



Plant regeneration from *Eucalyptus bosistoana* callus culture

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Abstract

Eucalyptus bosistoana is an important durable hardwood species selected by New Zealand Dryland Forest Initiative (NZDFI) for their adaptability to diverse environments and good quality wood. There is no report on *in vitro* methods for callus induction and plantlet micropropagation of this species, although these methods could assist further genetic improvement of plants. In this study, different combinations of α -naphthalene acetic acid (NAA) and benzyl adenine (BA) at concentrations ranging from 0.5 to 2 mg L⁻¹ were investigated for callus induction, shoot regeneration, shoot elongation and root formation. High frequency of callus induction was obtained when cotyledon explants were cultured on MS (Murashige and Skoog medium 1962) with 1.0 mg L⁻¹ NAA and 1.5 mg L⁻¹ BA. For shoot regeneration from callus, MS medium with 0.5 mg L⁻¹ NAA and 1.5 mg L⁻¹ BA was an effective medium. Shoot elongation was observed on MS medium supplemented with 0.5 mg L⁻¹ NAA, 1.0 mg L⁻¹ BA and 1.0 mg L⁻¹ GA₃. High frequency of adventitious root formation in micro-cuttings of *E. bosistoana* was observed on half-strength MS medium with 0.5 to 1.0 mg L⁻¹ NAA. Finally, pre-acclimatization of the micropropagated plantlets led to 100% survival under glasshouse conditions.

Keywords Callus induction · Plantlet acclimatization · Root formation · Shoot regeneration

Introduction

Eucalyptus spp. are useful for durable hardwood production due to their abilities of good adaptation to different environments, rapid growth and producing high-quality wood for the various purposes of fibre, saw timber, chipboard, charcoal, posts and building construction (Nakhoda and Mandiri 2016). After banning CCA (copper chrome arsenic) treatment in USA and Europe, hardwood *Eucalyptus* species are becoming popular as a source of wood preservative-free high-grade timber (Li *et al.* 2018). New Zealand grown durable eucalypts may be used to substitute CCA-treated wood for various usages and are also ideal for diverse land areas (Millner and Kemp 2012). In Australia, there are 101 million hectares of *Eucalyptus* plantations, making up 77% of the Australian native forest area (Australian Bureau of Agricultural and Resource Economics and Sciences 2019). In New Zealand, there are nearly 25,000 ha with *Eucalyptus* plants, resulting in only 1% of timber production in the country (Li *et al.* 2018).

Since 1988, *Eucalyptus bosistoana* has been recognized as the best species of special interest to the New Zealand Dryland Forests Initiative (NZDFI) project. *E. bosistoana* is known as Coast grey box or Gippsland grey box and a Class 1 durable hardwood which is resistant to termite damage and highly durable in underground condition up to 25 yr (Li *et al.* 2018). Therefore, NZDFI conducted varietal trials and developmental research on these economically important hardwood trees. Although there have been these research initiatives on genetic improvement and breeding of the eucalypts of interest to NZDFI (Li *et al.* 2018), there is only one report of successful *in vitro* establishment and micropropagation of this species (Leung 2017). *In vitro* methods are useful tools to assist tree improvement using biotechnology (Pijut *et al.* 2007).

Micropropagation may be used for rapid and mass clonal propagation of a chosen plant variety under aseptic conditions. After establishing a reproducible protocol for rapid micropropagation, healthy propagules can be produced in a small space without interruption due to any adverse seasonal effects. Moreover, the clonal plants are free from any pests and diseases. Uniformed plantlets are produced when grown under controlled culture environmental conditions such as light, temperature, nutrients and plant growth regulators (Gamborg and Phillips 1995). Callus is an undifferentiated or unorganized mass of parenchymatous cells, which can be

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induced from different parts of plant organs under *in vitro* conditions by adjusting optimum plant growth regulator (PGR) concentration and combination. Calli are useful for various purposes of basic and applied research including rapid multiplication of cell cultures, mass propagation through plantlet regeneration, somatic embryo production, secondary metabolic production, *in vitro* selection procedures and gene transformation for tree and crop improvement (Trigiano and Gray, 2011).

α -Naphthalene acetic acid (NAA) is a synthetic auxin frequently used in plant tissue culture media for callus and root induction, somatic embryo production through stimulation of cell division and tissue differentiation. Benzyl adenine (BA) is a synthetic cytokinin commonly used in micropropagation medium for shoot induction, multiplication and organogenesis. The concentration and combination of different PGRs, mainly auxin to cytokinin ratio in the medium often determine the response of explant tissue to organogenesis (Trigiano and Gray, 2005). However, plant genotype, endogenous hormone level, juvenility and hormone sensitivity of tissue also play different roles on the *in vitro* responses of explants. In many of *Eucalyptus* species such as *E. grandis*, *E. globulus*, *E. camaldulensis*, *E. urophylla*, *E. tereticornis* and *E. stricklandii* species, hypocotyls and shoots from seedlings were found to be desirable explant tissues, although stem and nodes from more mature trees were also used (Trueman *et al.* 2018). Since there is no prior study on *E. bosistoana* plantlets derived from callus culture, here the protocols involving the effective use of PGRs (low concentrations of NAA and BA from 0.5 to 2 mg L⁻¹) for rapid callus induction, multiplication, plantlet regeneration and acclimatization of *E. bosistoana* as well as the major changes occurring during each of these stages towards plant production are reported.

Materials and methods

Surface sterilization and germination of seeds *Eucalyptus bosistoana* seeds were purchased from NZ Seeds (Rangiora, New Zealand). Dark brown, larger seeds were selected from the commercially available seeds for surface sterilization and germination. For ease of surface-sterilization of the seeds, lots of one hundred selected seeds each were packed into a piece of cotton cloth (5 cm²) which was then folded up to form a sachet. The sachets were first dipped in 70% (v/v) ethanol for 30 s and then soaked in commercially available bleach containing 20 g L⁻¹ sodium hypochlorite (NaOCl) as the active ingredient for 30 min. Finally, the seeds were rinsed five times by immersing the sachets in sterile deionized water. The surface-disinfected seeds were spread on a circle of Whatman No. 1 filter paper (90 mm in diameter) wetted with 5 ml of sterile deionized water in a sterile Petri dish (90 × 15 mm). These Petri dishes were placed in a growth room at

24 ± 2°C under continuous lighting. After 10 d, the cotyledons were excised from the germinated seeds of *E. bosistoana* as explants for callus induction.

Callus induction In callus induction medium, the basal Murashige and Skoog (1962) or MS medium supplemented with 30 g L⁻¹ sucrose, 8 g L⁻¹ of agar (Oxoid Ltd., Basingstoke, UK), 20 g L⁻¹ insoluble polyvinylpyrrolidone (PVPP, Sigma, St. Louis, MO) and different concentrations of plant growth regulators (PGRs) were trialled to determine an optimum callus induction medium for *E. bosistoana*. The pH of the medium was adjusted to be between 5.6 and 5.7 before autoclaving at 121°C. Different concentrations (0.5, 1.0, 1.5 and 2.0 mg L⁻¹) of each of NAA and BA in combinations were investigated for callogenesis. Five explants were placed in each of the five Petri dishes in a treatment. The cultures were kept under continuous lighting (2890 lx) at 24 ± 2°C.

For determining subculture intervals of callus culture, cell viability was checked after staining with 0.25 g L⁻¹ Evans blue (Gahan 1984). Blue colour stained cells were considered as dead cells because the stain could penetrate only the dead or damaged cells. The number of cells without staining were calculated in relation to the total number of cells counted using a haemocytometer to calculate % of cell viability.

Shoot regeneration from callus Callus initiated from explants was subcultured and proliferated on the same callus induction medium. After three subcultures (each for a duration of 30 d), the callus was transferred to a shoot regeneration medium containing basal MS salts and vitamins, 30 g L⁻¹ sucrose, 8 g L⁻¹ agar and different combinations of NAA and BA ranging from 0.5, 1.0, 1.5 and 2.0 mg L⁻¹. There was a total of 12 treatments. Five pieces of calli (8 to 10 mm in diameter) were placed in each of the five Petri dishes in a treatment. Callus was maintained at 30-d intervals under continuous lighting at 24 ± 2°C until visible shoot initials (shoot-primordia) were produced.

Shoot elongation and root induction The whole callus showing shoot initials was transferred to a jar allowing more space for shoot elongation and rooting. For shoot elongation, basal MS medium was supplemented with 30 g L⁻¹ sucrose, 8 g L⁻¹ agar, 0.5 mg L⁻¹ NAA, 1.0 mg L⁻¹ BA and 1.0 mg L⁻¹ GA₃. After shoots had elongated to 1.5–2.0 cm in length with 6 to 8 leaves, individual shoots were transferred to a rooting medium containing half-strength basal MS medium supplemented with 30 g L⁻¹ sucrose, 8 g L⁻¹ agar and 0, 0.5, 1.0 or 1.5 mg L⁻¹ NAA. Plantlets were grown on the same medium until they were 3 to 5 cm tall with enough (10 to 15) leaves and (6 to 8) roots for transfer to *ex vitro* conditions.

Plantlet acclimatization The plantlets were divided into two groups: direct transplanting to greenhouse conditions and pre-

acclimated before transplanting to *ex vitro* condition. In the pre-acclimated system, polycarbonate jars (round jars with a total volume of 250 ml) purchased from LabServ, Palmerston North, New Zealand, were used. Inside a sterile laminar flow cabinet, the lids of the jars were regularly removed to get good gas exchange and ventilation. In this system, 1/10 MS liquid medium (15 ml) together with perlite (2 g) was added into a jar and the jars were autoclaved at 121°C for 15 min. Five plantlets were transferred into each jar, and there were 7 jars. In the pre-acclimated system, after 1 wk of transfer, the lids of the jars were removed inside a laminar airflow cabinet for 5 min in the first day and then the lid was put back on for another 2 d. After this, the lids of the jars were taken off for 10 min. Two more days later, the duration of opening was increased to 20 min with the aim of hardening the plantlets by reducing relative humidity gradually. The duration of opening the jars was increased by 10 min after 2 d of keeping the jars closed until 4 wk of pre-acclimation. All the plantlets were soaked in a dilute fungicide solution (1% Taratek 5 F, Tapuae Partnership, NZ) for 5 min before transfer to pots (7.5 cm × 9.5 cm) with potting mix of garden soil: sand: compost (1:1:1). High humidity was maintained with a plastic humidity dome in the first week of transplanting to the glasshouse. After 1 wk, the plastic humidity domes were removed, and plantlets were kept under automatic mist spraying system to keep high humidity. After 4 wk of transplanting, the plantlets were exposed to normal greenhouse environment. The % of survival, plant height and number of leaf and branches of plantlets were recorded.

Data collection and statistical analysis Callus induction percentage was calculated based on the number of explants that produced callus in relation to the total number of explants used in a PGR combination/concentration. The days to first visible sign of callus induction, callus types based on appearance and colours were also recorded. Through staining, cell viability and cell size and shapes of callus were observed under a light microscope. In the shoot regeneration experiment, the number

of callus pieces showing shoot formation, days to induce shoots and number of shoots formed per callus were recorded. Root formation percentage in micro-cuttings on the rooting medium and survival percentage of plantlets at 4 wk after transferring to greenhouse were also recorded. Data were statistically analysed using SAS software version 9.1. Analysis of variance (ANOVA) and mean comparison were performed by the least significant difference test (LSD_{0.05}) at 95% level of significant difference.

Results and discussion

Callus induction In preliminary experiments, callus formation was first observed in cotyledon explants excised from the germinated seeds of *E. bosistoana* after 14 d of culture on media containing different combinations of NAA and BA. On the T6 medium supplemented with (1.0 mg L⁻¹ NAA and 1.5 mg L⁻¹ BA), 95% of the cotyledon explants of *E. bosistoana* formed callus. There was no callus formation in the explants on the T11 medium supplemented with 2.0 mg L⁻¹ NAA and 1.0 mg L⁻¹ BA (Figs. 1 and 2a).

Callus induction was observed in various *Eucalyptus* species, for example, in leaf explants of *E. camaldulensis* (Mullins *et al.* 1997) and seed explants of *E. globulus* (Dobrowolska *et al.* 2017) on medium containing high NAA concentrations from 3 mg L⁻¹ to 5 mg L⁻¹. In another study, callus was initiated in hypocotyl explants of *E. urophylla* on medium supplemented with 5 mg L⁻¹ picloram (Arruda *et al.* 2000). When 2–3-mm pieces of *in vitro* shoots of the clones of *E. grandis* and *E. grandis* × *E. urophylla* were cultured in the dark at 24°C on basal MS medium supplemented with 5 mg L⁻¹ IAA and 0.25 mg L⁻¹ BAP, 100% of the explants formed callus (Hajari *et al.* 2006).

In the current study, the T6 medium supplemented with 1.0 mg L⁻¹ NAA and 1.5 mg L⁻¹ BA was suitable for callus induction in cotyledon explants of *E. bosistoana*. The callus induction protocols should be useful for future tree genetic

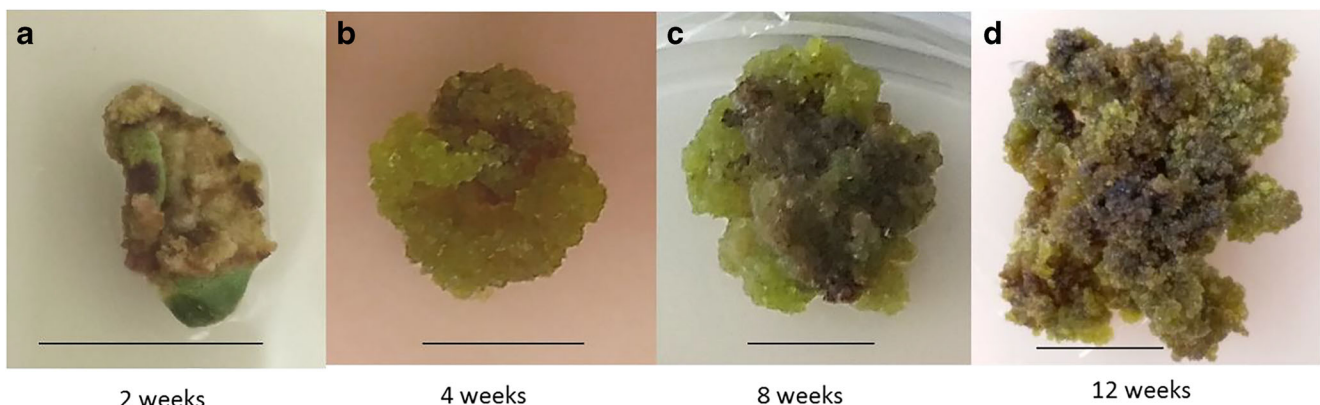


Fig. 1 Callus initiation and development in cotyledon explants of *E. bosistoana* (a–d) after 2 to 12 wk of culture. Bar = 1 cm

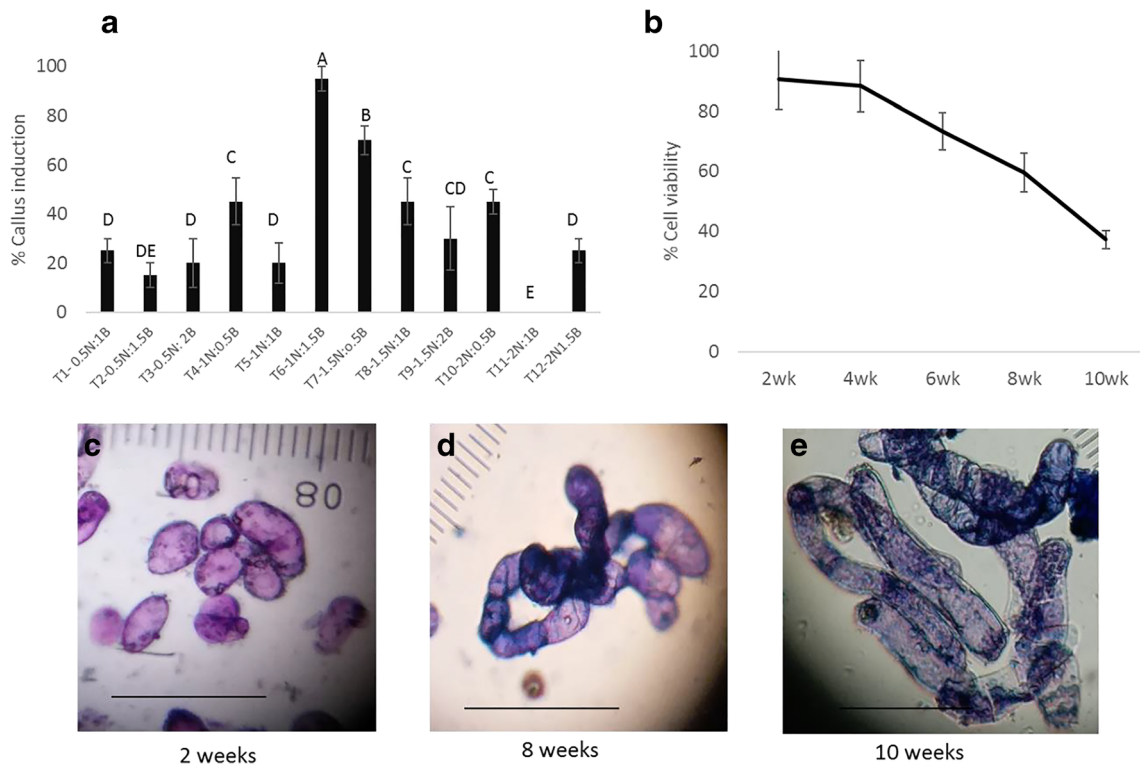


Fig. 2 (a) Callus induction (%) in cotyledon explant on callus induction medium with different NAA and BA combinations, bars = standard error, n = 25 callus, bars with different letters are significantly different at LSD 0.05. (b) Changes in viability (%) of callus cells of *E. bosistoana* during 10 wk of culture, bars = standard error. (c) Isodiametric shaped callus

cells (30–40 μm in diameter × 50–60 μm in length) after 2 wk of culture. (d) Elongated cells (40 μm in diameter × 80–100 μm in length) after 8 wk of culture. (e) The longest cells (40 μm in diameter × 120–150 μm in length) after 10 wk of culture, bars = 100 μm

improvement studies in this *Eucalyptus* species using *in vitro* cell line selection for isolation of somaclonal variants, gene transfer (plant transformation) and somatic embryo production.

The significant drop in cell viability of callus cultures was found after 6 wk (Fig. 2b). Around 90% of viable cells were observed after 4 wk of culture, but this declined to around 70% by the end of 6 wk of culture. After 8 wk of culture,

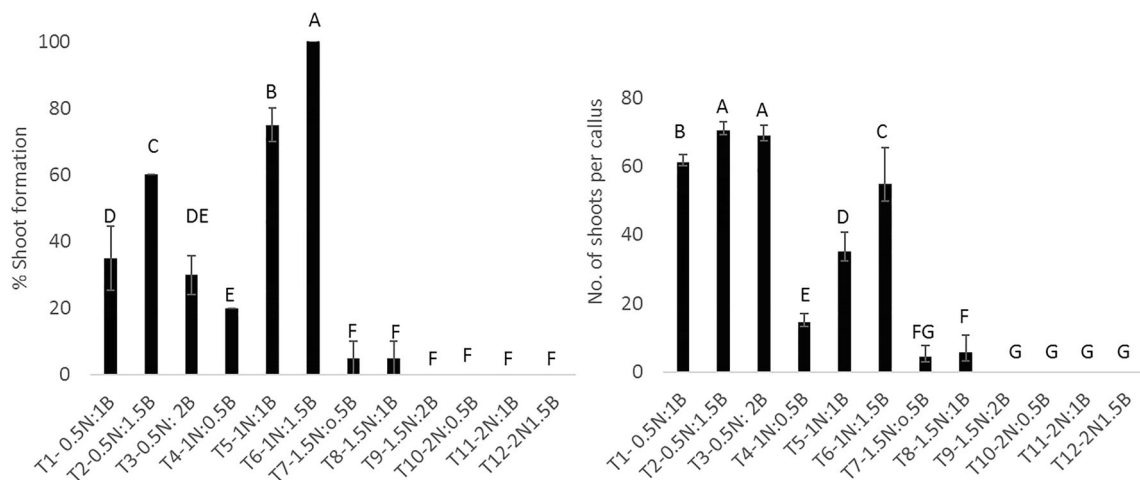


Fig. 3 Percentage of shoot regeneration in cotyledon-derived callus (a) and number of shoots per callus (b) of *E. bosistoana* on different media (T1–T12) with different concentrations of NAA and BA (for example, T1-0.5N-1B: 0.5 mg L⁻¹ NAA and 1 mg L⁻¹ BA) after 8 wk of culture on shoot induction medium. n = 5 Petri dishes; 5 calli per plate; bars =

standard error, and the bars with different letters are significantly different at LSD 0.05, cv % = 28.32, LSD = 11.035, Pr > F < 0.0001 for % shoot induction; cv % = 14.47, LSD = 5.48, Pr > F < 0.0001 for number of shoots per piece of callus

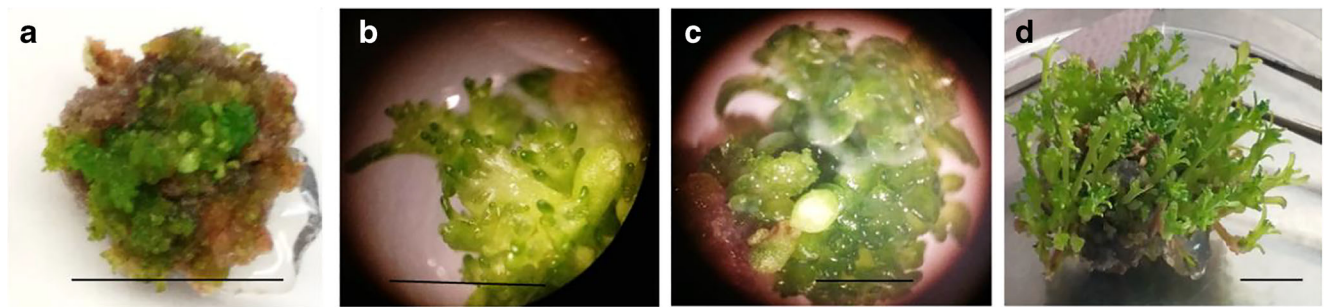


Fig. 4 Steps in shoot regeneration from calli of *E. bosistoana*: (a) green spot formation on callus after 4 wk, (b) shoot primordia are visible after bud-breaking at 6 wk, (c) shoot cluster formation after 12 wk of culture on

shoot regeneration medium and (d) elongated shoots at 4 wk on the shoot elongation medium with GA₃. Bars = 5 mm

the cell survival rate was observed to be below 60%. Therefore, regular subculture to a fresh medium every 6 wk of culture was used to maintain active cell growth and good survival rate.

Change in cell shape and sizes were observed until 10 wk of culture in the same medium without sub-culturing (Fig. 2c–e). After 2 wk of culture, the callus cells were ovate or isodiametric in shape. Their sizes ranged from 30 to 40 μm in diameter and 50–60 μm in length. However, their sizes changed during culture. After 8 wk of culture, cells of 40 μm in diameter and 80–100 μm in length were observed. After 10 wk of culture, the average diameter (40 μm) of the cells remained unchanged throughout the experiment but the lengths of the cells increased to 150 μm . Callus may consist of different cell types, and only some of them could be involved in organ regeneration (Feher 2019). Similarly, it was determined that shoot regeneration competence in tobacco internodal explants was diminished in more mature or elongated cells in the explants (Gilissen *et al.* 1996). In the present study, the elongated cells in *E. bosistoana* callus after 10 wk of culture could be mature cells that had lost shoot regeneration ability.

Shoot regeneration Shoots were formed in 100% of *E. bosistoana* callus cultured on the T6 medium (1.0 mg L⁻¹

NAA and 1.5 mg L⁻¹ BA), but in only 75% and 60% of the calli cultured on T5 medium (1.0 mg L⁻¹ NAA and 1.0 mg L⁻¹ BA) and T2 medium (supplemented with 0.5 mg L⁻¹ NAA and 1.0 mg L⁻¹ BA), respectively (Fig. 3a). A very low level of shoot regeneration (less than 10%) was observed in callus cultured on T7 and T8 media supplemented with a higher NAA level (1.5 mg L⁻¹). Shoot formation was not observed in callus cultured on the T9, T10, T11 and T12 media supplemented with 2 mg L⁻¹ of NAA.

The number of shoots formed per piece of callus was significantly different when the calli were cultured on the media supplemented with different NAA and BA combinations (Fig. 3b). The highest number of shoots (about 70 per piece of callus) was formed when the *E. bosistoana* calli were cultured on the T2 (0.5 mg L⁻¹ NAA and 1.5 mg L⁻¹ BA) and T3 (0.5 mg L⁻¹ NAA and 2.0 mg L⁻¹ BA) media.

In a recent study, a higher ratio of cytokinin to auxin (0.1 mg L⁻¹ NAA and 0.6 mg L⁻¹ BA) was used in medium for shoot multiplication of epicormic shoots (Silva de Oliveira *et al.* 2015). The offsprings of *E. benthamii* x *E. dunnii* produced a large number of buds per nodal explant on medium containing an even higher ratio of cytokinin to auxin (0.05 mg L⁻¹ NAA and 0.5 mg L⁻¹ BA; Brondani *et al.* 2011). In general, this is consistent with the concept that appropriate ratios of cytokinins and auxins, particularly higher

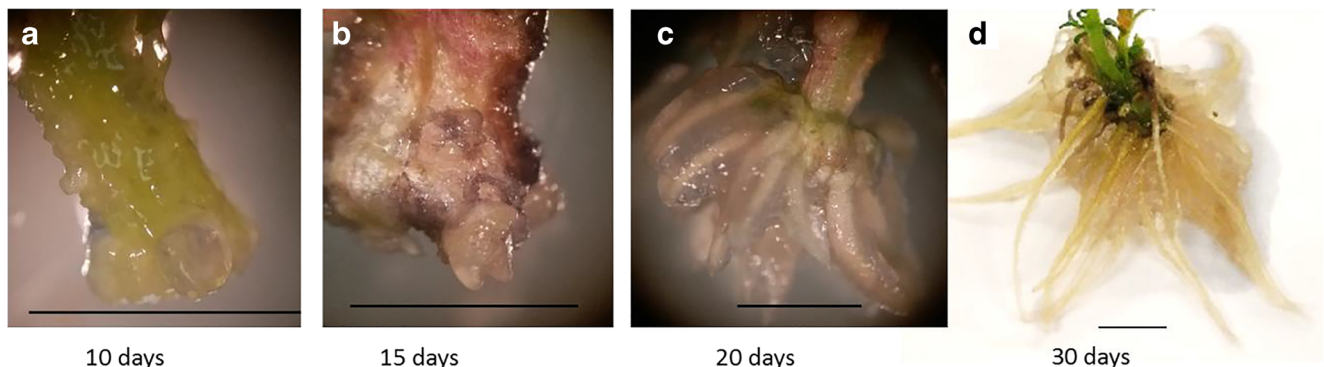


Fig. 5 Root initiation from shoot cuttings of *E. bosistoana*: (a) callus growth starting on the micro-cuttings after 10 d on the root induction medium, (b) root initials emerging from the wounded area on the stem

at 15 d after induction, (c) adventitious root development after 20 d of induction and (d) root elongation at 30 d of culture on root induction medium. Bars = 10 mm

Table 1 Effect of NAA level in the rooting medium (half-strength MS medium) on rooting in *E. bosistoana* micro-cuttings after 4 wk of culture

NAA	Days to induce root	Root induction %	Root number	Root length (mm)
0.0 mg L ⁻¹	24.4 ± 0.54 A	5.0 ± 11.18 C	0.4 ± 0.89 C	0.8 ± 1.87 B
0.5 mg L ⁻¹	17.8 ± 2.28 B	80.0 ± 20.92 AB	3.6 ± 1.77 B	6.4 ± 1.40 A
1.0 mg L ⁻¹	15.6 ± 0.54 C	90.0 ± 13.69 A	7.7 ± 1.11 A	3.4 ± 0.91 B
1.5 mg L ⁻¹	15.6 ± 0.54 C	70.0 ± 20.91 B	6.1 ± 2.03 A	2.9 ± 2.19 B
Pr > F	< .0001	< .0001	< .0001	0.0048
LSD _{0.05}	1.645	19.640	1.993	2.630
CV (%)	6.505	23.268	32.429	55.983

Means ± standard deviation ($n = 5$) followed by different letters at the same column are significantly different at LSD 95% level of significant difference

concentrations of cytokinin than auxin, in the shoot induction medium could favour shoot formation (Sugimoto *et al.* 2011).

Interestingly, a medium containing 5 mg L⁻¹ IAA and 0.25 mg L⁻¹ BA was found to be useful for both callus induction and shoot regeneration in *E. grandis* and its hybrid clones (Hajari *et al.* 2006). Similar to the study by Hajari *et al.*, 2006, the present study also shows that one medium (T6) is suitable for callus induction and shoot regeneration. T2 is effective in terms of number of shoots per callus.

Different steps of shoot formation from callus are illustrated in Fig. 4. Firstly, the green spot formation was observed on the surface of light brown or creamy coloured callus after 4 wk of transfer to the regeneration medium. The green spots seem to consist of meristematic cells in a globular or nodular structure. After 6 wk of culture, some of these globular structures started bud breaking and showing shoot primordia, which could be observed clearly with a binocular stereo-microscope under 10 times of magnification. The same pattern of globular or nodular structure formation was observed in *E. globulus* (Dobrowolska *et al.* 2017). Within 10–12 wk of culture, the differentiated shoots were growing, and axillary buds became visible. The whole callus was turned into a cluster with many shoots. However, the shoots were very compact with short internodes.

The whole shoot cluster was transferred to a jar containing 50–60 ml of shoot elongation medium (0.5 mg L⁻¹ NAA, 1.0 mg L⁻¹ BA and 1.0 mg L⁻¹ GA₃). After 4 wk on shoot elongation medium, the shoots elongated and nodes and internodes were visible (Fig. 4). The individual shoots of 1.5 to 2.5 cm tall were harvested and transferred to rooting medium (half-strength MS with 0, 0.5, 1.0 or 1.5 mg L⁻¹ of NAA).

Root induction from shoot micro-cuttings In the present study, rooting data were recorded after visible root formation from shoot micro-cuttings. During 5 to 9 d of culture on the rooting medium, wound healing and a thin layer of callus cells were found at the cut ends of the shoots (Fig. 5). However, the earliest sign of root initiation was observed after 15 d on the root induction medium supplemented with 1.0 mg L⁻¹ NAA (Table 1). With a lower NAA concentration (0.5 mg L⁻¹) in the medium, it took 17.8 d for root formation to occur. On the control medium in the absence of NAA, roots were initiated from shoots only after 24 d. In contrast, it was reported that in the stem cuttings of *E. camaldulensis* root initials were observed after 9 d of auxin treatments (Shanthi *et al.* 2015). Variation in time to rooting may be related to genotype-dependent response.

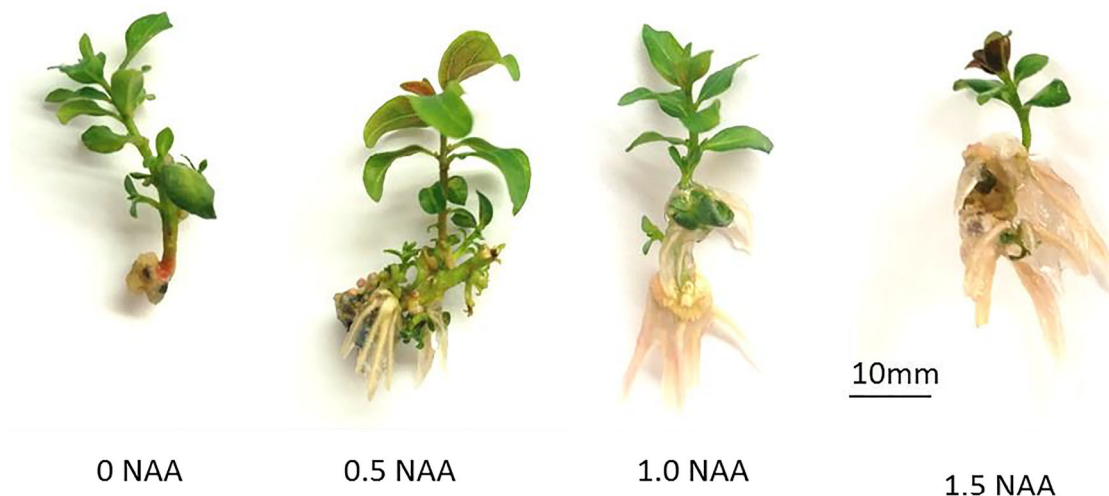


Fig. 6 Rooted micro-cuttings of *E. bosistoana* at 20 d after cultured on root induction medium; half-strength MS medium with 0 to 1.5 mg L⁻¹ NAA

Table 2 Survival and growth parameters of *E. bosistoana* plantlets kept under glasshouse conditions. The plantlets were from pre-acclimating and direct transplanting to the greenhouse. DAT, days after transplanting

	Survival%	Numbers of leaves			Plant height (cm)				
		30 DAT	0 DAT	10 DAT	20 DAT	30 DAT	0 DAT	10 DAT	20 DAT
Direct transplanting	86.6	8.2 ± 2.3 A	12.7 ± 5.0 B	15.5 ± 6.1 B	19.5 ± 7.5 B	2.6 ± 0.4 A	3.19 ± 1.3 A	3.64 ± 1.5 B	4.95 ± 1.9 B
Pre-acclimatizing	100	9.5 ± 3.1 A	16.6 ± 5.9 A	21.8 ± 7.5 A	27.2 ± 6.9 A	2.5 ± 0.5 A	3.67 ± 0.9 A	5.62 ± 0.8 A	6.22 ± 0.8 A
Pr > f		0.216	0.016	0.003	0.0001	0.854	0.1125	<.0001	0.0021
LSD _{0.05}		2.16	3.05	3.64	3.04	0.49	0.61	0.58	0.71
CV (%)		30.53	24.91	22.41	15.59	23.82	21.48	14.49	15.28

Means ± standard deviation ($n = 15$) followed by different letters at the same column are significantly different at LSD 95% level of significant difference. All pre-acclimated *E. bosistoana* plantlets

The best root formation response (7.7 roots per cutting) in the micro-cuttings of *E. bosistoana* was observed when they were cultured on the medium supplemented with 1.0 mg L⁻¹ NAA (Table 1). In a recent study, roots were initiated from shoot clusters of a *E. grandis* × *E. urophylla* clone on the medium supplemented with 0.5 mg L⁻¹ of IBA (indole butyric acid) (de Oliveira *et al.* 2017). However, for the rooting of *E. camaldulensis*, half-strength MS medium with 1.0 mg L⁻¹ IBA was reported as the most suitable rooting medium (Girijashankar 2012).

Although a shoot of *E. bosistoana* cultured on the medium supplemented with 0.5 mg L⁻¹ NAA produced only 3.64 roots, these roots were the longest (6.44 mm) among different treatments (Table 1). However, the longer roots could be easily broken or detached during exflasking and transplanting. It was found that *Eucalyptus* roots could recover rapidly from injury and produce more lateral roots (Fig. 6). The cankerous growth formation in a few leaves was also observed when the micro-cuttings were cultured on the root induction medium.

Plantlet acclimatization The acclimatization or hardening stage of *in vitro* plantlets is the last phase of micropropagation and is essential for the survival and successful establishment of plantlets in *ex vitro* condition (Kadleček *et al.* 2001). At the acclimatization stage, the plantlets have physiologically changed

from heterotrophic to autotrophic growth, and slow growth and plant mortality could occur (Kozai 1991). The Gas-Permeable-Culture system has many advantages for plantlet growth and high survival rate (Hew and Yong 2004; Chang 2008; Mohamed and Alsadon 2010). In the present study, normal tissue culture jars with the lids manually taken off regularly in a laminar flow were used to get the benefit of ventilated culture system for the pre-acclimated plantlet group.

After 30 d of transplanting, 100% pre-acclimated and 86.6% of directly transplanted *E. bosistoana* plantlets survived. The significant increase in the number of leaves and plant height were observed in pre-acclimated plantlets compared with directly transplanted ones after 10 and 20 d of transplanting to the glasshouse conditions (DAT), respectively (Table 2). Drying, necrotic cell death and falling of leaves that were formed during tissue culture were commonly observed in the directly transplanted plantlets (Fig. 7a), while those of the pre-acclimated plantlets were still alive (Fig. 7c). However, the new shoots started to come out after 10 DAT in both systems. The hardening effect in the pre-acclimated system could be helpful not only for stimulating start of the functioning of stomata but also for the establishment of cuticular waxy layer. These could be helpful for better preparing plantlet transition from heterotrophic to autotrophic mode of growth.

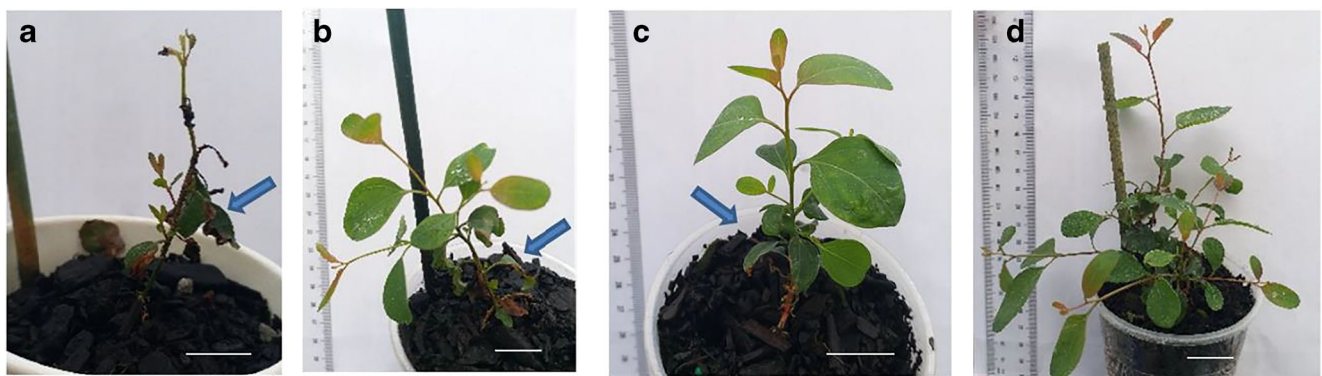


Fig. 7 A directly transplanted plantlet at 20 DAT (a), 30 DAT (b), a pre-acclimated plantlet at 20 DAT (c) and 30 DAT (d) of *E. bosistoana*. Arrows = leaves at the time of exflasking; bars = 10 mm; DAT = days after transfer to the greenhouse

Conclusion

Effective surface sterilization of *E. bosistoana* seeds was achieved as follows: washing in 70% ethanol for 30 s and then soaking in 2% NaOCl for 30 min before final 5 rinses with sterile water. The surface-disinfected seeds germinated under aseptic *in vitro* conditions giving rise to explants for callus induction. Culturing cotyledon explants of *E. bosistoana* on MS medium with 1.0 mg L⁻¹ NAA and 1.5 mg L⁻¹ BA resulted in high % of callus induction. The regular callus subculture to a fresh medium every 6 wk is advisable to maintain active cell growth and good survival rate.

For shoot regeneration from callus, MS medium with 0.5 mg L⁻¹ NAA and 1.5 mg L⁻¹ BA was selected as an effective medium because high % of shoot induction and maximum number of shoots per explant were observed. Shoot elongation was achieved upon transfer to MS medium supplemented with 0.5 mg L⁻¹ NAA, 1.0 mg L⁻¹ BA and 1.0 mg L⁻¹ GA₃. High frequency of adventitious root formation in microcuttings of *E. bosistoana* was observed on half-strength MS medium with 0.5 to 1.0 mg L⁻¹ NAA. Before plantlets were placed under greenhouse conditions, a period of gradual reduction of relative humidity by taking the lids of the tissue culture jars off from time to time inside a laminar flow cabinet was helpful for more successful acclimatization of the micropropagated plantlets.

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