcorys*, the genetic stability assessment was performed using 20 ISSR universal primers (Table 2). The PCR reactions were performed using a total amount of 13 µL having 30 ng of DNA template and 10 µL of PCR mix [1.5 mM of PCR buffer Phoneutria ®, 1.5 mM dNTP, 1 U Taq polymerase Phoneutria® (5 U μL^{-1}) and 0.2 mM of each primer and sterile ddH_2O]. Amplifications were performed in a thermocycler (GeneAmp PCR System

TABLE 2: Specifications of ISSR primers used in genetic fidelity tests of micropropagated materials of *Eucalyptus microcorys*.

Nr.	Primer	Sequence	Nr.	Primer	Sequence
1	Becky	$(CA)_7$-YC	11	UBC835	$(AG)_8$-YC
2	Chris	$(CA)_7$-YG	12	UBC840	$(GA)_8$-YT
3	John	$(AG)_7$-YC	13	UBC841	$(GA)_8$-YC
4	Manny	$(CAC)_4$-RC	14	UBC842	$(GA)_8$-YG
5	UBC807	$(AG)_8$-T	15	UBC848	$(CA)_6$-RG
6	UBC808	$(AG)_8$-C	16	UBC880	$(GGAGA)_3$
7	UBC810	$(GA)_8$-T	17	UBC889	DBD$(AC)_7$
8	UBC814	$(CT)_8$-TG	18	UBC898	$(CA)_6$-RY
9	UBC825	$(AC)_8$-T	19	UBC901	$(GT)_6$-YR
10	UBC827	$(AC)_8$-G	20	UBC902	$(GT)_6$-AY

R = purine (A or G) e Y = pyrimidine (C or T).

9700) with an initial denaturation of DNA at 94°C for 5 min, followed by 30 s denaturation at 94°C, and 45 s annealing at 42°C and 1 min extension at 72°C, the final extension of 10 min at 72°C and a holding temperature of 4°C. The amplified products were separated in agarose gel (1.5%) and stained with GelRedTM (Uniscience). The amplified products were compared with a marker of known molecular weight (Ladder 1Kb Plus). The gel images were photographed using the gel doc system (Bio-Rad, USA). The occurrence of polymorphism in each sample was assessed using only well-defined bands.

Statistical analysis

The data were analysed by analysis of variance (ANOVA, $p < 0.05$) according to the previously described randomised factorial designs. The means of significant factors were compared with Tukey's test ($p < 0.05$). Homogeneity of variance between factors and normal distribution were assessed with Hartley ($p > 0.05$) and Shapiro-Wilk ($p > 0.05$) tests, respectively. A polynomial regression analysis was conducted to determine the relationship between the number of explants and time. Data were transformed when required using Box-Cox transformations. All statistical analyses were performed in R (version 3.5.2, R Core Team) using the package ExpDes (Ferreira et al. 2021).

Results

Epicormic shoots

There was no difference in the number of buds or epicormic shoots in relation to the diametric classes of the branches collected from the selected mature tree (Table 3). Even though a decreasing trend in the number of buds with increasing diameter class was observed, this difference was not significant, with an overall mean of 9 buds and 10 shoots per tree branch.

Due to the high coefficient of variation (CV) for both number of buds and number of shoots, a transformation was performed. This was due to the large variation in the number of buds and shoots per branch within the same diametric class, while some branches only had one or two buds and shoots, others had more than 20. A total of 150 epicormic shoots were collected for *in vitro* inoculation, reflecting the good quality of the selected mature tree.

In vitro establishment and multiplication

The asepsis implemented in the *in vitro* establishment phase of *E. microcorys* explants resulted in an average establishment of 40.6% of nodal segments after 30 days. Contamination due to fungal, bacterial and phenolic oxidation of tissues was 49.8%, 6.2% and 3.4%, respectively.

The multiplication phase resulted in 11 subcultures lasting 275 days. The polynomial regression analysis provided an adequate estimate for the mean values of the number of explants throughout the multiplication phase, with an R^2 of 0.98 (Figure 4). The *E. microcorys* explants had a satisfactory multiplication percentage resulting in an exponential growth from the 6th subculture, at 150 days (Figure 4).

TABLE 3: Average number of buds and epicormic shoots according to diametric class of branches collected from *Eucalyptus microcorys* kept in greenhouse for 45 days.

Diametetric class	Bud per explant(1)	Shoot per explant(1)
1 - 3 cm	9.80[a] (±1.76)	12.20[a] (±2.63)
3 - 5 cm	8.60[a] (±1.07)	9.70[a] (±0.99)
5 - 7 cm	7.80[a] (±1.98)	10.40[a] (±2.13)
Mean	8.67	10.50
CV (%)	3.4	4.0

Means followed by different letters in the same columns differ statistically according to Tukey's test ($p < 0.05$). (1) Transformed data 1/EXP[(n+0.5)/100], where n = sampled data. CV = coefficient of variation. Values are mean ± standard error.

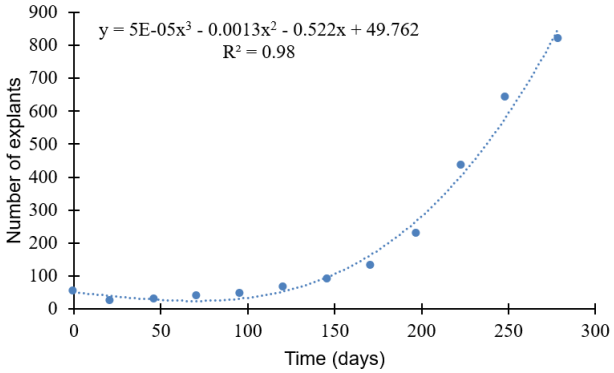

FIGURE 4: Number of explants as a function of subculture time during the multiplication phase of tissues derived from *Eucalyptus microcorys* selected mature tree.

Eucalyptus microcorys responded positively to the multiplication medium developed for *Eucalyptus benthamii*, adapted from Brondani et al. (2012) with BAP (0.5 mg L^{-1}) and NAA (0.05 mg L^{-1}), resulting in 820 explants with an average of 12 (± 0.36) buds per explant (Figure 1D) over a period of 275 days.

In vitro elongation

The plant growth regulators affected the number of shoots and the number of elongated shoots (> 1 cm) of *E. microcorys* explants produced during the shoot elongation phase (Table 4). The highest mean number of shoots, ranging from 90 to 112, were observed with 1 mg L^{-1} NAA, augmented with varying concentrations of IBA. Supplementing the WPM with 2 mg L^{-1} NAA led to an increase in the mean number of shoots compared with no added NAA but a decrease in the mean number of shoots compared with the addition of 1 mg L^{-1} NAA. The lowest mean number of shoots was observed in media not supplemented with NAA (Table 4).

The highest mean number of elongated shoots were observed after supplementation with 1 mg L^{-1} NAA and 1 mg L^{-1} NAA + 1 mg L^{-1} IBA (Figure 1E), with no significant difference observed between these two treatments, which resulted in approximately 18 elongated shoots per explant. The lowest mean number of elongated shoots resulted from the treatments without NAA, with fewer than 8 elongated shoots per explant (Table 4).

TABLE 4: Mean number of shoots and number of elongated shoots (> 1 cm) from explants derived from a mature individual of *Eucalyptus microcorys* exposed to varying concentrations of plant growth regulators (NAA and IBA) at 120 days after various multiplication subcultures.

NAA (mg L^{-1})	IBA	Number of Shoots	Elongated shoots (> 1cm)
0	1	40.00^{c} (±5.78)	6.75^{c} (±0.80)
0	2	39.25^{c} (±4.60)	7.25^{c} (±1.03)
1	0	104.25^{a} (±5.41)	18.35^{a} (±0.56)
1	1	112.00^{a} (±9.03)	18.65^{a} (±0.98)
1	2	90.00^{ab} (±6.57)	13.75^{b} (±1.50)
2	0	69.00^{b} (±5.75)	12.75^{b} (±1.00)
2	1	67.90^{b} (±2.36)	11.50^{b} (±0.68)
2	2	70.00^{b} (±4.27)	12.00^{b} (±0.78)
Mean		70.03	13.77
CV (%)		10	8.8

Means followed by different letters in the same columns differ statistically according to Tukey's test ($p < 0.05$). Values are mean ± standard error. CV = coefficient of variation.

In vitro adventitious rooting and acclimatisation

The various microvessel types tested affected survival, rooting and acclimatisation of *E. microcorys* microcuttings. The microcuttings placed in microvessels, M2 and M3, had a significantly higher survival percentage than those in M1, mirroring the success of these two microvessels during the rooting phase (Table 5). Similarly, microvessels M2 and M3 provided better acclimatisation of microcuttings (Table 5, Figure 1G). As shown in Figure 5, micro-plantlets in microvessel types M2 and M3 were the tallest after 30 days in the greenhouse.

Most of the mortality in *E. microcorys* microcuttings during rooting was due to fungal infection of plant tissues. The most affected being the microcuttings placed in microvessels M1, with 13.3% survival, and only 6.7% with adequate rooting.

TABLE 5: Percentage of microcuttings survival and adventitious rooting during the rooting and acclimatisation phases of a mature individual of *Eucalyptus microcorys* according to microvessel types.

Microvessel type	Survival (%)	Rooting (%)	Acclimatisation (%)
M1	13.3^{b} (±9.1)	6.7^{b} (±6.7)	6.7^{b} (±6.7)
M2	53.3^{a} (±13.3)	40.0^{ab} (±13.1)	33.3^{ab} (±12.7)
M3	58.9^{a} (±12.7)	45.7^{a} (±12.9)	45.7^{a} (±12.9)
Mean	42.2	31.1	28.9
CV (%)	21.9	19.9	19.4

Means followed by different letters in the same columns differ statistically according to Tukey's test ($p < 0.05$). Values are mean ± standard error. CV = coefficient of variation.

FIGURE 5: Micro-plantlets derived from the micropropagted epicormic shoots of an *Eucalyptus microcorys* selected mature tree, and acclimatised in a greenhouse for 30 days, displaying the difference in the growth of shoots between three types of microvessels tested (Table 1). Bar corresponds to 1 cm.

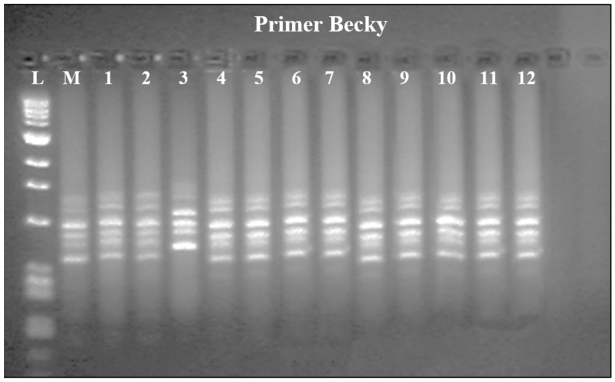

FIGURE 7: Becky primer electrophoresis of tissue cultured *Eucalyptus microcorys* in relation to the mature selected tree from which the cultures were initiated. L = 1Kb Plus DNA Ladder marker; M = mature selected tree; 1 to 12 = micropropagated clonal plants.

Histological analysis

The anatomical cuts indicated the base of the microcutting at the point where the adventitious roots originate. The cuts exposed the organisation of the vascular bundles forming a cylindrical ring around the stem (Figure 6A). Figure 6B provides a detailed view of the vascular connection established between the adventitious root and the stem cambium.

Genetic fidelity

Of the 20 primers used, 18 resulted in amplification of the DNA of the propagated materials from *E. microcorys*, with primers ranging from 3 to 8 bands. The size of the amplified markers ranged from 200 to 2,500 bp, based on the standard marker for comparison (Figure 7). The profiles of the bands of the ISSR markers used indicated that all propagated plants from *E. microcorys* were genetically similar to those of the original selected mature tree, thus guaranteeing the clonal nature of all plants. The difference in band mobility observed in some samples is expected due to the running of the samples (Figure 7).

Discussion

The induction of epicormic shoots from pruned branches provided shoots with satisfactory quality and quantity for *in vitro* introduction, suggesting this was a viable technique for the micropropagation of the selected *E. microcorys* individual. This method has been used effectively for woody tree species such as *Ilex paraguariensis* (19-years old) (Wendling et al. 2013), *E. benthamii* (13-years old) (Baccarin et al. 2015) and *Eucalyptus cloeziana* (26-years old) (Oliveira et al. 2015). One of the major challenges related to clonal propagation of woody tree species is the ontogenetic aging of plant tissues, which may lower regeneration and rooting of propagules (Hartmann et al. 2011; Oliveira et al. 2015; Wendling et al. 2013). In this study, despite

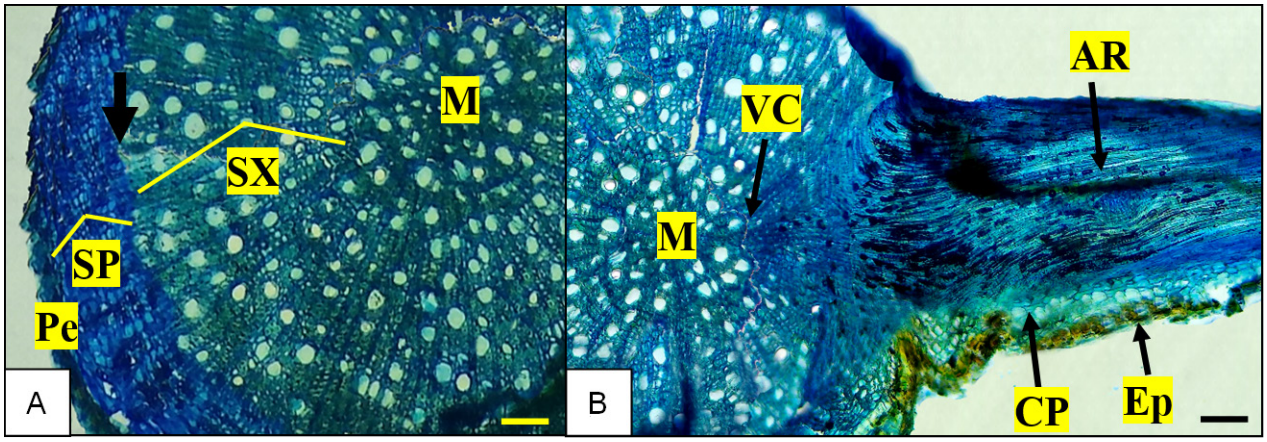

FIGURE 6: Anatomical sections of the acclimatized microcuttings of *Eucalyptus microcorys*. (A) Stem section exposing the organisation of the vascular bundles. (B) Vascular connection between the adventitious root and the stem cambium. Pe = periderm; SP = secondary phloem; (arrow) = cambium; SX = secondary xylem; M = medulla (pith); VC = vascular cambium; CP = cortical parenchyma; Ep = epidermis; AR = adventitious root. Bars correspond to 100 µm.

collecting branches from the lowest portion of the crown to minimise the ontogenetic age effect, there were likely variations in the physiological and genetic potential of the selected tree.

A large loss of explants due to fungal contamination was observed during the *in vitro* explant establishment phase. Fungal contamination is commonly reported during establishment; for instance Brondani et al. (2009) reported over 40% fungal contamination in *E. benthamii* × *E. dunnii* explants. Besides the age and physiological state of the selected tree, sterilisation time and concentration of the antiseptic solution strongly affect the level of bacterial and fungal contamination of explants (Navroski et al. 2014; Baccarin et al. 2015). Fungicide application on the shoots while in the greenhouse a few days before collection would have probably rendered the sources of contamination and increased the micropropagation success of the studied species. Nevertheless, despite the loss of nearly 60% of the materials inoculated *in vitro* in this study, only a few explants with contamination-free buds are required to successfully conduct micropropagation, as the risk of contamination in the next phases is lower (Trueman et al. 2018). In the present study, the established axillary buds of *E. microcorys* were of excellent quality, and thus satisfactory for use in the next phases of the micropropagation protocol.

During the shoots and buds' multiplication phase, high concentrations of plant growth regulators, such as cytokinin, are generally used to stimulate the development of axillary buds, triggering the growth and cell divisions, which will introduce new shoots. BAP is the most used cytokinin during *in vitro* multiplication of tree species and is often combined with NAA (Davey & Anthon 2010; Oliveira et al. 2013). In this study, *E. microcorys* responded positively to the multiplication medium developed for *E. benthamii*, adapted from Brondani et al. (2012), supplemented with BAP (0.5 mg L^{-1}) and NAA (0.05 mg L^{-1}). These concentrations have been successfully implemented in the micropropagation of several species of *Eucalyptus* and *Corymbia*, for instance *E. tereticornis* × *E. camaldulensis* (Bisht et al. 1999), *E. tereticornis* (Sharma & Ramamurthy 2000), *E. tereticornis* × *E. grandis* (Joshi et al. 2003), *E. camaldulensis* × *E. tereticornis* and *E. torelliana* × *E. citriodora* (Arya et al. 2009), *E. benthamii* × *E. dunnii* (Brondani et al. 2011) and *E. benthamii* (Baccarin et al. 2015; Brondani et al. 2012). At the end of the multiplication phase, many *E. microcorys* explants were obtained in the 11th subculture. According to Alfenas et al. (2009), the rejuvenation or at least the rooting ability is usually restored after 10 – 12 subcultures. Similar results were also reported in Xavier et al. (2007), in which a minimum of 12 subcultures were recommended when the objective is tissue rejuvenation of selected mature eucalypts and to improve adventitious rooting.

The aim of the *in vitro* shoot elongation phase is to gather shoots of suitable size (at least 1 cm) in the shortest time (Hartmann et al. 2011). In the current study, the culture medium supplemented with NAA and the combination of NAA and IBA provided the largest number of elongated shoots of *E. microcorys*, with the use of auxins instead of commonly used gibberellins. Due to the high maturation of the source material, auxins are expected to promote rooting in addition to shoot elongation, thus contributing to greater cloning success (Souza & Pereira 2007; Rocha et al. 2009; Trueman et al. 2018). A satisfactory number of elongated shoots were reported by Navroski et al. (2014) using NAA (0.5 mg L^{-1}) for shoot elongation of *E. dunnii*, while in Brondani et al. (2009), the highest number of elongated shoots of *E. benthamii* × *E. dunnii* were achieved with a combination of NAA (0.25 and 0.75 mg L^{-1}) and BAP (0.05 mg L^{-1}). A combination of plant growth regulators belonging to the auxin and cytokinin groups is also recommended for many species of the *Eucalyptus* and *Corymbia* genera, for instance *E. grandis* × *E. nitens* and *E. grandis* × *E. urophylla* (Watt 2014), *E. grandis* × *E. nitens* and *E. grandis* (Nakhooda et al. 2012) and pure *E. grandis* (Nakhooda et al. 2011).

The anatomical studies conducted showed that adventitious rooting occurred, indicating a successful connection to the vascular cambium of the stem. Once this connection is established, the plant ceases to depend only on the nutritional reserves of the aerial part but is also supplied by the chemical elements assimilated by the root. This ultimately enables the acclimatisation of the propagated microcuttings.

Problems associated with rooting and acclimatisation phases following standard micropropagation protocols can be minimised by using microvessels. In this study, microvessels were placed in a mini-incubator based on the acclimatisation success for *E. benthamii* (Brondani et al. 2012) and for *E. cloeziana* (Baccarin et al. 2015; Oliveira et al. 2015). Yield losses usually occur during *in vitro* to *ex vitro* transplantation of micro-plantlets due to root and shoot stress. The use of microvessels helps to minimise plant stress, as there is no need to transplant rooted shoots. In addition, they decrease operational time in the nursery (the need to further transfer rooted plants out of the microvessels) since they are biodegradeable and thus degrade over time. Moreover, *ex vitro* rooting using a growth substrate is more cost-effective and removes the need to use plant growth regulators, such as IBA (Brondani et al. 2012; Brondani et al. 2018).

The adventitious rooting and survival of the microcuttings in this study were at best 46% and 59%, respectively, even though the overall microcutting rooting and survival of eucalypt trees is at least 85% (Almeida et al. 2007; Brondani et al. 2010b; Borges et al. 2011). However, several studies report different results in adventitious rooting when using juvenile compared to mature plant material. Differences may be due to the physiological condition of the selected trees and their ontogenetic age (Brondani et al. 2009; 2010a; 2012; Wendling et al. 2013; Hung & Trueman 2011; Trueman et al. 2018). Therefore, these factors may have contributed to the lower rooting percentage of *E. microcorys* in this study. Additionally, the high mortality of *E. microcorys* microcuttings was partially due to fungal contamination. Microcuttings growing in M1, the smallest microvessel

tested, were the most affected, probably due to their proximity to the moisture accumulating in the bottom of the mini-incubator. All microvessel types were designed with elongated slits and perforations to facilitate vessel degradation in addition to aiding root expansion, substrate aeration and prevention of moisture accumulation. However, due to the lower number and height of slits in the M1 microvessel, the growth and expansion of the microcutting radial system was hindered. Taken together, these results suggest that microvessels can be suitable for rooting and acclimatisation of *E. microcorys*, especially using microvessels of at least 5.5 cm height and with a minimum of 8 slits, and studies on their viability in the micropropagation of other eucalypt species are encouraged.

In this study, the acclimatised plants had 100% genetic uniformity with the original parent plant (i.e., selected tree), demonstrating genome stability with the manipulations that occurred during the micropropagation phases and the time of *in vitro* cultivation. Genetic fidelity studies using ISSR markers to test the integrity of eucalypt plant clones via micropropagation of *E. microcorys* have not been performed, even though it has been used before in several species, for example *E. tereticornis* (Aggarwal et al. 2010), *E. globulus* (Oliveira et al. 2017) and *E. camaldulensis* (Shanthi et al. 2015). Assessment of genetic fidelity for *in vitro* micropropagated plants is crucial to identify the multiplied clones and to ensure their quality, as somaclonal variations may occur during prolonged *in vitro* cultivation, producing different morphological characteristics in the clones compared to their selected tree (Shen et al. 2007).

Characterisation of epigenetic diversity is important for our increased understanding of plant responses to changes in the environment, as well as for the full use of productive potential through breeding programmes and to ensure greater control in the homogeneity of clonal plantations. In the past decade, studies have been undertaken to unravel the different genetic mechanisms of micropropagation and to overcome challenges when carrying out plant propagation, such as epigenetic modification. Epigenetic diversity may occur in response to environmental variations resulting in phenotypic changes in propagated individuals without the occurrence of changes in the DNA sequencing (Elwell et al. 2011; Rasmann et al. 2012; Vivas et al. 2013; Vivas et al. 2017). Although the results of this study did not indicate genetic diversity among the propagated *E. microcorys* individuals, the tissue rejuvenation of the propagated materials suggest that epigenetic diversity may have occurred, mainly due to the *in vitro* cultivation, as different individuals may present different levels of expression according to the environment where they developed (Vivas et al. 2019; Wang et al. 2020).

Conclusions

The proposed micropropagation protocol incorporating microvessels was considered satisfactory to produce *E. microcorys* micro-plantlets. The use of epicormic shoots as explants were suitable for *in vitro* cultivation with an establishment percentage higher than 40% and total multiplication of 820 explants with 12 buds per explant by the 11th subculture. Shoot elongation was higher in growing media supplemented with the combination of BAP (0.05 mg L^{-1}) + NAA (1 mg L^{-1}) as well as the aforementioned concentrations with the addition of IBA (1 mg L^{-1}). Use of microvessels M2 (volume = 2.60 cm^3) and M3 (volume = 5.30 cm^3) provided better rooting and acclimatisation of the microcuttings, forming normal adventitious roots with a direct connection to the vascular cambium. The *in vitro* micropropagation lasted 490 days and genetic fidelity was confirmed by ISSR markers. Considering all six phases of *E. microcorys* micropropagation evaluated here, especially rooting and acclimatisation using microvessels, the proposed protocol (535 days) could potentially be integrated into future mechanisation of *in vitro* propagation of *E. microcorys*, and extended to other genotypes of *E. microcorys*, or other forest species.

Competing interests

The authors declare that they have no competing interests.

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Abbreviations

ANOVA: analysis of variance
BAP: 6-benzylaminopurine
CTAB: cetyltrimethylammonium bromide
CV: coefficient of variation
DNA: desoxyribonucleic acid
dNTP: deoxynucleotide triphosphate
FAA: formalin–acetic acid–alcohol
IBA: indole-3-butyric acid
ISSR: inter simple sequence repeat
NAA: α-naphthaleneacetic acid
PCR: polymerase chain reaction
PLA: polylactic acid
RH: relative humidity
WPM: Woody Plant Medium (Lloyd & McCown 1980).

Author Contributions

Each author contributed to the present manuscript as follows: JCTF and GEB contributed to the design and development of the study, establishment of the experiment, data collection, processing and statistical analysis, writing, review and construction of figures and tables. CR-K contributed to the discussion of the results,

text revision, and helped translate the manuscript to English. DC, JEBPP and ARSN contributed to the laboratory analysis, interpretation of the data, especially from the genetic analysis, and discussion of the results. RSRC and EMN contributed to the establishment of the experiment, data collection and processing, discussion of results and text revision. All authors read and approved the final manuscript.

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