



Eucalyptus nitens plant regeneration from seedling explants through direct adventitious shoot bud formation

Paula G. Ayala¹ · Elsa A. Brugnoli¹ · Claudia V. Luna¹ · Ana M. González¹ · Raúl Pezzutti² · Pedro A. Sansberro¹

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Abstract

Key message A protocol for true-to-type plantlets production by direct adventitious bud proliferation from *Eucalyptus nitens* hypocotyl segments was developed.

Abstract Hypocotyls, cotyledons, and expanded leaves of *Eucalyptus nitens* were cultured in Murashige and Skoog (MS) medium containing naphthalene acetic acid or indole-3-acetic acid (IAA) and 6-benzyl adenine (BA). The most effective treatment for shoot bud formation, 0.5 μM IAA and 2.5 μM BA, was used in additional experiments. After 30 days of culture, the highest rate of regeneration ($40 \pm 5.8\%$) and the maximum number of buds differentiated per hypocotyl explant (10.3 ± 4) were obtained when explants were subjected to darkness for the first 10 days and then transferred to light-emitting diode lights mimicking daylight for 20 days of incubation. Histological examination confirmed direct shoot regeneration of *E. nitens*. For shoot growth, half-strength MS plus 0.09 M sucrose under forced ventilation yielded elongated shoots after 1 month. After 12 weeks from the onset of an experiment, $40 \pm 11.5\%$ of elongated shoots produced 5.3 ± 0.8 roots after pretreatment with indole-3-butyric acid aqueous solution and culture on basal medium without plant growth regulators under a temporary immersion system. Inter simple sequence repeat (ISSR) marker analysis revealed the genetic uniformity among the in vitro raised plants, demonstrating the reliability of the procedure.

Keywords Direct shoot bud proliferation · Lighting · Organogenesis · Shining gum

Introduction

Eucalyptus nitens (Deane and Maiden) Maiden, commonly known as silver top or shining gum, is grown spontaneously in south-eastern Australia under cold to warm, humid-to-subhumid conditions (Orwa et al. 2009). Owing to the physical properties of its wood, *E. nitens* is mainly planted for kraft pulpwood, and to a lesser extent, for lumber and/or veneer production in the high-rainfall temperate regions of Australia, Chile, New Zealand, and Southern Africa

(Hamilton et al. 2011; Vega et al. 2016). Field experiments performed outside its centre of origin have shown its capacity to grow well in regions 600–1000 m above sea level (MASL), where winter temperatures are too low for other fast-growing commercial *Eucalyptus* species (Nakhoda and Jain 2016). One of the main traits that characterises its performance is a relatively high tolerance to freezing and moderate resistance to drought (Orwa et al. 2009). Since cold tolerance is considered a quite highly heritable trait, it has been used in interspecific hybrid combinations to produce cold-tolerant cultivars. Notable hybrids include *E. globulus* \times *E. nitens* and *E. grandis* \times *E. nitens*, which combine the fast growth rates of *E. globulus* and *E. grandis* with the cold tolerance of *E. nitens* (Tibbits et al. 1991; Denison and Kietzka 1993; Harwood 2015).

E. nitens is usually propagated by seeds (RIRDC 2009); however, it requires long periods for seed maturation, resulting in low and irregular germination rate (Turnbull and Doran 1987; Humara et al. 2000). Vegetative propagation of *E. nitens* using conventional and tissue culture procedures has previously been carried out; however, these

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✉ Pedro A. Sansberro
sansber@agr.unne.edu.ar

¹ Instituto de Botánica del Nordeste (IBONE-CONICET), Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste, Sargento Cabral 2131, W3402BKG Corrientes, Argentina

² Forestal Bosques del Plata S.A., Av. J.M. Fangio 3873, CP: 3300, Posadas, Misiones, Argentina

methods need further research before they can be applied commercially. Maile and Nieuwenhuis (1996) reported a macropropagation protocol for *E. nitens* using stem cuttings from juvenile plants, and micropropagation was achieved with 4-, 8-, and 10-week-old seedling cuttings (Gomes and Canhoto 2003). Indirect organogenesis from hypocotyl and cotyledon calli proliferation was reported by Bandyopadhyay et al. (1999). Sporadic formation of somatic embryos was also observed on the underside of wound callus that developed at the base of regenerated shoots (Bandyopadhyay and Hamill 2000).

In this study, we described the procedures developed to induce direct regeneration of shoots and subsequently true-to-type plantlet production from hypocotyl and cotyledon culture of *Eucalyptus nitens*. To our knowledge, direct regeneration of *E. nitens* has not been reported to date.

Materials and methods

Plant material and in vitro seed germination

Toorong Plateau provenance *Eucalyptus nitens* (H. Deane and Maiden) Maiden seeds obtained from an orchard established in the Biobio region of Chile were used for all experiments. The seeds were collected in 2016 and stored in a refrigerator at 4 °C until needed.

Under aseptic conditions, seeds were disinfected in 70% (v/v) ethanol for 2 min, transferred to 1.8% (w/v) NaOCl and 0.1% Triton X-100 for 20 min, and rinsed three times with sterile distilled water. Afterward, the seed coat was removed, and the zygotic embryos were cultured in 11 ml glass tubes (3 seeds/tube) containing 3 ml Murashige and Skoog (MS, Murashige and Skoog 1962) medium with 0.09 M sucrose (germination medium). The pH of the medium was adjusted to 5.8 before the addition of 6.5 g l⁻¹ agar A-1296 (Sigma-Aldrich, St. Louis, MO, USA). The medium was autoclaved at 121 °C and 1.4 kg m⁻² pressure for 20 min. The cultures were incubated in a growth room at 27 ± 2 °C, in the dark for 7 days, and then placed under light-emitting diode (LED) lights mimicking daylight (14 h photoperiod, 116 μmol m⁻² s⁻¹ photosynthetic photon flux density, PPFd).

Organogenesis and plant regeneration

Hypocotyl, cotyledon, and expanded leaf segments from in vitro 15-day-old seedlings were used for the regeneration experiments. Each seedling parts was cut into 3–4 mm segments and cultured for 30 days in an 11 ml glass tubes containing 3 ml semisolid MS medium with 0.09 M sucrose, supplemented with indole-3-acetic acid (IAA; 0–0.5 μM), naphthaleneacetic acid (NAA; 0–0.5 μM), and 6-benzylaminopurine (BA; 0.5–13.5 μM). The cultures were kept in the

dark for 1 or 6 days, and then transferred to the light conditions described above until the 30th day. In a second experiment, hypocotyl and cotyledon explants were cultured on the best regeneration medium (MS plus 0.5 μM IAA and 2.5 μM BA). In this case, the explants were subjected to darkness for the first 6 or 10 days of incubation. Subsequently, they were transferred to low (58 μmol m⁻² s⁻¹ PPFd) or high (116 μmol m⁻² s⁻¹ PPFd) light intensity. Two control treatments were included in which the explants were cultured under complete darkness or light (low or high light intensity) for the entire experimental period.

To stimulate shoot elongation, regenerating explants were subcultured into 200 ml crystalline flasks (5 bud clusters per flask), each containing 50 ml half-strength MS (½ MS) supplemented with 0.09 M sucrose. The culture vessels were closed with polypropylene caps that were equipped with 50 mm nylon membrane filters of 0.22 μm pore size, which enabled gas exchange. Four treatments combining different gelling agents (either agar or gellan gum) and ventilation were tested: 6.5 g l⁻¹ agar (A-1296, Sigma-Aldrich) with diffusive ventilation (ADV); 6.5 g l⁻¹ agar with forced ventilation (AFV); 3.5 g l⁻¹ gellan gum (Phytigel[®], Sigma-Aldrich) with diffusive ventilation (PDV); and 3.5 g l⁻¹ gellan gum with forced ventilation (PFV).

Finally, the elongated shoots were rooted by pretreatment in a 5 mM indolebutyric acid (IBA) solution for 30 min, before being transferred to either agar, gellan gum, vermiculite, or liquid medium (temporary immersion), containing ½ MS lacking plant growth regulators under a forced ventilation system. The twin flask bioreactors (BIT[®]) developed by Lorenzo et al. (1998) were used, consisting of two transparent glass flasks connected by silicone hosepipes. The hosepipes are equipped with nylon syringe filters (0.45 μm pore size, 55 mm diameter) to avoid contamination and were connected to air compressors to transfer liquid medium from one flask to the other for a stipulated period. The flow was reversed at the end of this period, for the same amount of time. For the forced ventilation system, the air was injected with a pressure of 0.5 bar for 1 min every 4 h. For the temporary immersion programme, explants were in contact with the medium for 1 min every 4 h. The cultures were incubated in a growth room under high light conditions (14 h photoperiod, 116 μmol m⁻² s⁻¹ PPFd).

Acclimatization

30-day-old plantlets were transferred to 150 cc pots containing sphagnum peat moss (Sunshine[®] Premix 6, Sun Gro Horticulture Distribution Inc., Agawam, MA, USA) and controlled-release micro-fertilizer (Osmocote[®] 18-5-9, 0.6 mg cm⁻³ substrate). They were grown for 4 weeks in a growth chamber that maintained the day air temperature below 30 °C and the substrate temperature at 22–25 °C with

a 12 h photoperiod (sunlight conditions reduced to 65%); the relative humidity ranged from 90 to 60%.

Histological analysis

Histological observations were made according to González and Cristóbal (1997). Samples of explants cultured in MS plus 0.5 μM IAA and 2.25 μM BA were fixed in a formalin:ethanol:acetic acid (FAA) solution and dehydrated with a Biopur[®] series. Transverse and longitudinal serial sections 10 μm thick were stained with safranin–astra blue (Sigma-Aldrich) and mounted in synthetic Canada balsam (Sigma-Aldrich). Photomicrographs were taken with a Leica DMLB2 photomicroscope equipped with a Leica ICC50HD digital camera (Leica, Wetzlar, Germany). Samples were harvested daily from the 1st to the 25th days of the induction phase.

Inter simple sequence repeat (ISSR) marker analysis

Genomic DNA was isolated from leaves of ten plants regenerated from hypocotyl segments and ten plants obtained by in vitro germination of seeds, using the EasyPure[®] plant genomic DNA kit (TransGen Biotech, Beijing, China). A total of eight primers were used for ISSR amplification, with the reactions being performed as described by Brugnoli et al. (2013). Amplification products were separated by 2% agarose gel electrophoresis in 1 \times TAE buffer at 70 V. The DNA amplification fragment obtained for each plant was introduced into a binary-data matrix. ISSR markers were scored for the presence (1) and absence (0) of homologous DNA bands of the same molecular size. Fragments with the same molecular size were considered to be analogous amplicons representing the same locus. The resulting binary-data matrix was analysed using the Info-Gen software package (Balzarini and Di Rienzo 2013). Genetic stability among samples was measured by Jaccard's dissimilarity coefficient (I-S). A dendrogram was created based on a matrix of distance, using the unweighted pair group method with arithmetic averages (UPGMA).

Experimental design and statistical analysis

The experiments were performed in triplicate, using a randomised block design. In the regeneration experiments, ten explants were singly cultured per treatment, while for the elongation and rooting phases, each culture vessel containing five bud clusters/elongated shoots was considered an experimental unit. For shoot elongation and rooting, cultures from all treatments were randomly placed along the compressed air line. The regeneration rate and the number of buds regenerated per explant were determined after 30 days of incubation. For the elongation phase, the shoot length,

number of shoots ≥ 1 cm in length, number of phytomers, and chlorosis of leaves were assessed at the end of the experiment. The rooting percentage and number of roots per rooted shoot were quantified after 20 days of culture.

All data were expressed as the mean \pm standard error of the mean (SEM). Statistical comparisons were performed using Tukey's multiple comparisons test, and *P* values < 0.05 were considered to be statistically significant. Statistical tests were performed with the help of Graph Pad software version 8.1.0 (San Diego, CA, USA) and InfoStat (Córdoba, Argentina).

Results

Organogenesis and plant regeneration

Initially, the effect of BA, alone or in combination with NAA or IAA, on adventitious shoot bud formation from hypocotyl, cotyledon, and leaf explants, was tested. The explants were incubated in the dark for 1 or 6 days, and transferred to the light until 30 days of incubation had taken place. Although shoot bud formation was achieved from all explants, the regeneration rate was dependent on plant growth regulators (PGR) supplementation and light conditions, ranging from 0 to $33.3 \pm 17.6\%$ (Table 1). The best results were obtained when hypocotyl segments were cultured on MS medium plus 0.5 μM IAA and 2.25 BA μM , and incubated in the dark for the first 6 days. In such conditions, the regenerating explants also produced the highest number of shoot buds per explant, an average of 12 ± 2 . The addition of NAA in the induction medium negatively affected the formation of shoot buds due to callus proliferation with bud numbers varying from one to seven per explant. The absence of auxins inhibited the morphogenic process in hypocotyl segments and, to a lesser extent, in leaf explants.

The best regeneration medium was chosen to then extend the pretreatment in the dark to 10 days and to evaluate the effect of different light conditions on the morphogenic responses of hypocotyl and cotyledon segments. After 30 days of culture on MS medium plus 0.5 μM IAA and 2.25 μM BA, the highest regeneration rate of $40 \pm 5.8\%$ was achieved when hypocotyl explants were incubated in the dark for 10 days and transferred to high light intensity. Statistical analysis of the data showed significant differences when compared with cotyledon explants undergoing the same light treatments (Table 2). The number of adventitious shoot buds per regenerating explant varied between 1 and 10.3 ± 4 , being higher from hypocotyl segments.

Principal component analysis (PCA) showed differences in the responses of hypocotyl segments treated with different light conditions. The first principal component (PC1) explained 42.6% of the total variation in the data

Table 1 Effects of NAA, IAA, and BA on the adventitious shoot bud formation from hypocotyl, cotyledon, and leaf segments cultured on MS medium

PGRs (μM)				Explants		
NAA	IAA	BA	Dark period ⁱ	Hypocotyl	Cotyledon	Leaf
0.05	–	0.5	1	13.3 \pm 13.3 ab ⁱⁱ	0 b	0 b
			6	0 b	0 b	6.7 \pm 6.7 ab
0.05	–	2.25	1	6.7 \pm 6.7 ab	6.7 \pm 6.7 ab	6.7 \pm 6.7 ab
			6	6.7 \pm 6.7 ab	0 b	0 b
0.05	–	4.5	1	0 b	6.7 \pm 6.7 ab	0 b
			6	0 b	6.7 \pm 6.7 ab	0 b
0.05	–	13.5	1	0 b	0 b	0 b
			6	0 b	0 b	0 b
0.5	–	0.5	1	13.3 \pm 6.7 ab	0 b	0 b
			6	6.7 \pm 6.7 ab	0 b	6.7 \pm 6.7 ab
0.5	–	2.25	1	0 b	0 b	0 b
			6	0 b	0 b	0 b
0.5	–	4.5	1	0 b	0 b	0 b
			6	6.7 \pm 6.7 ab	0 b	6.7 \pm 6.7 ab
0.5	–	13.5	1	0 b	0 b	0 b
			6	0 b	0 b	0 b
–	–	0.5	1	0 b	0 b	0 b
			6	0 b	0 b	13.3 \pm 6.7 ab
–	–	2.25	1	0 b	13.3 \pm 13.3 ab	0 b
			6	0 b	6.7 \pm 6.7 ab	0 b
–	–	4.5	1	0 b	0 b	0 b
			6	0 b	6.7 \pm 6.7 ab	0 b
–	–	13.5	1	0 b	0 b	0 b
			6	0 b	0 b	0 b
–	0.5	0.5	1	20 \pm 0 ab	0 b	0 b
			6	20 \pm 11.5 ab	0 b	6.7 \pm 6.7 ab
–	0.5	2.25	1	6.7 \pm 6.7 ab	6.7 \pm 6.7 ab	6.7 \pm 6.7 ab
			6	33.3 \pm 17.6 a	6.7 \pm 6.7 ab	0 b
–	0.5	4.5	1	0 b	0 b	0 b
			6	6.7 \pm 6.7 ab	0 b	0 b
–	0.5	13.5	1	0 b	0 b	0 b
			6	6.7 \pm 6.7 ab	0 b	0 b

ⁱDark period: the explants were incubated in the dark for 1 or 6 days and transferred to light until completing 30 days of incubation

ⁱⁱValues represent the mean percentage (\pm SEM) of three independent experiments ($n=10$). Means followed by the same letters are not significantly different at $p \leq 0.05$, according to Tukey's multiple comparison test

and allowed light treatments to be separated, while PC2 explained 25.8% of the variation (Fig. 1). Pre-culture in the dark for the first 10 days and transfer to high light intensity was the treatment with the highest correlation in PC1, and constituent the treatment most strongly correlated with the bud regeneration rate and the number of adventitious shoot buds per explant. In contrast, these variables were negatively affected by continuous exposure to darkness or high light intensity, due to the proliferation of callus and browning of the explants, respectively. PC1 explained 58.3% of the total variation from cotyledon explants and allowed the tested

treatments to be separated, while PC2 explained 21.5% of the variation (Fig. 2). Incubation in the dark for the first 6 days and subsequent transfer to light conditions was the treatment that showed the highest correlation in PC1.

Histological examination confirmed direct shoot regeneration of *E. nitens* (Fig. 3a–f). Features typical of the stem were observed in cross sections of initial explants (Fig. 3a), including uniseriate epidermis, cortex, a cylindrical core of xylem surrounded by a ring of phloem (ectophloic siphonosteles) and parenchymal pith. Adventitious shoot buds were originated from meristemoid regions formed after 10 days

Table 2 Effect of different light treatments on the adventitious shoot bud regeneration from hypocotyl and cotyledon explants cultured on MS medium plus IAA 0.5 μM and BA 2.25 μM

Treatments	Light source	Hypocotyls	Cotyledons
Light (30 ds)	LLI	16.7 \pm 3.3 bc ⁱⁱ	6.7 \pm 3.3 bcd
	HLI	3.3 \pm 3.3 d	0 d
Dark ⁱ (6 ds) + light (24 ds)	LLI	16.7 \pm 3.3 bc	3.3 \pm 3.3 cd
	HLI	20.0 \pm 10.0 b	16.7 \pm 6.7 bc
Dark (10 ds) + light (20 ds)	LLI	13.3 \pm 3.3 bcd	3.3 \pm 3.3 cd
	HLI	40 \pm 5.8 a	6.7 \pm 6.7 bcd
Dark (30 ds)	–	0 d	3.3 \pm 3.3 bcd

ⁱDark period: the explants were incubated in the dark for 6, 10, or 30 days and transferred to either low (LLI) or high (HLI) LEDs' light intensity until completing 30 days of incubation

ⁱⁱValues represent the mean percentage (\pm SEM) of three independent experiments ($n=10$). Means followed by the same letters are not significantly different at $p \leq 0.05$ according to Tukey's multiple comparison test

of incubation and located just below the hypocotyl cut end (Fig. 3b). Meristemoids were originated in the cortical parenchyma from subepidermal cells. Meristematic cells were found to be much smaller than the surrounding cells,

with dense cytoplasm, small vacuoles, and a conspicuous nucleus. During organogenesis, successive mitotic divisions produced hemispheric domes with the tunica-carpus organization characteristic of the shoot apical meristem. The leaf buttress originated from the lateral flanks of the apical meristem, resulting from periclinal and anticlinal cell divisions in the outer carpus and tunica layers, respectively (Fig. 3c–d). Completely developed shoots were intimately coupled with the explants through the formation of a vascular connection (Fig. 3e). The *in vitro* regenerated leaves also had subepidermal oil secretory cavities characteristic of *Eucalyptus spp.* (Fig. 3f).

The 30-day-old bud clusters originated from hypocotyl segments subjected to darkness for 10 days, with transferring to high light intensity were chosen to stimulate shoot elongation. The bud clusters were subcultured on semisolid $\frac{1}{2}$ MS medium plus 0.09 M sucrose with either agar or gellan gum, under a diffusive or forced ventilation system. Even though none of the treatments tested affected the elongation of the stems (Table 3), the interaction of gellan gum and forced ventilation slightly promoted the differentiation of phytomers, and produced healthy shoots without chlorosis. Both parameters were considered the most appropriate treatment for the elongation step.

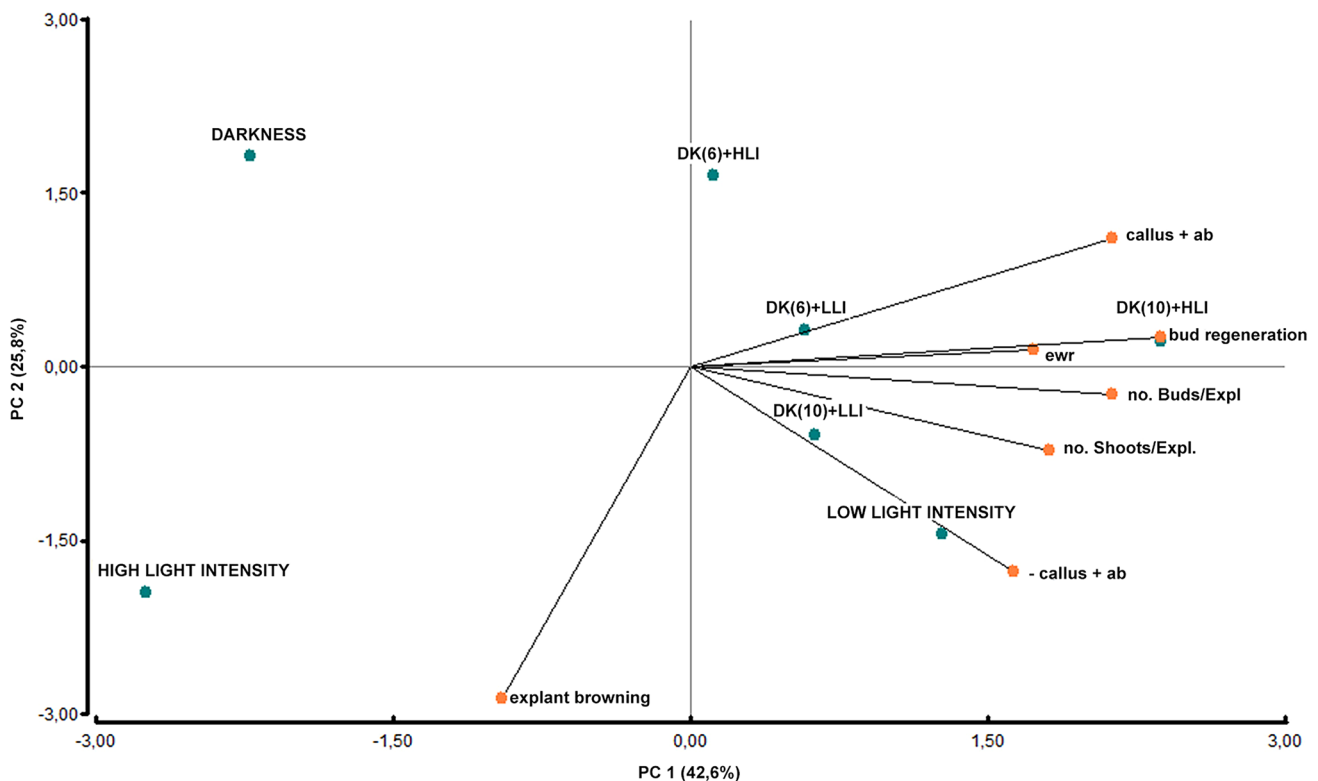


Fig. 1 Biplot of PCA of light conditions and morphogenic responses of hypocotyl explants cultured on MS medium plus μM IAA 0.5 and 2.25 μM BA. *CP1* principal component 1, *CP2* principal component

2, *Dk* dark (number of days in parentheses), *HLI* high light intensity, *LLI* low light intensity, *ab* adventitious buds, *ewr* explants without responses (the explant remained green)

Finally, the elongated shoots were pretreated with a 5 mM IBA solution for 30 min and transferred to agar, gellan gum, vermiculite, or liquid media, containing $\frac{1}{2}$ MS plus 0.09 M sucrose, under forced ventilation. Pretreatment with IBA stimulated direct root formation in shoots from organogenic cultures of *E. nitens*. The adventitious roots emerged from stems after 7 days of culture. The percentage of rooting increased from 6.7 ± 6.7 to $40 \pm 11.5\%$ after pretreatment with IBA, while the number of adventitious roots per shoot varied between 1.8 ± 0.5 and 5.3 ± 0.8 (Fig. 4). Although there were no statistically significant differences for any of the parameters measured, the temporary immersion system provided the more stable results. More than 70% of rooted plantlets transferred to pots continued the growth.

Genetic stability assessment of regenerated plants

The oligonucleotide primers used in the amplification reactions generated 74 bands on gel electrophoresis, between 250 and 2000 base pairs (bp) in length. All primers produced precise and reproducible patterns (Fig. 5). Eighteen (24.3%)

Fig. 3 Direct organogenesis from hypocotyl explants of *E. nitens*. **a** Transverse section of an initial explant showing the tissue arrangement. **b** Section of regenerating hypocotyl explant after 10 days of culture in the regeneration medium, showing *de novo* subepidermal formation of meristemoid region composed of an unistratified epidermis and small, densely staining, meristem-like cells. **c** Adventitious shoot bud with two small leaf primordia (arrows). **d** Longitudinal section showing leaf primordia originating at the flanks of the shoot meristem from periclinal and anticlinal divisions in the outer corpus cells and tunica layer, respectively. **e** Longitudinal section through a 25-day-old hypocotyl segment showing a young shoot primordium. **f** Longitudinal section showing several subepidermal secretory cavities (arrow) formed in the leaves of young adventitious shoots. *acd* anticlinal cell division, *ec* epidermic cells, *ex* explant, *lp* leaf primordium, *mr* meristemoid region, *pc* cortical parenchyma cells, *pcd* periclinal cell division, *sc* oil secretory cavities, *vc* vascular cylinder

of the fragments were monomorphic bands, while the rest were polymorphic, a total polymorphic index of 75.7%. The polymorphic information for the selected markers varied from 0.19 ± 0.03 to 0.31 ± 0.01 , and therefore, the markers were classified as highly informative (Table 4). The genetic distances among the regenerated plants, measured by the Jaccard's dissimilarity coefficient (1-S) ranged from 0.17

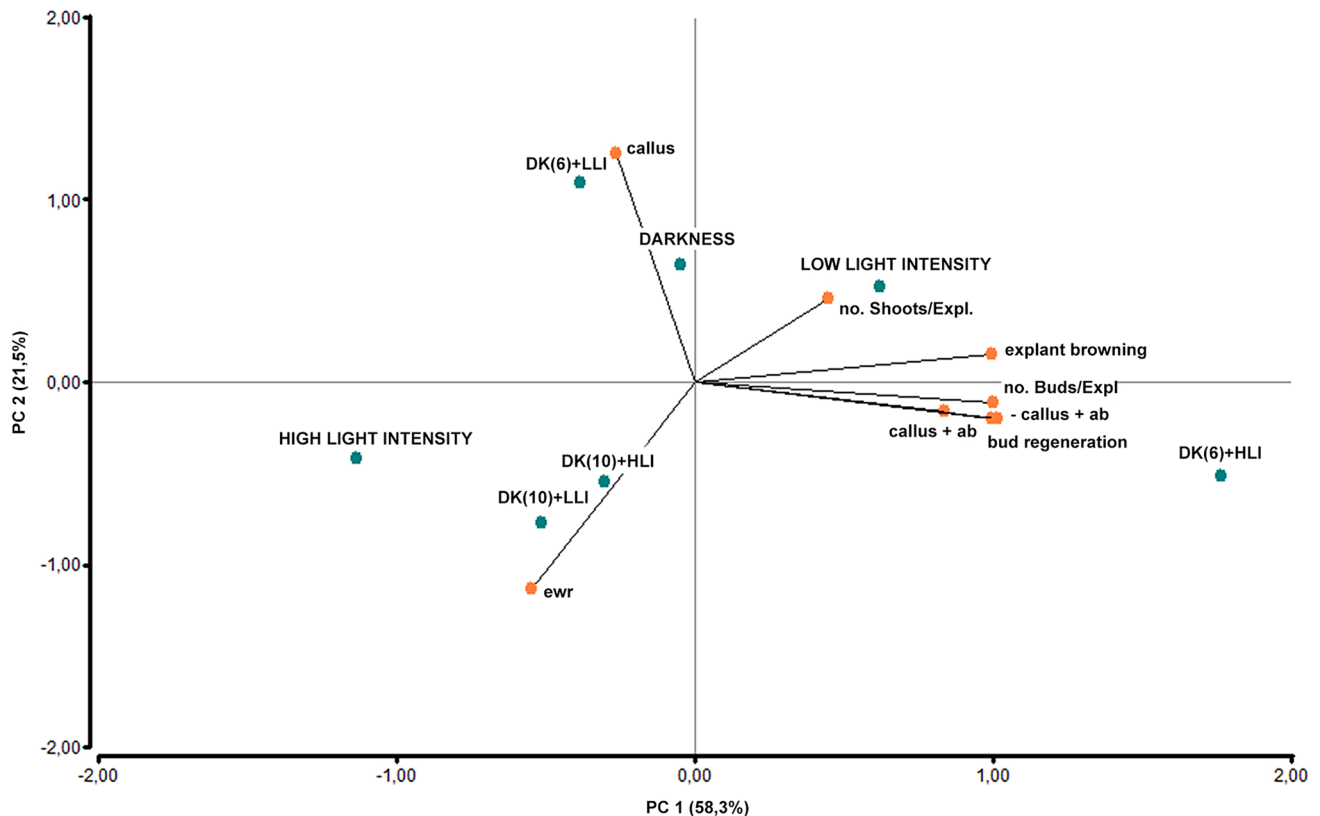


Fig. 2 Biplot of principal component analysis of light conditions and morphogenic responses of cotyledon explants cultured on MS medium plus 0.5 μ M IAA and 2.25 μ M BA. *CP1* principal com-

ponent 1, *CP2* principal component 2, *Dk* dark (number of days in parentheses), *HLI* high light intensity, *LLI* low light intensity, *ab* adventitious buds, *ewr* explants without responses

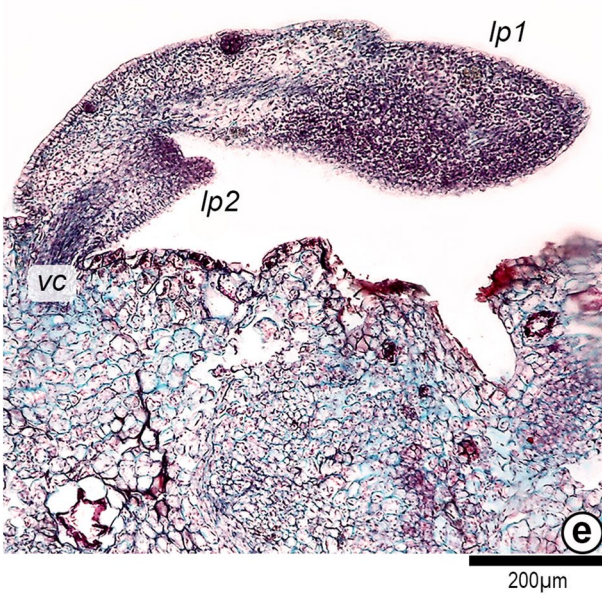
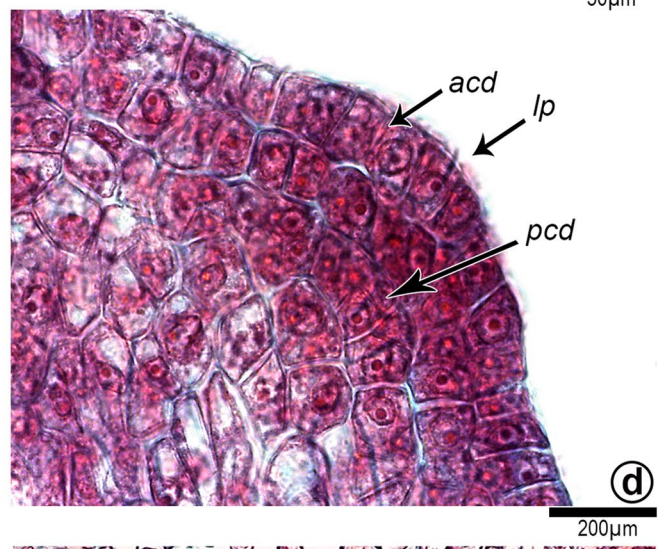
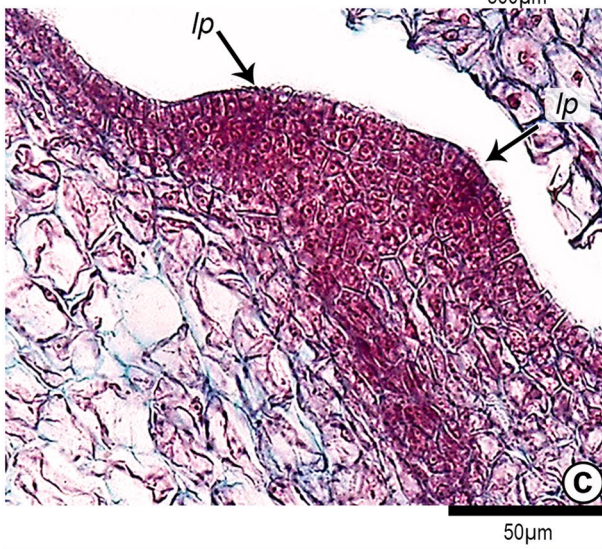
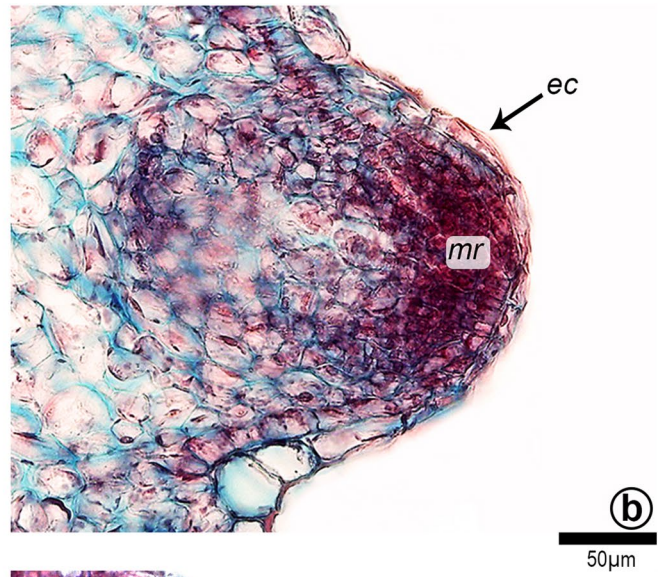
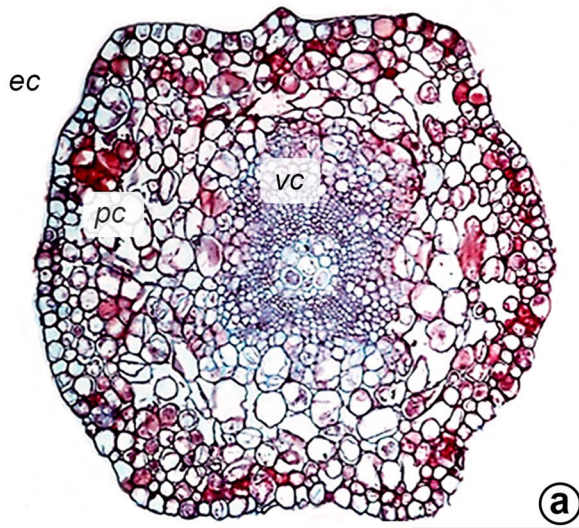


Table 3 Effect of gelling agent and ventilation on shoot development and chlorosis of *E. nitens* adventitious bud clusters grown on ½ MS plus sucrose 0.09 M

Ventilation type ⁱ	Gelling agent	No. of phytomers	Shoot length (mm)	No. of shoots with more than 10 mm in length	Shoot chlorosis (%)
Diffusive	Agar	2.3±0.7 a	8.3±2.3 a	1.5±0.4 a	31.2±6.9 b ⁱⁱ
Forced	Agar	4.0±0.5 a	14.1±3.4 a	3.0±0.7 a	6.2±3.3 a
Diffusive	Gellan gum	5.0±2.3 a	8.0±2.3 a	1.8±0.5 a	5.0±0.3 a
Forced	Gellan gum	7.0±2.3 a	8.0±2.7 a	2.0±0.8 a	0 a

ⁱVentilation type: in both cases, the culture vessels were closed with polypropylene caps that contain two 50 mm nylon membrane filters which enabled gas exchange. For forced ventilation, the air from a pneumatic pumping system was injected with a pressure of 0.5 bar for 1 min every 4 h

ⁱⁱMeans followed by the same letters are not significantly different at $p \leq 0.05$ according to Tukey's multiple comparisons test ($n=3$)

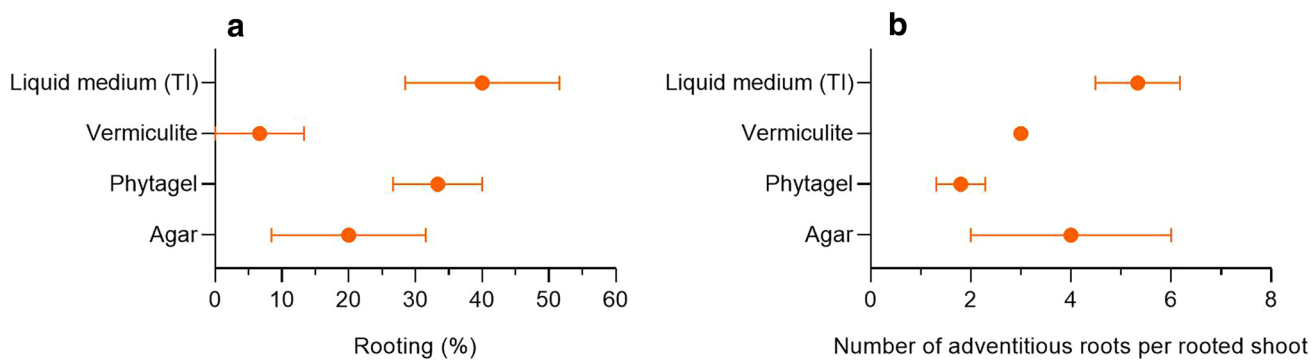
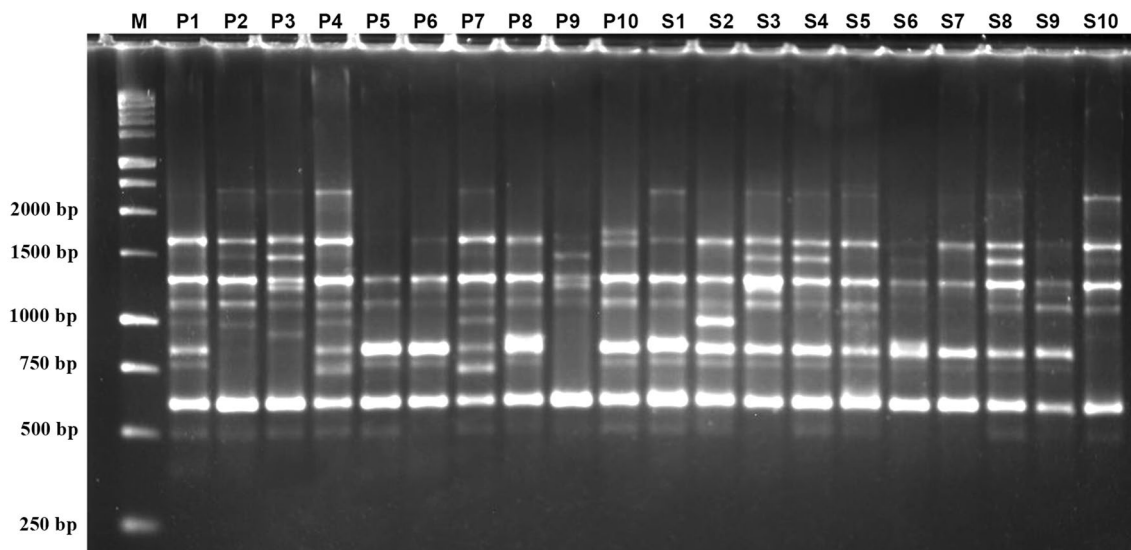
**Fig. 4** Influence of substrates on in vitro root formation of *E. nitens*. **a** Rooting percentage; **b** number of roots differentiated per rooted shoot**Fig. 5** Agarose gel electrophoresis of ISSR fragments of *E. nitens* plants from seedlings and in vitro plantlets generated with primer (CT)8G. Lane M, molecular marker (1 kb); lanes P1–P10, regenerated plantlets; lanes S1–S10, seedlings

Table 4 ISSR molecular characterization of seedlings and regenerated plantlets

Primer repeat (5'→3')	PB	MB	TB	PIC	PI (%)
(TC)8A	6	4	10	0.29±0.04	60
(CT)8G	8	3	11	0.23±0.03	73
(GC)8C	5	3	8	0.31±0.01	63
(GT)8TC	3	2	5	0.26±0.02	60
(AC)8T	10	2	12	0.20±0.02	83
(GA)8T	8	3	11	0.19±0.03	73
(AGAC)4GC	6	1	7	0.20±0.03	86
(CAG)7CA	10	0	10	0.26±0.02	100
Total	56	18	74		

PB number of polymorphic bands, *MB* number of monomorphic bands, *TB* number of total amplified bands, *PIC* polymorphism information content, *PI* polymorphism index

to 0.50. Furthermore, comparison between pairs of plants revealed ≥ 65% similarity between the seedlings and the regenerated plantlets (Table 5). The dendrogram of ISSR markers (Fig. 6) showed a total genetic distance between all plants close to 0.4, and separated the samples tested into five clusters that revealed a genetic distance ≤ 0.3, without distinction between seedlings and plantlets.

Discussion

Various tissue culture techniques had been developed for the vegetative propagation of *Eucalyptus* species, including shoot culture, organogenesis, and somatic embryogenesis, which were recently reviewed by Nakhoda and Jain (2016) and Trueman et al. (2018). In this study, we describe a procedure to propagate *E. nitens* based on adventitious bud formation from juvenile plants. Even though an organogenic protocol was reported by Bandyopadhyay et al. (1999), the adventitious buds were formed from callus, presenting a risk of genetic instability (Leva et al. 2012). Callus has previously been obtained from hypocotyl and cotyledon explants cultured on MS basal medium supplemented with coconut water, NAA, and BA under low light conditions (16 h photoperiod, 25 μmol m⁻² s⁻¹ PPF), and shoot proliferation was apparent 7–8 weeks after transfer to the regeneration medium composed of MS plus NAA and BA under 30–35 μmol m⁻² s⁻¹ PPF (Bandyopadhyay et al. 1999).

By contrast, combining an appropriate regeneration medium with an initial dark incubation period before transfer to 116 μmol m⁻² s⁻¹ PPF (14 h photoperiod) resulted in the production of *E. nitens* adventitious buds through a direct pattern of formation. Although the combination of NAA and BA on MS medium was widely used in the regeneration protocols of *E. camaldulensis* (Dibax et al. 2005),

Table 5 Matrix-based on the Jaccard's dissimilarity coefficient (1-S) of *E. nitens* from regenerated plantlets (P) and seedlings (S)

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
P1	0																			
P2	0.43	0																		
P3	0.34	0.45	0																	
P4	0.38	0.47	0.31	0																
P5	0.32	0.44	0.30	0.31	0															
P6	0.39	0.57	0.33	0.28	0.30	0														
P7	0.42	0.43	0.33	0.16	0.36	0.30	0													
P8	0.32	0.45	0.31	0.32	0.26	0.30	0.40	0												
P9	0.38	0.42	0.14	0.22	0.30	0.26	0.30	0.26	0											
P10	0.41	0.40	0.40	0.42	0.37	0.43	0.47	0.37	0.44	0										
S1	0.30	0.47	0.31	0.30	0.26	0.25	0.38	0.22	0.26	0.39	0									
S2	0.40	0.45	0.39	0.33	0.40	0.29	0.37	0.41	0.39	0.43	0.41	0								
S3	0.38	0.50	0.38	0.42	0.36	0.38	0.46	0.36	0.39	0.44	0.35	0.51	0							
S4	0.34	0.43	0.31	0.32	0.27	0.28	0.35	0.24	0.24	0.39	0.24	0.40	0.3	0						
S5	0.30	0.42	0.29	0.31	0.23	0.31	0.38	0.23	0.23	0.37	0.24	0.39	0.34	0.27	0					
S6	0.26	0.41	0.30	0.32	0.24	0.30	0.40	0.21	0.24	0.30	0.24	0.36	0.35	0.26	0.17	0				
S7	0.31	0.47	0.28	0.31	0.25	0.31	0.37	0.26	0.22	0.44	0.27	0.39	0.33	0.28	0.21	0.20	0			
S8	0.34	0.46	0.30	0.34	0.25	0.28	0.40	0.26	0.24	0.40	0.21	0.40	0.26	0.20	0.29	0.28	0.26	0		
S9	0.27	0.46	0.38	0.26	0.27	0.33	0.40	0.14	0.24	0.38	0.19	0.43	0.39	0.18	0.17	0.19	0.24	0.24	0	
S10	0.36	0.46	0.28	0.32	0.29	0.39	0.35	0.31	0.23	0.44	0.31	0.49	0.41	0.34	0.26	0.29	0.22	0.33	0.25	0

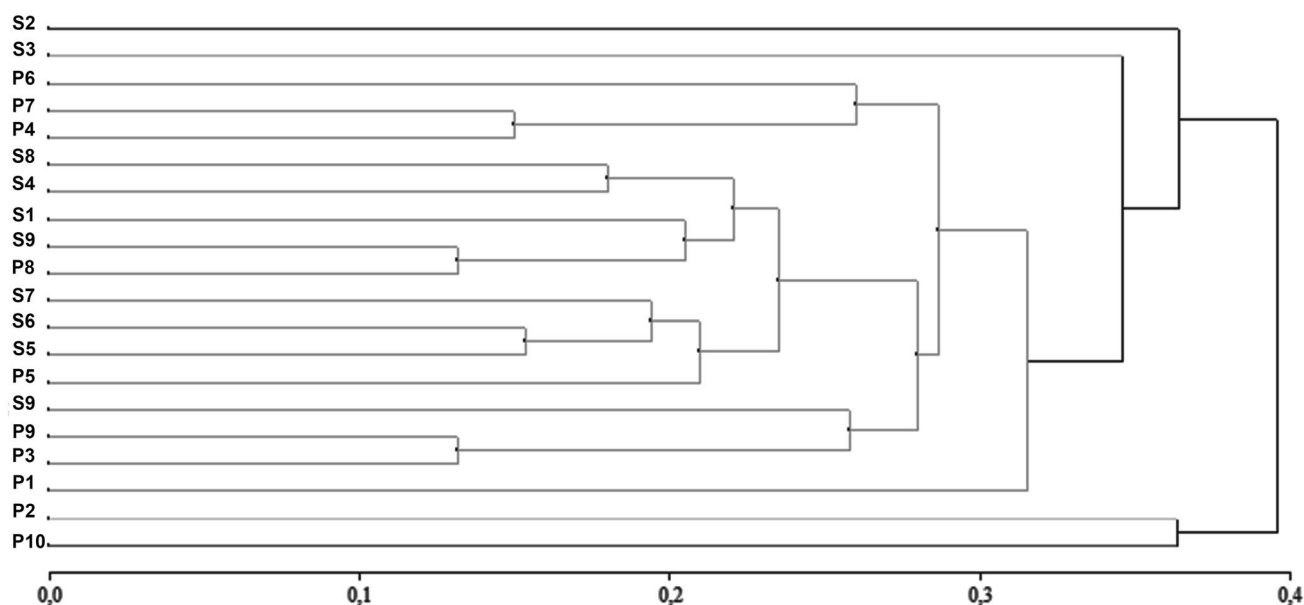


Fig. 6 UPGMA dendrogram derived using Jaccard's coefficient of similarity, demonstrating the genetic similarity between seedlings and plantlets obtained by direct organogenesis

E. erythronema (Glocke et al. 2005), *E. saligna* (Silva et al. 2015), *E. stricklandii* (Glocke et al. 2005), and *E. urophylla* (Bunn et al. 2007), our results suggested that the substitution of NAA by IAA prevented the proliferation of calli from *E. nitens* explants. It is well known that auxins have a dominant role in the formation of the apical meristem. More specifically, auxins and downstream transcriptional regulation interfere with the structural elements of the cell wall to induce specific morphogenetic events (Traas 2019). Moreover, cross-talk with other signaling pathways, in particular those of cytokinins, is essential for correct organ initiation (Vernoux et al. 2011; Huang et al. 2014). Our histological studies revealed that the meristemoid regions originated from cortical and subepidermal cells located just below the hypocotyl cut ends. During the elongation phase in $\frac{1}{2}$ MS free medium with gellan gum under forced ventilation, the sprouted buds differentiated successive units of phytomers containing leaves with normal aspects. Proper aeration improved the anatomical and histological features of *Ilex paraguariensis* leaves, particularly under temporary immersion systems, and promoted photosynthesis during hardening (Luna et al. 2017). Unlike other procedures developed for in vitro rooting of regenerated shoots from *E. globulus* (Azmi et al. 1997; Bandyopadhyay et al. 1999) and *E. nitens* (Bandyopadhyay et al. 1999), in the current study, rooting from elongated shoots was promoted with

a short pretreatment of IBA and transfer to $\frac{1}{2}$ MS lacking plant growth regulators.

Finally, as genetic stability of the propagated shoots was of paramount importance for establishing a cloning method with predictable results, we analysed ISSR markers of the regenerated shoots. The ISSR analysis showed monomorphic banding patterns and revealed a low polymorphism index among the seedlings and the in vitro plantlets, suggesting that the developed regeneration procedure did not greatly affect the genetic stability of *E. nitens* germplasm. Notably, we observed a natural genetic variation within the cultivar using the ISSR primers, which could be used to determine genetic variation in domesticated populations of *E. nitens* (data not shown).

In conclusion, we reported a simple and reliable procedure to regenerate *E. nitens* plants (Fig. 7), which comprised four culture steps: (i) in vitro germination of seeds (explant source); (ii) adventitious bud formation on MS medium containing IAA and BA; (iii) shoot elongation on $\frac{1}{2}$ MS medium lacking plant growth regulators under forced aeration; and (iv) in vitro rooting of the elongated shoots by means of pretreatment with IBA, and transfer to a basal medium of similar composition and forced ventilation.

The procedure took about 3 months to produce true-to-type *E. nitens* plants from hypocotyl explants. The absence

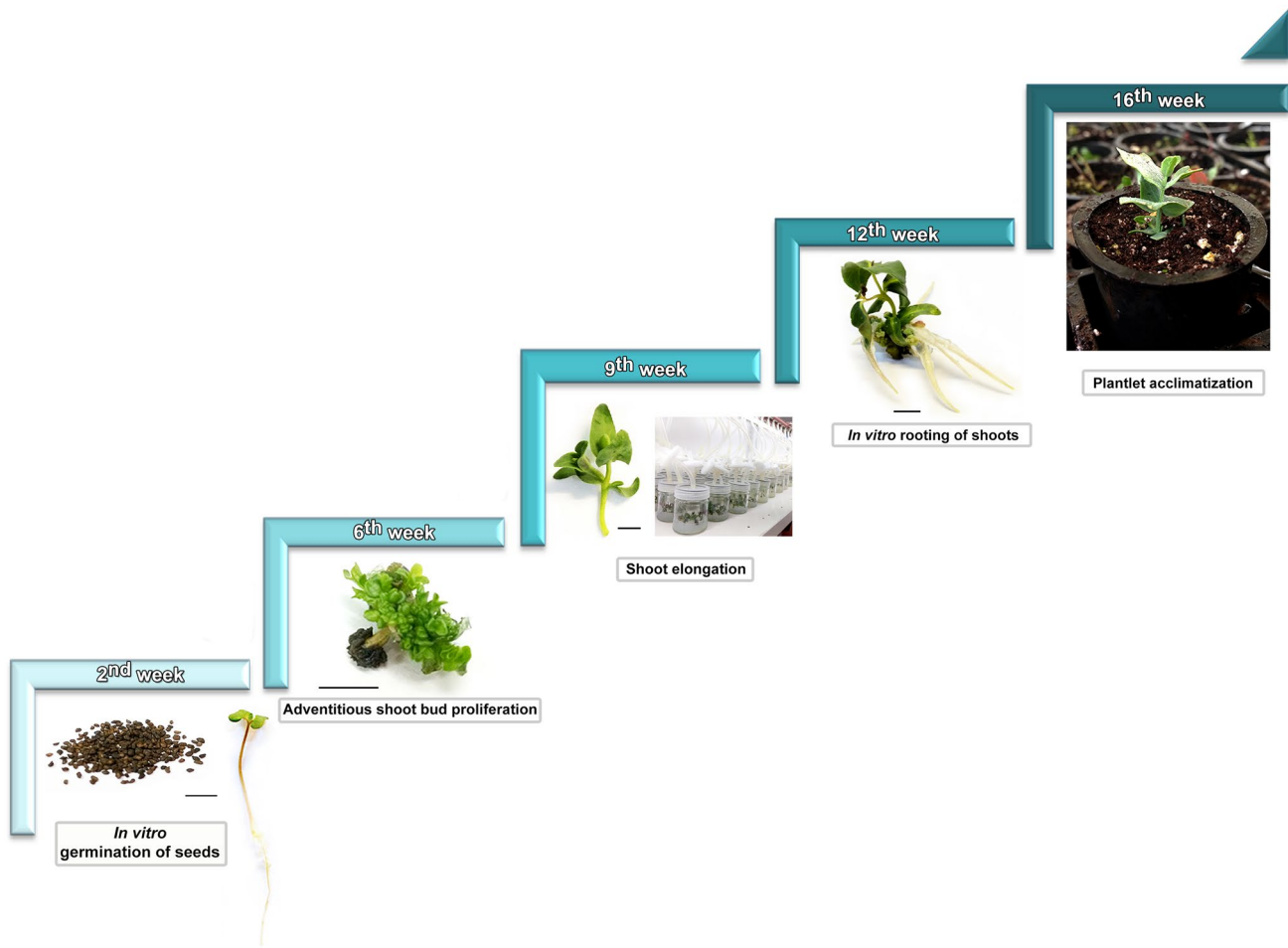


Fig. 7 Plantlet regeneration from *E. nitens* juvenile explants. The whole process took about 3 months to produce true-to-type plants and comprise the in vitro germination of seeds in semisolid MS medium (a), adventitious shoot bud induction from seedlings explants cultured on MS plus IAA and BA (b), shoot elongation on semisolid ½ MS

with gellan gum under a forced ventilation system (c), and rooting of the elongated shoot through a pretreatment with IBA solution and culture on liquid ½ MS medium under a temporary immersion system (d). Finally, plantlets are transferred to substrate and submitted to a period of acclimatization. Bars indicate 5 mm

of an intermediate callus phase in the organogenic process was the most significant advantage of this protocol.

Author contributions GA and PS conceived and designed the experiments. GA, EB, CL, AG, and RP performed the research. PS wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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