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Optimisation of micropropagation protocols for temperate eucalypt hybrids in South Africa, with a focus on auxin transport proteins

Rafael Keret[1], Muhammad Nakhooda[2,3], Nicoletta B Jones[4] and Paul N Hills[1]*

[1] Institute for Plant Biotechnology, Department of Genetics, Stellenbosch University, Stellenbosch, South Africa
[2] Department of Biotechnology and Consumer Sciences, Cape Peninsula University of Technology, Cape Town, South Africa
[3] School of Life Sciences, University of KwaZulu-Natal Westville Campus, Durban, South Africa
[4] Forests – Research, Planning and Nurseries Department, Sappi Shaw Research Centre, Howick, South Africa
** Corresponding author: phills@sun.ac.za*

Globally the demand for forestry resources is booming, and consequently innovative approaches to cultivate valuable hardwoods, such as eucalypts, are crucial. Although micropropagation offers a means to clonally propagate desirable cultivars, this technique is often variety-specific for eucalypts. Thus, recalcitrant genotypes are often excluded from micropropagation programmes as development requires large investments of time, money and expertise. In this study, a minimal maintenance medium was developed to reduce subculturing frequency (~8 days) and to evaluate each micropropagation stage independently. Furthermore, a generic protocol for use across different varieties was developed by assessing the *in vitro* performance of three different *Eucalyptus grandis* x *Eucalyptus nitens* varieties (varieties 1–3) across various treatments. The purpose of this protocol was to ease the *in vitro* establishment of novel eucalypt varieties by providing a foundation for future protocol optimisation. During multiplication, medium containing 0.5 mg l^{-1} *meta*-topolin with 0.1 mg l^{-1} of indole acetic acid (IAA) induced the highest bud proliferation and shoot elongation for varieties 1 (59.1 ± 2.9; 1.2 cm ± 0.08), 2 (62.3 ± 2.7; 1.5 cm ± 0.09) and 3 (58.1 ± 3; 1.2 cm ± 0.07). Following multiplication, the rooting treatment that achieved the most consistent rooting percentages among varieties 1 (33.4%), 2 (43.5%) and 3 (34.3%) incorporated 0.029 mg l^{-1} of *racemic*-GR24 with 0.5 mg l^{-1} of IAA. Additionally, root vigour was assessed by measuring the root number and length, which varied considerably based on the variety and treatment in question. Overall, variety 2 was the most amenable to plant growth regulators and upon further investigation was found to possess equal expression levels of the auxin transporters *PIN1* and *AUX1*. Conversely, the other varieties displayed unequal ratios of these transporters. Considering that auxins are principal media components, these expression profiles may serve as markers to identify eucalypt cultivars amenable to micropropagation.

Keywords: plant growth regulators, indole-3-acetic acid, indole-3-butyric acid, *meta*-topolin, *Eucalyptus grandis* × *E. nitens*, strigolactone, *racemic*-GR24, *PIN1*, *AUX1*

Introduction

Globally, the demand placed on the forestry industry and its associated products continues to escalate. Owing to its high productivity, versatile wood characteristics and adaptability to a wide range of climatic conditions, the economically valuable *Eucalyptus* genus has been established as a major plantation crop internationally, with over 20 million hectares of land cultivated for commercial applications (Trueman et al. 2018). Eucalypt forestry stands have served as a source of raw materials for industrial products, including timber, pulp and paper, lignocellulosic compounds, biorefinery feedstock, biofuels and essential oils (Turnbull 1999; Hinchee et al. 2009; Trueman et al. 2018). Continuous improvement through tree breeding efforts has enabled hybridisation programmes to combine desirable economic traits of various eucalypt species, which, in South Africa, has enabled plantations to be established in marginal land areas (Bennet 2010).

Improved genotypes, however, need to be effectively propagated and deployed to rapidly realise genetic gains. Vegetative propagation is advantageous over traditional methods (i.e. via seed) as superior germplasm is conserved (Bonga 1982), resulting in clonal proliferation of genotypes that favour high yields and desirable pulp properties. True-to-type clonal propagation of *Eucalyptus* spp. has mostly been achieved via vegetative propagation using macro cuttings and, since the 1990s, also includes use of mini- and micro-propagation systems (de Assis et al. 2004; Trueman et al. 2018). However, certain *Eucalyptus* spp., including many of the temperate eucalypts, remain recalcitrant to adventitious rooting (Li et al. 2009; Hartmann et al. 2011; Nakhooda et al. 2011; Brondani et al. 2012; Pijut et al. 2012).

Micropropagation provides a means to enhance adventitious rooting of eucalypt genotypes, as explant material is grown under highly controlled conditions that can be optimised for ideal rooting performance, high proliferation rates and conservation of superior germplasm (Debergh and Read 1991; Nakhooda and Jain 2016). *In vitro* development of eucalypt explants requires careful manipulation of plant growth regulators (PGRs), most notably cytokinins and auxins. The growth medium is supplemented with PGRs to stimulate regeneration of the desired plant organs (Fehér et al. 2003),

with auxins generally favouring root induction and cytokinins resulting in shoot induction (Akiyoshi et al. 1984; Rayle et al. 1992; Riou-Khamlichi et al. 1999). *In vitro* conditions are often variety-specific and therefore require optimisation of PGRs based on the genotype in question (Mankessi et al. 2009).

Typically, following explant decontamination, *in vitro* establishment and multiplication is achieved via axillary and apical bud stimulation in growth medium containing low concentrations of auxin, such as indole acetic acid (IAA) or indole butyric acid (IBA), in combination with an elevated level of cytokinin, which may include kinetin, 6-benzylaminopurine (BAP), trans-zeatin or thidiazuron (Jones and van Staden 1997; Trueman et al. 2018). Cytokinin concentration and type requires careful consideration as persistence of stable cytokinins, such as kinetin, has been shown to have antagonistic effects on adventitious root formation in the latter stages of *in vitro* propagation (Nakhooda et al. 2012; Nakhooda and Jain 2016). Once explants are sufficiently established and proliferated *in vitro*, elongation is often necessary to condition explants for rooting (Le Roux and Van Staden 1991; Joshi et al. 2003; Nakhooda et al. 2011).

Following multiplication and elongation, adventitious *in vitro* rooting typically incorporates auxins, responsible for cell expansion and root development (Rayle et al. 1992; Enders and Strader 2015). Use of IBA has been shown to stimulate *in vitro* rooting in many eucalypt species (Gupta et al. 1983; Jones and van Staden 1993; Fett-Neto et al. 2001; Brondani et al. 2012). In most cases, IBA provides more favourable results than IAA (James 1983; Gantait et al. 2009). This response may be related to the higher relative stability of IBA, which is five-times less readily photo-oxidised than IAA (Nissen and Sutter 1990). Others have suggested that this may be because of the slow conversion of IBA to IAA, serving as a steady release source of easily metabolised IAA (Forgaça and Fett-Neto 2005).

The underlying mechanisms behind these observed variations in clonal responses to PGRs are poorly understood (de Almeida et al. 2010). The influence of PGRs on eucalypt varieties may be attributed to the genotype's ability to transport these hormones (de Almeida et al. 2015). Auxins are central to adventitious root development, and varietal responses may depend on the endogenous concentration and transport rate of this regulator across cells (Forgaça and Fett-Neto 2005). Auxins are distributed basipetally through the plant by active transport, which is conducted via the combination of influx (*AUX1*) and efflux (*PIN*) carriers (Muday and de Long 2001; Benjamins and Scheres 2008; Petrášek and Friml 2009). Collectively, the expression of these proteins may affect the overall *in vitro* responses of eucalypt varieties to auxins.

This study was aimed at creating a protocol that will be suitable for the micropropagation of eucalypt varieties that display varying degrees of recalcitrance to *in vitro* propagation. Consequently, three *Eucalyptus grandis* x *Eucalyptus nitens* temperate hybrids, ranging from easy to difficult to propagate, were selected as candidates for protocol development. Maintenance medium (Mm) was developed firstly to reduce the subculture frequency, and secondly to enable independent testing of each treatment. Refinement of the multiplication and rooting stages was conducted to develop a generalised protocol for the rapid *in vitro* and subsequent *ex vitro* establishment of eucalypts. Furthermore, the expression of genes involved in auxin transport (*AUX1*/*PIN1*) was quantified in response to auxin supplementation. The gene expression patterns for each eucalypt variety were linked to their observed *in vitro* responses, to formulate an understanding of why various genotypes often respond differently to *in vitro* treatments.

Materials and methods

Parental material preparation for culture initiation

Cuttings of three different *E. grandis* x *E. nitens* hybrid varieties (variety 1, 2 and 3) were obtained from Sappi Forests in KwaZulu-Natal Province, South Africa. Each parent plant was approximately 6 months old. Decontamination involved immersing the trimmed cuttings in a fungicide solution (1 g l^{-1} of Benlate®; 1 g l^{-1} of boric acid; 0.1% [v/v] Tween 20) for 30 min, followed by a $HgCl_2$ solution (0.2 g l^{-1} $HgCl_2$; 0.01% [v/v] Tween-20) for 2–4 min. Next, cuttings were rinsed in 500 ml of sterile milli-Q water for 5 min before being placed into a calcium hypochlorite (CaOCl) solution (10 g l^{-1} CaOCl; 0.01% [v/v] Tween-20) for 5 min. Finally, the cuttings were rinsed in 500 ml of sterile water and blotted dry on sterile filter paper before being placed onto multiplication medium 5 (Mt5) (Table 1). The resulting shoots were subcultured on Mt5 every 28 days to develop sufficient stock plants for experimentation.

In vitro *explant establishment*

Following decontamination, the explants were transferred from Mt5 to the Murashige and Skoog (MS) (1962)-based maintenance medium (Mm) (Table 1) with 12 shoots per magenta vessel, and sub-cultured every 4–5 weeks. The explants were maintained on Mm for the remainder of the study and provided material for the subsequent multiplication and rooting experiments.

The pH of all *in vitro* media was adjusted to 5.8, using 0.1 M KOH. Subsequently, 2.8 g l^{-1} of Gelrite® was added to the culture medium prior to autoclaving at 121 °C (~1.0 kgf cm^{-2}) for 20 min. After autoclaving, all media were allowed to cool to 60 °C, prior to the addition of phytohormones and vitamins, which were filter-sterilised with a Millex-GP® filter (0.22-µm sterile membrane). Incubation of the eucalypt cultures was conducted in a controlled growth room at 23 °C (± 2 °C), under cool white fluorescent lighting (Osram L 58V/740) with a 16-h photoperiod (50 µmol photons $m^{-2}s^{-1}$ of luminosity) followed by an 8-h dark period.

Culture maintenance

To investigate the effectiveness of the Mm at reducing the subculturing frequency, 9 cultures were initiated for Mm and Mt5 for eucalypt variety 2 (i.e. a total of 18 cultures). These cultures contained six shoots each that were grown *in vitro* until signs of necrosis and chlorosis developed. The number of days it took for these stress responses to show from culture initiation was documented.

In vitro *multiplication*

Shoot explants were trimmed to contain five buds (four axillary and one apical) per shoot and to a size of between 0.7 cm and 1.6 cm. Explants were then placed into magenta vessels containing 100 ml of medium. Five multiplication media

(Mt1–Mt5) were investigated (Table 1). The multiplication experiment was performed in a randomised complete block design, testing different media with each of the three eucalypt varieties. Each experiment consisted of 3 replicates with 12 shoots per replicate (n = 36). Following a 28-day culture period, the change in shoot length and bud proliferation was measured.

In vitro *rooting*

Seven rooting media treatments (Rt1–Rt7) were investigated (Table 2). Cytokinins were omitted from the rooting medium, with most treatments containing a reduction in concentration of MS to quarter strength and sucrose to 15 g l^{-1}. Furthermore, auxin concentrations were increased in the rooting medium, as opposed to in the multiplication medium, with a more stable auxin, namely IBA being included. Single shoots were trimmed and those exceeding 1.2 cm were transferred into culture tubes (49.09 cm^3) containing 15 ml of rooting medium. Following a 28-day growth period, the plants were carefully extracted from the rooting medium to measure the root length and number. The rooting experiment was performed in a randomised, complete block design. All rooting experiments included 36 shoots. The top three rooting treatments were assessed in terms of yielding consistent rooting percentages of above 30% for all varieties. In cases where the rooting percentage failed to consistently overcome the 30% threshold, the rooting vigour (i.e. root number, length and structure) was considered. These top-performing treatments were repeated in triplicate (n = 108).

Expression analysis of genes involved in auxin transport: total plant gene expression analysis

After 48 h of growth in Rt4 medium, total RNA was independently extracted from each variety, using the Maxwell® 16 Cell LEV Total RNA Purification Kit (Promega, Madison, Wisconsin), according to the manufacturer's protocol. The RNA samples were subsequently treated with a DNase I kit (Thermo Fisher Scientific, USA). The concentration, purity and integrity of the RNA was measured using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA) and via subjecting 300 ng of RNA to gel electrophoresis on a 1% (w/v) TBE agarose gel at 100V. The RNA extracts were subjected to first-strand cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) as per the manufacturer's protocol. The final cDNA products were diluted 10-fold, prior to use in reverse transcriptase-quantitative PCR (RT-qPCR).

The primer sequences were obtained from prior studies by de Almeida et al. (2010, 2015). The primers used included auxin transporters *PIN1* and *AUX1* as well as the housekeeping genes *H2B* and *TUA*. RT-qPCR was performed using the PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, USA), as per the manufacturer's recommendations. The reactions were performed in a 0.1 ml, MicroAmp™ Fast Optical 96-Well Reaction Plate (Applied Biosystems, USA) using a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, USA), in accordance with the manufacturer's instructions. The incubations for the RT-qPCR reactions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Subsequent to the final RT-qPCR cycle, a heat dissociation curve was generated, from 60 °C to 95 °C. The gene expression data from the QuantStudio™ design and analysis software (Thermo Fisher Scientific, USA) were analysed via the comparative Ct method (Schmittgen and Livak 2008) and calculation of relative expression was conducted with each reference gene (*H2B* and *TUA*), separately (de Almeida et al. 2010, 2015). The normalisation procedure revealed similar relative expression results for both reference genes. Consequently, *H2B* was selected for normalisation to determine the fold expression.

Table 1: Respective concentrations of various plant growth regulators (PGRs) and supplements incorporated into the maintenance medium (Mm) and multiplication media (Mt1–Mt5)

	Culture medium composition					
	Mm	Mt1	Mt2	Mt3	Mt4	Mt5
PGRs (mg l^{-1})						
IAA	0.05	0.1	0.1	0.1	0.1	0.1
BAP	0.025	0.05	0.25	0.1	–	0.5
Kinetin	–	0.5	0.25	0.1	–	0.1
Meta-topolin	–	–	–	–	0.5	–
Supplements (g l^{-1})						
MS	4.4	4.4	4.4	4.4	4.4	4.4
Vitamin B5 (mg l^{-1})	0.1	0.1	0.1	0.1	0.1	0.1
Biotin (mg l^{-1})	0.1	0.1	0.1	0.1	0.1	0.1
Sucrose (g l^{-1})	20	20	20	20	20	20

BAP = 6-Benzylaminopurine
IAA = Indole acetic acid
MS = Murashige and Skoog (1962) basal medium with vitamins

Table 2: Respective concentrations of various plant growth regulators (PGRs) and supplements incorporated into the rooting media (Rt1-Rt7)

	Culture medium composition						
	Rt1	Rt2	Rt3	Rt4	Rt5	Rt6	Rt7
PGRs (mg l^{-1})							
IAA	0.1	0.5	0.1	0.5	–	–	0.5
IBA	–	–	–	–	0.1	0.5	–
rac-GR24	–	–	–	–	–	–	0.029
Supplements							
MS (g l^{-1})	4.4	4.4	1.1	1.1	1.1	1.1	1.1
Vitamin B5 (mg l^{-1})	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Biotin (mg l^{-1})	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sucrose (g l^{-1})	20	20	15	15	15	15	15

IAA = Indole acetic acid
rac-GR24 = *racemic*-GR24, synthetic strigolactone
IBA = Indole butyric acid
MS = Murashige and Skoog (1962) basal medium with vitamins

Statistical analysis: culture maintenance, multiplication, rooting and qPCR data analysis

All the datasets were assessed for normality via the Shapiro–Wilk test. Normally distributed data sets for the multiplication experiment were analysed via two-way ANOVA with Tukey's HSD to distinguish statistically significant differences. Normally distributed culture maintenance and RT-qPCR data were analysed with independent T-tests and one-way ANOVA, followed by Tukey's HSD. Significant differences were assessed at $\alpha = 0.05$ ($p \leq 0.05$).

The rooting experiment data were distribution-free, and thus the Friedman test with a two-stage linear step-up procedure of Benjamini et al. (2006) was used to distinguish statistically significant differences at $\alpha = 0.05$ ($p \leq 0.05$) (Benjamini et al. 2006). Percentage data were analysed via the Chi-squared test of independence, followed by a pairwise test to discern differences between treatments.

Results

In vitro *explant establishment*

The decontamination protocol yielded 45% explant survival (i.e. 45% of the shoots were free from contaminants).

Culture maintenance

This experiment clearly demonstrated that shoots reared on Mm could be maintained in culture for a prolonged period when compared with medium Mt5. On average, shoots grown on Mm displayed signs of necrosis and chlorosis 43 days after culture initiation, whereas shoots placed into Mt5 exhibited these stress responses 35 days post-initiation. This demonstrated that the Mm maintained healthier shoots for a significantly ($p = 0.0025$) longer time as opposed to the Mt5 medium. Furthermore, the shoots cultured on Mm displayed less basal callus.

Multiplication

In response to all five multiplication treatments implemented, variety 2 displayed the highest mean bud proliferation (59.36 ± 5.8), followed by variety 3 (47.94 ± 4.7), and lastly variety 1 (37.17 ± 7.8) being the most recalcitrant (Figure 1a). In terms of treatment consistency, treatments incorporating high concentrations of *meta*-topolin (Mt4) and BAP (Mt5) at 0.5 mg l^{-1}, induced significantly higher levels of bud proliferation, with 59.1 (±2.9) and 51.7 (±2.42) buds per shoot for variety 1, 62.3 (±2.7) and 68 (±2.7) for variety 2, and 58.1 (±3) and 54.9 (±2.2) for variety 3 (Figure 1a).

In terms of shoot lengthening, variety 2 was again the most responsive to *in vitro* stimulation, demonstrating an average increase in shoot length over the five treatments, of 1.51 cm (± 0.1), which was significantly taller than varieties 1 (0.72 cm ± 0.15) and 3 (0.98 cm ± 0.077) (Figure 1b). All varieties responded positively to the supplementation of *meta*-topolin at 0.5 mg l^{-1} into the growth medium when compared with the other PGRs tested, and this was consequently the multiplication treatment that produced the tallest shoots for varieties 1 (1.2 cm ± 0.08), 2 (1.8 cm ± 0.08) and 3 (1.2 cm ± 0.07) (Figure 1b).

Rooting

An improvement in *in vitro* adventitious root formation was accomplished by decreasing the sucrose concentration in the rooting medium from 20 to 15 g l^{-1}, and MS from full to quarter strength. Furthermore, the percentage of adventitious roots increased when the concentration of IAA was raised from 0.1 mg l^{-1} to 0.5 mg l^{-1}, for all three varieties (Table 3). The rooting percentage was further improved for varieties 1 and 2 with 0.1 mg l^{-1} of IBA over IAA, whereas variety 3 displayed a significant improvement in response to 0.5 mg l^{-1} of IBA (Table 3). Interestingly, elevating the IBA concentration from 0.1–0.5 mg l^{-1} resulted in an observable increase in root-hair production for all the varieties tested (Figure 2a,b). This observation may have implications for acclimatisation, as the root hairs formed in Rt6 will drastically increase the root surface area, and consequently presumably enhance nutrient sequestration (Gonçalves et al. 1998; Ubalua and Nsofor 2017). Treatment of the shoots with Rt7, containing *racemic*-GR24 (*rac*-GR24, a synthetic strigolactone), appeared to delay explant senescence and increased shoot length considerably when compared with the other treatments (Figure 5).

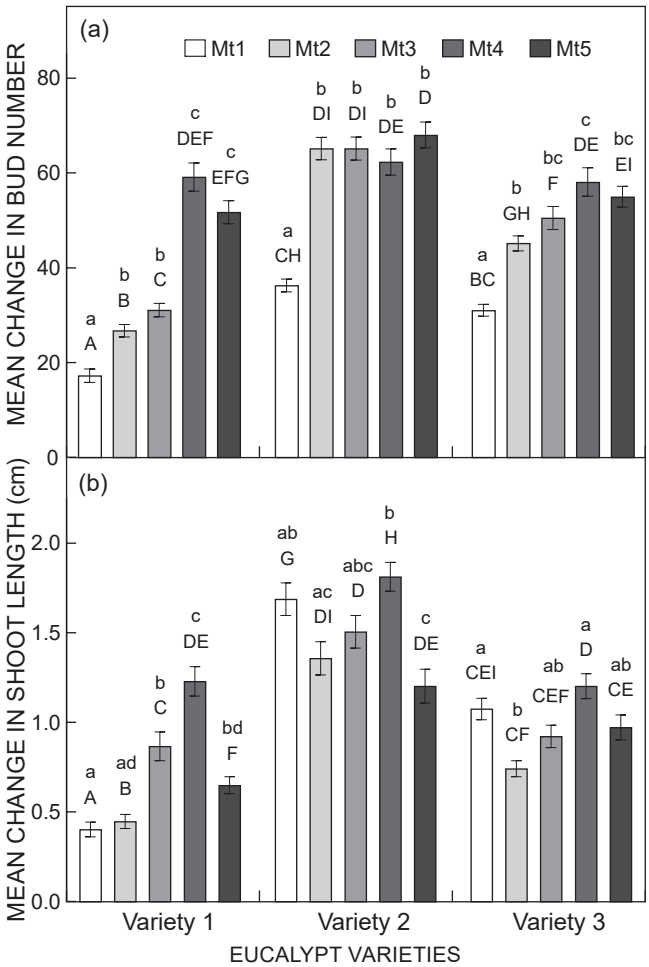

Figure 1: Mean changes in (a) bud number (i.e. bud proliferation) and (b) shoot length for three *Eucalyptus* varieties subject to five multiplication treatments (Mt1–Mt5). The data were analysed using square-root-transformed values. Error bars represent standard error of the mean; lowercase letters denote significant differences between treatments within a variety, and uppercase letters indicate significant differences between the varieties, analysed via Tukey's HSD with a 95% confidence interval ($p \leq 0.05$). The graphs display the untransformed data values

To determine the optimal rooting treatment for each eucalypt variety, the top-three rooting experiments, Rt4, Rt6 and Rt7, were repeated. These were selected based on those that produced the highest rooting percentages most consistently across all three eucalypt varieties. Treatments producing rooting percentages of more than 30% for all three varieties were considered consistent. In addition to

Table 3: Rooting percentages obtained for *Eucalyptus grandis* x *Eucalyptus nitens* varieties 1, 2 and 3 grown on seven different rooting media (Rt1–Rt7). The Chi-squared test of independence revealed that a significant difference based on the rooting treatment was present. To further elucidate between which treatments the significance lies, a *post hoc* pairwise test was conducted at $\alpha = 0.05$

	Percentage rooting per treatment						
Variety	Rt1	Rt2	Rt3	Rt4	Rt5	Rt6	Rt7
1	8.33^{a}	11.1^{ab}	27.8^{ac}	36.1^{c}	41.7^{c}	13.9^{ab}	30.6^{bc}
2	8.33^{a}	11.1^{a}	22.2^{ab}	33.3^{bc}	61.1^{d}	52.8^{cd}	44.4^{bd}
3	8.33^{ab}	5.56^{a}	25.0^{bc}	38.9^{cd}	19.4^{ac}	58.3^{d}	30.6^{c}

Supercript lower-case letters denote significant differences between the rooting treatments for each variety

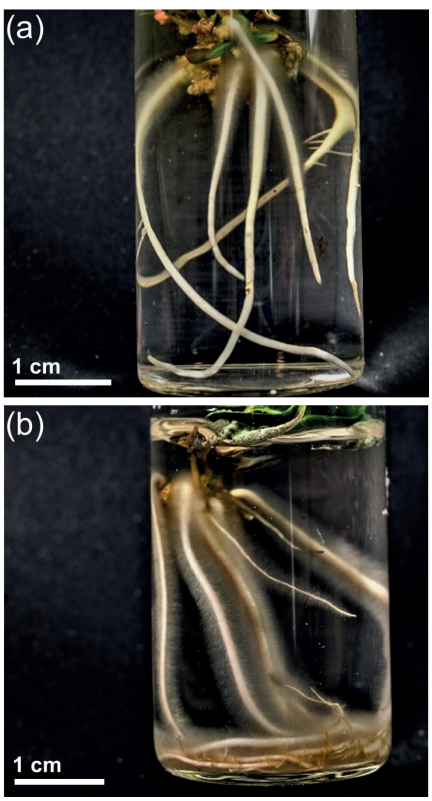

Figure 2: (a) Illustration of the *Eucalyptus* phenotype lacking root hairs, obtained with the 0.1 mg l^{-1} indole butyric acid (IBA) treatment, versus (b) an enhancement in root-hair production, induced by the 0.5 mg l^{-1} IBA treatment. Scale bars represent 1 cm

rooting percentage, parameters such as root length and number were also measured (Figure 3a,b). Although not reflected in the root number, variety 1 displayed the greatest root length of 3.24 cm (±0.6) and percentage (33.4%) in the presence of *rac*-GR24 (Rt7). Upon IBA treatment, varieties 2 and 3 produced the highest number of roots, with 1.29 (±0.2) and 1.44 (±0.2) roots per shoot, respectively (Figure 3a,b). In addition, the highest rooting percentages for variety 2 were achieved with IBA (44.6%) and *rac*-GR24 (43.5%) treatment, whereas variety 3 clearly responded best to IBA (47.2%) (Table 4). The greatest root length of 5.02 cm (± 0.9) was achieved for variety 3 when subjected to IBA treatment

Table 4: Mean ($\bar{x}$) rooting percentage of the three best-performing rooting treatments following a 4-week growth period for *Eucalyptus grandis* x *E. nitens* varieties 1, 2 and 3. All treatments were replicated in triplicate (n = 108). The Chi-squared test of independence revealed that a significant difference based on the rooting treatment was present. To further elucidate between which treatments the significance lies, a *post hoc* pairwise test was conducted at $\alpha = 0.05$

	Percent rooting per treatment		
Variety	Rt4	Rt6	Rt7
1	32.5 ^{a}	19.6 ^{b}	33.4 ^{a}
2	30.6 ^{a}	44.6 ^{b}	43.5 ^{b}
3	32.5 ^{a}	47.2 ^{b}	34.3 ^{ab}

Supercript lower-case letters denote significant differences between the rooting treatments for each variety

(Figure 3a). However, when considering all three varieties, the optimal treatment that generated the most consistent results for root induction contained *rac*-GR24 (Rt7), which also produced considerably healthier shoots than those cultured in its absence, as these shoots displayed fewer signs of necrosis and chlorosis (Figure 5). During the rooting stage some of the shoots appeared to present basal callus formation. However, this did not seem to adversely affect the functionality of the roots as the eucalypt plants were successfully acclimatised, with an average survival of 87.5% for each variety.

Gene expression

The relative expressions of *PIN1* to *AUX1* were determined for each of the eucalypt varieties, via qPCR, to identify whether an endogenous bias or ratio of *PIN1*:*AUX1* (efflux:influx) may exist within each variety. Varieties 1 and 3 showed a *PIN1* bias over *AUX1* (Figure 4a,c). In contrast, the relative expressions of *PIN1* and *AUX1* were statistically equivalent in variety 2 (Figure 4b).

Discussion

In vitro *culture maintenance*

During the study, a Murashige and Skoog (MS)-based maintenance medium (Mm) containing low PGR concentrations was developed with the premise of storing eucalypt shoot material in culture for a prolonged period.

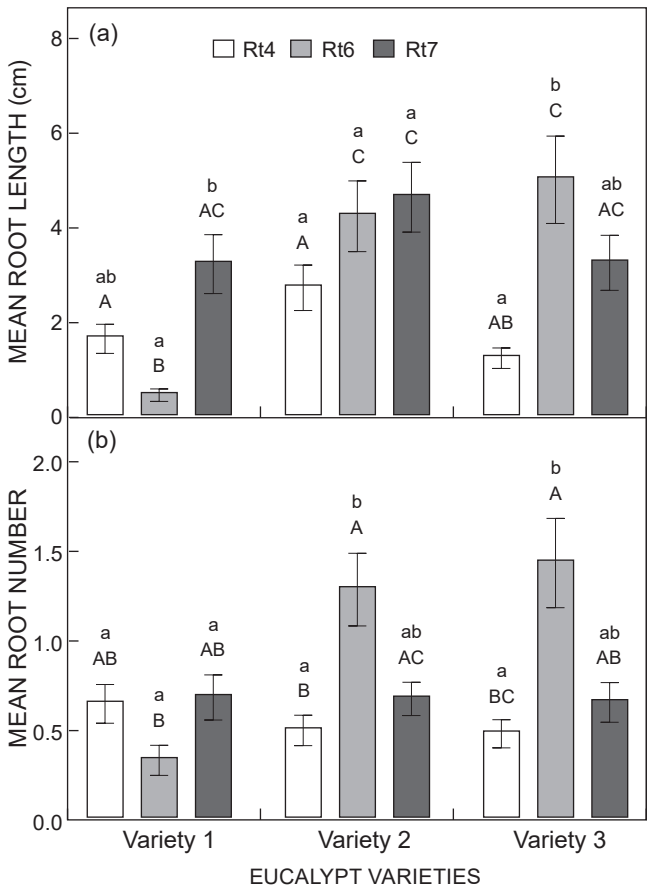

Figure 3: Comparison of the mean (a) root length and (b) root number of three eucalypt varieties subject to the top three rooting treatments (Rt4, Rt6, Rt7), following a 28-day growth period and repeated in triplicate. Error bars represent standard error of the mean; lowercase letters denote whether significant differences existed between the treatments for a given variety, and uppercase letters indicate a significant difference among the varieties, analysed via the two-stage linear step-up procedure of Benjamini et al. (2006), with a 95% confidence interval ($p \leq 0.05$)

The clear benefits would be a reduction in time and costs associated with frequent subcultures. The Mm successfully prolonged the time in culture by 8 days as compared with multiplication medium 5 (Mt5). This finding may be related to basal callus formation, which was more prevalent in treatments with Mt5 than with Mm. Since callus is a mass of unorganised cells, this may have served as a physical barrier that prevented the flow of nutrients from the medium to the shoots in Mt5, and hence resulted in nutrient deprivation leading to earlier signs of stress (Ikeuchi et al. 2013). It is clear from the literature that elevated PGR concentrations can exacerbate the formation of basal callus, and thus the lower concentration in the Mm may have reduced the prevalence of basal callus (Gaspar et al. 1996).

In vitro *multiplication of Eucalyptus grandis x E. nitens explants*

During multiplication, the bud proliferation response is primarily stimulated under the influence of cytokinins, which reduce apical bud dominance leading to an increase in axillary bud proliferation (George et al. 2008). In this investigation, multiplication was used to rapidly establish three commercially valuable eucalypt varieties *in vitro*. This was best achieved with treatments containing an elevated BAP and reduced kinetin concentration in the growth medium or alternatively with an elevated concentration of *meta*-topolin (Figure 1a).

This result is supported by various studies that report enhanced multiplication performance with the use of BAP as opposed to kinetin in the growth medium (Trindade et al. 1990; Muhammad et al. 2007). The disparate clonal responses to the various cytokinin types tested may be attributed to the ability of the particular genotype to either transport, degrade or inactivate cytokinins (Blakesley 1991; Mok and Mok 2001). The conjugation of PGRs, via sugars or amino acids, serves to inactivate them, which influences the availability of their physiologically active free forms (Kamínek 1992; Klemŝ et al. 2000). The superior bud proliferation observed with BAP, compared with kinetin, may be attributed to a reduced tendency of BAP to form conjugates (Buah et al. 2010), which translates into a greater stability and presence of BAP in either the free or ionised form. This observation is supported by Klemŝ et al. (2000) who reported an enhanced stability of BAP compared with other purine-type cytokinins.

Similarly, improved bud proliferation was obtained in the presence of the aromatic cytokinin *meta*-topolin. The positive effects of *meta*-topolin might be attributable to the hydroxyl group present in the side chains of topolins that allow for the formation of *O*-glucoside conjugates which are easily translocated and converted into free active forms, when required by the shoots (Amoo et al. 2011; Amoo and van Staden 2013). In most cases *meta*-topolin resulted in significantly taller shoots compared with BAP and kinetin treatment, for all varieties tested.

In vitro *rooting of Eucalyptus grandis x E. nitens explants*

The stimulation of adventitious roots often requires the use of exogenously supplied auxin as the sole PGR in the growth medium (George et al. 2008; Durbak et al. 2012). A reduction in MS salts and sucrose significantly enhanced the rhizogenic response in all three eucalypt varieties (Table 3). Comparable observations have been documented previously in the literature, where a reduction in MS salt concentration to quarter strength was shown to significantly enhance the induction of adventitious roots in multiple plant species *in vitro* (Dalal and Rai 2004; Pijut et al. 2012). This observation may be attributed to the reduced salinity levels in the medium resulting from the use of quarter-strength MS, which has been shown to be beneficial to adventitious rooting in *E. tereticornis* (Das and Mitra 1990) and *E. grandis* hybrids (Warrag et al. 1990). Sucrose influences the osmotic potential and thus the uptake of components from the growth medium (Cheng et al. 1992; Nourissier and Monteuuis 2008). In the present investigation, a reduction in the osmotic potential, with a lower sucrose concentration, significantly enhanced adventitious rooting in the hybrid varieties (Table 3).

Increasing the concentration of exogenously supplied IAA enhanced the rooting percentage in all eucalypt varieties (Table 3). IAA is relatively unstable and is more easily photo-oxidised or conjugated into inactive forms, so higher concentrations are required compared with more stable auxins such as IBA

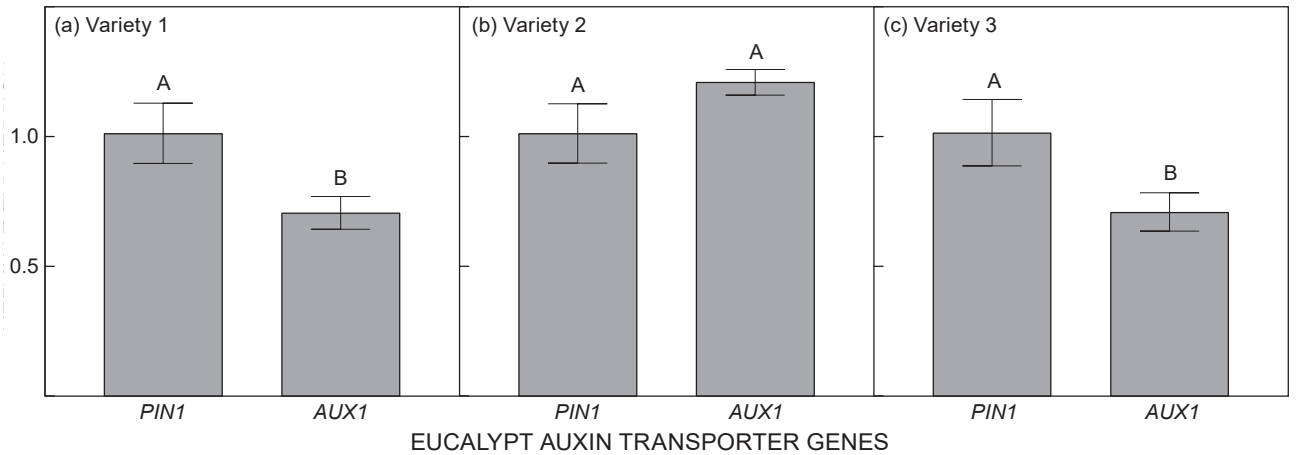

Figure 4: Comparisons of the mean relative expression of PIN1 versus AUX1, for varieties (a) 1; (b) 2; and (c) 3. Error bars represent the standard error of the mean and the letters denote whether significant differences in gene expression exist between the PIN1 and AUX1 (Tukey's HSD with a 95% confidence interval, p≤0.05). The PIN1 gene expression for each of the varieties tested was used as the control and thus set at one. The expression of AUX1 was, therefore, determined in relation to PIN1 for each of the varieties

(Blakesley 1994; de Klerk et al. 1999; Phillips and Garda 2019). A study by Nissen and Sutter (1990) showed that, after 20 days, up to 97% of the exogenously supplied IAA may be subjected to non-biological degradation in the growth medium. Therefore, the use of an elevated IAA concentration may have presented more auxin in the free form, to sustain adventitious rooting in the tested eucalypt varieties.

Treatment with IBA resulted in a significantly improved rooting percentage in the eucalypt varieties tested, in comparison to IAA. While the concentrations of exogenously supplied IBA had a significant impact on varieties 1 and 3, the concentration of IBA did not seem to significantly affect the rooting percentage of variety 2 (Table 3), confirming genotypic effects of PGRs (Mankessi et al. 2009; Brondani et al. 2012). The improved rooting of the eucalypt varieties in response to IBA may be attributed to the higher relative stability of IBA over IAA (Ludwig-Müller 2000). IBA can serve as a precursor of IAA, which, through fatty acid β-oxidation, can be converted into free active IAA (Strader and Bartel 2011; Frick and Strader 2018). Since IBA is less susceptible to degradation than IAA, this auxin may have remained in the growth medium for an extended period, and, through conversion to IAA, served as a sustained *in vitro* regulator of adventitious rooting (Nissen and Sutter 1990; Forgaça and Fett-Neto 2005). Similar findings have been observed with the hybrid *E. urophylla* x *E. grandis*, where an enhanced rooting response was obtained with IBA than with IAA (Nourissier and Monteuuis 2008). A discernible increase in root-hair development was observed with IBA concentrations greater than 0.1 mg l^{-1} for all three varieties (Figure 2a,b). A lack of root hairs, such as from treatment with 0.1 mg l^{-1} IBA, can lead to a reduction in water and mineral nutrient uptake, which may be detrimental to survival during acclimatisation (Gonçalves et al. 1998; Ubalua and Nsofor 2017).

Strigolactones are a class of PGRs that play a crucial role in the regulation of cell division and elongation (Hoffmann et al. 2014; Al-Babili and Bouwmeester 2015). Although no significant enhancement in rooting response was observed compared with treatment with IAA alone, shoots treated with *rac*-GR24 appeared taller and healthier than those under other treatments, with fewer visible signs of necrosis and chlorosis (Figure 5). Increases in internode length, accompanied by an inhibition of shoot branching, has been observed with the use of *rac*-GR24 (Gomez-Roldan et al. 2008; de Saint Germain et al. 2013), similar to the present observations. Although strigolactones have been linked to the acceleration of leaf senescence (Snowden et al. 2005) by enhancing the action of ethylene (Ueda and Kusaba 2015), the inclusion of *rac*-GR24 into the rooting medium in the present study resulted in reduced necrosis and chlorosis across varieties when compared with treatments without strigolactone.

Although rooting treatments with IBA yielded some of the best rooting responses, the purpose of the present investigation was to develop the most-high-yielding generic media for the eucalypt varieties currently tested. Evidently, the treatment that produced the greatest rooting responses in the most consistent manner was with *rac*-GR24 in combination with IAA (Rt7). Furthermore, Rt7 produced visibly healthier and elongated shoots, with reduced branching and fewer signs of necrosis. The use of a strigolactone in combination with IAA or IBA might therefore be a valuable generic treatment for various varieties, while having further beneficial effects in terms of enhancing explant health.

Regardless of the presence of basal callus, the eucalypt shoots were successfully acclimated and grown in soil, with 87.5% survival. Despite this result, studies have reported that the presence of basal callus can adversely affect acclimatisation by serving as a physical barrier between the shoot and root vasculature that will ultimately prevent the transfer of nutrients from roots to shoot (Joshi et al. 2003; Bunn et al. 2005). To overcome this, some have achieved rooting success on hormone-free medium as the endogenous auxin content within the shoots are adequate to stimulate rooting (Jana and Shekhawat 2011). Lastly, some studies report the use of *ex vitro* rooting procedures, which can mitigate the formation of basal callus (Behera et al. 2015).

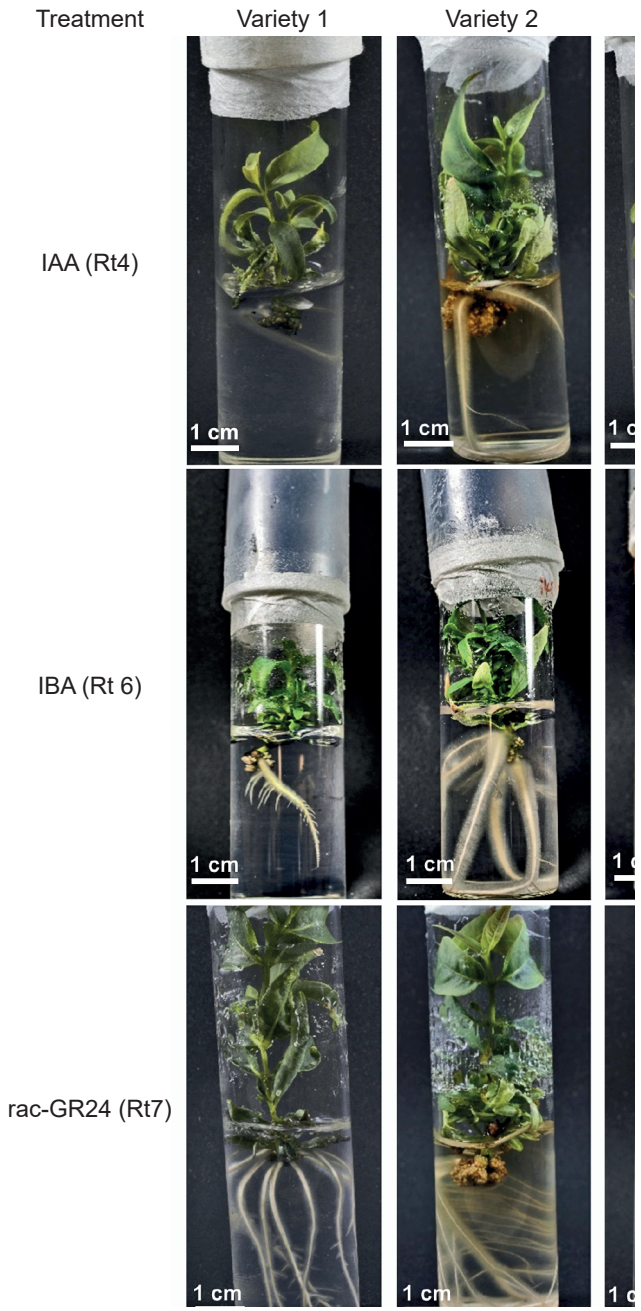

Figure 5: Depiction of the rooting response induced by the top-three rooting treatments on each of the eucalypt varieties tested: Rt4 with indole acetic acid (IAA); Rt6 with indole butyric acid (IBA); and Rt7 with rac-GR24. Scale bars represent 1 cm

Gene expression studies offer clues to in vitro *performance*

Given the disparate genotypic responses to auxins *in vitro*, an investigation into the expression of auxin transport genes (*PIN1/AUX1*) was undertaken. Synthesis of auxins occurs in the shoot apex, and thus efficient polar transport is essential for growth and development, as it regulates regions of auxin accumulation (Blakeslee et al. 2005). This has been shown to stimulate the elongation of hypocotyls in *Brassica oleracea* (Esmon et al. 2006), lateral rooting in *Arabidopsis thaliana* (Laskowski et al. 2008) and adventitious root formation in *E. grandis* (Nakhooda et al. 2011). The influx of auxin into cells is modulated by members of the *AUX* family of transport membrane proteins, while the efflux of auxin from cells is controlled by the *PIN*-formed family of membrane proteins (Vanneste and Friml 2009; Swarup and Péret 2012; Ahkami et al. 2013). Both *PIN1* and *AUX1* expression was detected in all the eucalypt varieties tested, but genes were differentially expressed in each variety, in response to exogenous auxin *in vitro*.

Variety 2 displayed an equal relative gene expression for the auxin transporters *PIN1* and *AUX1*, which suggests that these transporters may be equally abundant within the cells of variety 2 (Figure 4b). With a comparable ability to facilitate auxin influx and efflux, it is suggested that auxin transport in variety 2 may be more capable of maintaining the optimum required auxin equilibrium, in comparison to varieties 1 and 3 which displayed a *PIN1* bias. As a result, auxin influx might not match efflux, creating an imbalance in the auxin transport requirement for growth and physiology (Delbarre et al. 1996).

Conclusions

Micropropagation has proven to be a valuable technique to clonally propagate numerous eucalypt varieties, especially those that are recalcitrant to other forms of vegetative propagation (Nakhooda and Jain 2016). However, since many protocols tend to be clone-specific, developing or adjusting protocols can drive up production costs (Mascarello et al. 2017). The reduced PGR content in the Mm effectively reduced the sub-culturing frequency and enabled direct transfer of the eucalypt shoots into multiplication media and rooting media, with no adverse effects of carry-over hormones. Exogenous treatment with *meta*-topolin during multiplication was shown to simultaneously lengthen and multiply eucalypt shoots, thus omitting the need for an elongation stage. During rooting, the incorporation of *rac*-GR24 in combination with IAA induced the most-consistent rooting response among the three varieties and was consequently considered to be the optimum generic treatment. The overall enhanced amenability of variety 2 to *in vitro* conditions was shown to correlate to an equivalent expression of auxin transporters *PIN1* and *AUX1*. Such gene expression patterns may serve as indicators to predict how various eucalypt varieties may respond to growth regulators *in vitro*.

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ORCIDS

Rafael Keret — https://orcid.org/0000-0003-4582-4516
Paul Hills — https://orcid.org/0000-0002-7473-0466

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