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# Genotypic-unspecific protocols for the commercial micropropagation of *Eucalyptus grandis* × *nitens* and *E. grandis* × *urophylla*

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**Abstract**: Clones of *Eucalyptus grandis* × *nitens* and *E. grandis* × *urophylla* were subjected to various culture conditions at every culture stage leading to plantlet regeneration directly from axillary buds. The objective was to determine the possibility of using the same protocol for all clones. Although genotypic effects were evident, generally most clones responded similarly to the tested variations in each of the protocol stages, i.e. bud break, multiplication, and rooting. Estimated yields for the clones of one of the tested *E. grandis* × *nitens* hybrids ranged from 24 to 90 acclimatised plants per explant, but those of a natural hybrid were much lower (8 to 15 plants). The tested clones of *E. grandis* × *urophylla* produced approximately 10 to 27 plants per explant. It is suggested that such an all-purpose protocol has applications in a commercial environment, such as the production of hedge plants and retrieval from in vitro storage, when large numbers of plants are not required. This would save time, labour, and other costs as compared with having to develop and implement clone-specific protocols.

**Key words:** Clonal propagation, eucalyptus hybrids, forestry, tissue culture

## 1. Introduction

Eucalyptus plays a pivotal role in the forestry industry in South Africa, and in many other parts of the world, in serving the wood, paper, pulp, and charcoal industries (Eldridge et al., 1994; Turnbull, 1999; Watt et al., 2003b). To meet the increasing demands for these products it is necessary to develop approaches and technologies that can ensure a constant and reliable production of the resource. In this regard, it is crucial to be able to propagate improved genetic material produced through breeding and clonal programmes effectively and inexpensively. The standard vegetative propagation method for eucalypts is that of stem cuttings (macro- and mini-cuttings) (de Assis et al., 2004). However, their yields are limited by the availability of coppice shoots and nodes within the stock plants and the rooting capacity of the clones, which is highly variable and decreases with age of the parent plant (Eldridge et al., 1994; de Assis et al., 2004). Micropropagation potentially addresses such shortcomings by providing a highly controlled environment that yields high shoot multiplication rates, improved potential, speed, and quality of rooting (Warrag et al., 1990; Le Roux and Van Staden, 1991; de Assis et al., 2004). It is also often the only feasible method of propagating difficult-to-root genotypes, many of which are hybrid clones (Mokotedi et al., 2000; Watt et al., 2003b; Yasodha et al., 2004; Nourissier and Monteuuis, 2008).

Natural hybridisation between *Eucalyptus* spp. is common (Butcher et al., 2005), and this has been exploited in genetic improvement programmes for the production of hybrids with value-added traits for plantations in marginal lands. Examples include *E. grandis* × *urophylla* in the Republic of Congo (Nourissier and Monteuuis, 2008), *E. camaldulensis* × *tereticornis* and *Eucalyptus torelliana* F.Muell. × *E. citriodora* in India (Arya et al., 2009), and hybrids of *E. globulus* in Brazil (Borges et al., 2011), amongst others. In South Africa, eucalyptus is grown in many different geographical and climatic regions of the country, and rigorous clonal programmes are employed to match clones to sites. In this regard, hybrids of *E. grandis*, such as *E. grandis* × *nitens* and *E. grandis* × *urophylla*, are important for plantations in cool and dry marginal areas, where pure species cannot be grown (Denison and Kietzka, 1993).

Despite the potential advantages of micropropagation, the propagation of commercially important hybrids is still mainly undertaken through cuttings, with in vitro multiplication being used primarily for the production of parent plants for clonal edges (Alpoim et al., 2004; de Assis et al., 2004). This is mainly due to the genotypic variation

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amongst clones of each hybrid, requiring the optimisation and implementation of clone-specific protocols (Le Roux and van Staden, 1991; Watt et al., 2003b). As this is time-consuming and labour-intensive, propagators and laboratory managers in the industry are faced with the decision of whether to optimise the protocol for each clone in production or to use a general protocol and accept lower yields (or compensate by initiating more material). Hence, the aim of this study was to investigate the feasibility of establishing hybrid-specific, rather than clone-specific, protocols in terms of yields suitable for commercial activities.

## 2. Materials and methods

### 2.1. Parent plants and explant preparation

Cutting-derived 1-year-old potted plants of clones from 3 hybrids were obtained from Mountain Home, Mondi Business Paper, Hilton, South Africa. They were *E. grandis* × *nitens* produced by a controlled cross between a female *E. grandis* and a male *E. nitens* (GN1, GN9, GN15, GN108, and GN121), a natural hybrid of *E. grandis* × *nitens* (NH0, NH58, and NH70), and *E. grandis* × *urophylla* produced by crossing a female *E. grandis* with a male *E. urophylla* (clones GU21, GU151, GU244, and GU297). The parent plants were maintained in the greenhouse and treated with fungicides as previously reported (Watt et al., 1996, 2003a) to remove endogenous contaminants. The plants were cut back every 3 to 4 weeks to stimulate coppice growth, and shoots with 3 nodes were excised and immersed in a solution of 1 g $L^{-1}$ Benlate (benomyl; Effekto SA), 0.5 mL $L^{-1}$ Bravo (chlorothalonil; Shell SA), 1 g $L^{-1}$ boric acid, and 0.2 mL Tween 20 (surfactant) for 15 min (Watt et al., 2003a). This was followed by transfer to a laminar flow cabinet where the shoots were trimmed to produce nodal explants, each comprising the nodal section of the stem and 1 or 2 leaves trimmed to two-thirds of their original size. These were then decontaminated for 10 min in 0.2 g $L^{-1}$ $HgCl_2$ and then 10 min in 10 g $L^{-1}$ CaOCl, with 3 washes in sterile water in between the sterilant solutions.

### 2.2. Bud break, multiplication, and elongation

Axillary buds are the most commonly used explants for micropropagation of eucalyptus (Le Roux and van Staden, 1991; Watt et al., 2003b; Arya et al., 2009; Borges et al., 2011) as they are responsive to culture conditions and are the appropriate vegetative material for the clonal multiplication of superior genotypes. Hence, in the present study, 3 methods were tested for culture initiation. Method 1 involved placing nodal explants on Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962), 0.1 mg $L^{-1}$ biotin, 0.1 mg $L^{-1}$ calcium pantothenate, 0.04 mg $L^{-1}$ 1-naphthalene acetic acid (NAA), 0.11 mg $L^{-1}$ 6-benzylaminopurine (BAP), 0.05 mg $L^{-1}$ kinetin, 20 g $L^{-1}$ sucrose, and 3.5 g $L^{-1}$ Gelrite, pH 6.2, for 2 weeks, before they were transferred to multiplication media. In Methods 2 and 3, the explants were placed directly onto multiplication media, omitting the bud break stage; nodal explants were used for Method 2, and excised axillary buds were the explants for Method 3.

For multiplication, 4 media were tested, all containing MS salts and vitamins, 0.1 mg $L^{-1}$ biotin, 0.1 mg $L^{-1}$ calcium pantothenate, 0.04 mg $L^{-1}$ NAA, 0.11 mg $L^{-1}$ BAP, 0.05 mg $L^{-1}$ kinetin, and 3.5 g $L^{-1}$ Gelrite. They also contained: 0.2 mg $L^{-1}$ BAP and 20 g $L^{-1}$ sucrose (M1); 0.2 mg $L^{-1}$ BAP, 0.01 mg $L^{-1}$ NAA, and 25 g $L^{-1}$ sucrose (M2); 0.5 mg $L^{-1}$ BAP, 0.2 mg $L^{-1}$ NAA, and 20 g $L^{-1}$ sucrose (M3); 0.1 mg $L^{-1}$ BAP, 0.01 mg $L^{-1}$ NAA, 0.2 mg $L^{-1}$ kinetin, and 25 g $L^{-1}$ sucrose (M4).

After 6 weeks of multiplication, individual shoots were excised from shoot clumps and transferred to elongation medium containing MS salts and vitamins, 0.1 mg $L^{-1}$ biotin, 0.1 mg $L^{-1}$ calcium pantothenate, 0.35 mg $L^{-1}$ NAA, 0.1 mg $L^{-1}$ indole-3-butyric acid (IBA), 0.1 mg $L^{-1}$ kinetin, 20 g $L^{-1}$ sucrose, 3.5 g $L^{-1}$ Gelrite, and maintained under either constant darkness or a photoperiod regime (as below) for 3 weeks.

For all culture stages, 4 explants were placed into 12.5 mm (diameter) × 100 mm (height) culture bottles containing 10 mL oh medium at pH 5.8. The environmental conditions were 25 ± 2 °C day/21 °C night, 16 h light/8 h dark, and 66 µmol $m^{-1}$ $s^{-1}$ photosynthetic photon flux density (PPFD). The exception was the constant dark treatment for elongation at 25 ± 2 °C.

### 2.3. Rooting and acclimatisation

Elongated shoots (approximately 20 mm long) were each transferred to 10 mL of the tested media, which each contained 0.1 mg $L^{-1}$ biotin, 0.1 mg $L^{-1}$ calcium pantothenate, 15 g $L^{-1}$ sucrose, and 3.5 g $L^{-1}$ Gelrite, with the following additions: half-strength MS salts and vitamins, 1 mg $L^{-1}$ IBA (R1); half-strength MS salts and vitamins, 0.1 mg $L^{-1}$ IBA (R2); half-strength MS salts and vitamins, 0.1 mg $L^{-1}$ IBA, 0.5 mg $L^{-1}$ NAA (R3); quarter-strength MS, 0.1 mg $L^{-1}$ IBA, 0.22 g $L^{-1}$ $CaCl_2.2H_2O$, 0.185 g $L^{-1}$ $MgSO_4.7H_2O$ (R4). All media were at pH 5.8. Cultures were initially maintained in the dark at 25 °C for 72 h, after which they were transferred to 25 ± 2 °C day/21 °C night, 16 h light/8 h dark, and 37 µmol $m^{-1}$ $s^{-1}$ PPFD for 7 days, followed by 21 days at 25 ± 2 °C day/21 °C night, 16 h light/8 h dark, and 66 µmol $m^{-1}$ $s^{-1}$ PPFD.

Plantlets (>40 mm in shoot height) were transferred to a sterile potting mixture of 1:9 river sand and pine bark (v $v^{-1}$) in 78 $cm^3$ pots, enveloped in transparent plastic bags, and kept in the greenhouse. After 1 week, holes were punched in the bags to reduce the humidity, and the bags were removed 1 week later.

### 2.4. Analyses of results

Cultures were in the growth room in a randomised configuration. The mean and standard error were

determined from the average of 15–25 replicates per treatment, repeated 3 times. Data were analysed using a one-way analysis of variance (ANOVA) and means were contrasted using Scheffe's multiple range test (95% confidence). The estimated final yields were calculated as follows:

(% bud break / 100) × no. buds produced per explant) × no. shoots / bud × multiplication rate at multiplication 1 × (% rooting / 100) × (90% acclimatisation / 100).

## 3. Results

### 3.1. Bud break

Contamination and phenolic production are problems often encountered when culturing woody species (George et al., 2008). However, both were negligible, with fewer than 10% of the explants being contaminated and less than 5% producing phenolics from the cut end of the explant.

Regarding the clones of the *E. grandis* × *nitens* (GN) hybrid, bud break Method 3 failed to elicit bud break in any of the tested clones. For the other 2 methods, bud break and shoot yields did not differ significantly amongst most of the clones of each hybrid. The exceptions were GN1 and GN108, which when subjected to Method 2 had significantly higher levels for % bud break than the other clones. Furthermore, for each method, the number of shoots produced per bud was not different amongst the clones of this hybrid. A comparison of the effect of the 3 methods on each clone indicated that Method 1 was the best choice for this hybrid: bud break values for GN9, GN15, and GN121 for Method 1 were significantly higher than with Methods 2 and 3, and for all 5 clones the highest shoot yields were obtained using Method 1.

There were no significant differences observed amongst the clones of the natural hybrid *E. nitens* × *nitens* (NH), other than higher bud break for NH0 with Methods 1 and 2. A comparison of the 3 methods for each clone indicated that Method 3 significantly decreased % bud break in NH0 and NH70, but shoot yield was not affected by the culture method.

Amongst the *E. grandis* × *urophylla* (GU clones), there were no significant differences for bud break and shoot yield with Methods 1 and 3. Similar results were obtained for Method 2, other than shoot yield being highest in GU244. In terms of the effect of the methods on each clone, there were no differences, except that Method 3 significantly decreased % bud break in GU151 and GU244 compared with the other methods.

A comparison amongst the 3 hybrids emphasised that the averages of % bud break of NH clones was significantly lower ($P \leq 0.05$) than for the other clones, which were not significantly different. This is due not only to the lower % bud break but also the clonal variation amongst NH clones (Table 1).

### 3.2. Multiplication and elongation

The main objective of the multiplication stage is to produce a high number of viable shoot propagules, arising from the action of cytokinins overcoming the dominance of the apical buds and promoting axillary bud shoot proliferation. For each hybrid, shoots were initiated from nodal explants using Method 1 (Table 1), and the objective was to establish shoot multiplication (Table 2) and elongation (Table 3) conditions suitable for all clones of each hybrid.

With the GN clones, the multiplication results for shoot yield reflected a measure of clonal specificity, with significantly different responses amongst the clones to each medium tested (Table 2). However, a comparison of the effect of all 4 multiplication media on each individual clone showed that all but GN1 responded best to M2. Similarly, with NH, M2 was the only medium for which shoot yields did not differ significantly amongst clones. However, M1 (medium with only cytokinin) was best for 2 (NH0 and NH70) out of the 3 clones tested for this hybrid. Clonal specificity was also recorded for all tested clones of GU concerning the effect of the multiplication media on shoot proliferation, except as a response to M3, where the yields were not significantly different. No significant differences were observed when comparing the effect of the 4 media on each clone, other than the significantly lower shoot yields for GU297 with M1.

The average shoot multiplication for all the clones of each hybrid showed no significant differences ($P \leq 0.05$) amongst hybrids except for the significantly lower values for GU with M1 and M2.

After multiplication, shoots were transferred to the same medium and the effect of a dark period (3 weeks of constant dark vs. 3 weeks of photoperiod) was investigated (Table 3). The dark treatment inhibited elongation but the shoots elongated adequately under the dark/light treatment, except for NH58. Other than for this clone, no significant differences were obtained for elongation amongst the clones.

### 3.3. Rooting and acclimatisation

Shoots of all clones were multiplied on M2 and elongated under a photoperiod prior to rooting on 4 different media (Table 4). As for the previous culture stages, the genotype affected the % rooting responses to each medium. There were significant differences in % rooting in media R2 and R3 and in media R1, R2, and R3 amongst clones of GN and GU, respectively, and in all media for NH clones. In terms of the effect of each of the 4 media on individual GN clones, only % rooting of GN9 and GN15 was significantly inhibited by R3. Similarly, R4 was the only medium that resulted in significantly low % rooting for NH58 and higher % rooting for GU21 and GU297. However, R4 was the medium that resulted in relatively high % rooting with

**Table 1.** Effect of bud break methods on % bud break and shoot yields from different clones of *E. grandis* × *nitens* (GN and NH) and *E. grandis* × *urophylla* (GU). Method 1 = nodal explants placed on bud break medium for 2 weeks, before being transferred to multiplication medium; Method 2 = nodal explants were placed directly on multiplication medium; Method 3 = excised buds were placed directly on multiplication medium.

| Hybrid | Clone | Method 1 | | Method 2 | | Method 3 | |
|---|---|---|---|---|---|---|---|
| | | % bud break | No. shoots/buds | % bud break | No. shoots/buds | % bud break | No. shoots/buds |
| *grandis* × *nitens* | GN1 | 90.3 ± 3.7$^{a}$ | 3 ± 0.1$^{a,*}$ | 85.5 ± 1.4$^{b}$ | 2 ± 0.1$^{a}$ | 0 | 0 |
| | GN9 | 90.3 ± 1.4$^{a,*}$ | 3 ± 0.2$^{a,*}$ | 79.8 ± 0.6$^{ab}$ | 1 ± 0.1$^{a}$ | 0 | 0 |
| | GN15 | 87.5 ± 2.4$^{a,*}$ | 2 ± 0.1$^{a,*}$ | 72.9 ± 3.2$^{ab}$ | 1 ± 0.2$^{a}$ | 0 | 0 |
| | GN108 | 88.9 ± 1.4$^{a}$ | 3 ± 1.0$^{a,*}$ | 82.6 ± 3.1$^{b}$ | 1 ± 0.1$^{a}$ | 0 | 0 |
| | GN121 | 90.3 ± 3.7$^{a,*}$ | 4 ± 0.3$^{a,*}$ | 67.1 ± 2.4$^{a}$ | 2 ± 0.2$^{a}$ | 0 | 0 |
| Average | | 89.5 ± 0.9 | 3 | 77.6 ± 5.7 | 1.4 | 0 | 0 |
| *grandis* × *nitens* (natural hybrid) | NH0 | 75.3 ± 9.6$^{b}$ | 2 ± 0.1$^{a}$ | 66.7 ± 1.4$^{b}$ | 2 ± 0.2$^{b}$ | 13.9 ± 5.6$^{a,*}$ | 2 ± 0.2$^{a}$ |
| | NH58 | 33.3 ± 4.2$^{a}$ | 1 ± 0.04$^{a}$ | 22.2 ± 0.6$^{a}$ | 1 ± 0.1$^{a}$ | 15.3 ± 1.4$^{a}$ | 1 ± 0.1$^{a}$ |
| | NH70 | 20.2 ± 8.1$^{a}$ | 2 ± 0.03$^{a}$ | 9.7 ± 5.0$^{a}$ | 1 ± 0.2$^{a}$ | 2.8 ± 2.8$^{a,*}$ | 1 ± 0.03$^{a}$ |
| Average | | 42.4 ± 17 | 1.6 | 32.8 ± 20 | 1.3 | 10.7 ± 4.8 | 1.3 |
| *grandis* × *urophylla* | GU21 | 97.0 ± 1.4$^{a}$ | 2 ± 0.3$^{a}$ | 91.6 ± 4.8$^{a}$ | 2 ± 0.4$^{a}$ | 73.7 ± 13$^{a}$ | 2 ± 0.03$^{a}$ |
| | GU151 | 84.3 ± 6.9$^{a}$ | 3 ± 0.8$^{a}$ | 80.6 ± 7.4$^{a}$ | 2 ± 0.1$^{a}$ | 55.5 ± 7.3$^{a,*}$ | 1 ± 0.08$^{a}$ |
| | GU244 | 90.6 ± 0.6$^{a}$ | 1 ± 0.2$^{a}$ | 91.7 ± 4.8$^{a}$ | 3 ± 0.4$^{b}$ | 59.7 ± 9.7$^{a,*}$ | 2 ± 0.5$^{a}$ |
| | GU297 | 93.9 ± 1.7$^{a}$ | 2 ± 0.3$^{a}$ | 86.6 ± 3.3$^{a}$ | 1 ± 0.2$^{a}$ | 81.7 ± 4.4$^{a}$ | 1 ± 0.03$^{a}$ |
| Average | | 91.5 ± 3.7 | 2 | 87.6 ± 3.5 | 2 | 67.7 ± 9.5 | 1.5 |

$^{a, b}$: Values represent mean ± standard error; mean values followed by different superscript letters within the same column within the same hybrid indicate significant differences amongst clones ($P \leq 0.05$).
*: Significant differences for the parameter amongst treatments for each clone (rows).

the least variation in percentage amongst the tested clones of each hybrid. This medium was developed for another clone of *E. grandis* × *nitens* by Mokotedi et al. (2000). All media resulted in root lengths of greater than 4 cm (results not shown), and on medium R4 the clones of GN, NH, and GU exhibited roots longer than 4 cm, 17 cm, and 28 cm, respectively. Acclimatisation success was greater than 90% in all cases and no significant differences were observed amongst clones (results not shown), as routinely observed in our laboratories.

### 3.4. Common protocol: plant yields and applications

The feasibility of applying the results of this study to a commercial situation has to be based on the predicted plantlet yield from a recommended protocol. In the present case, the conditions found to be suitable to both GN and GU clones are shown in the Figure and calculated yields in Table 5. The protocol involves sterilisation as per Watt et al. (2003a), which reduces contamination and phenolic production by the explants, followed by a short culture period (1–2 weeks) to induce the buds to develop (Figure). These are then transferred to a multiplication medium where the levels of sucrose and the cytokinin BAP are increased. After multiplication, the shoots are removed from the clumps, individually transferred to a medium containing auxins, and maintained for 3 weeks under the same photoperiod conditions as for the previous culture stages. Shoots are rooted on quarter-strength MS nutrients and decreased levels of sucrose, following a 3-step light regime: 72 h dark, then transferred to 16 h light/8 h dark at 37 μmol $m^{-1}$ $s^{-1}$ PPFD for the first 7 days, and then 66 μmol $m^{-1}$ $s^{-1}$ PPFD for 21 days (Figure). The estimates of yield (Table 5) were based on 100 nodal explants at culture initiation and data are presented in Tables 1, 2, and 4. In addition, 2 cycles in multiplication media were applied to the calculations, as this is a common practice in most laboratories (research and commercial), and 90% acclimatisation success was achieved for all clones.

## 4. Discussion

Many authors have found it necessary to induce bud break on a medium that is distinct from that used for multiplication (Le Roux and van Staden, 1991; Watt et al., 2003b; Arya et al., 2009; Borges et al., 2011), and the present results for % bud break of the clones of *E. grandis* × *nitens* (GN and NH) with Method 1 are consistent with those findings. For the GU clones tested in this study,

**Table 2.** Effect of multiplication media composition on number of shoots/bud from different clones of *E. grandis* × *nitens* (GN and NH) and *E. grandis* × *urophylla* (GU). M1 = 0.2 mg $L^{-1}$ BAP and 20 g $L^{-1}$ sucrose; M2 = 0.2 mg $L^{-1}$ BAP, 0.01 mg $L^{-1}$ NAA, and 25 g $L^{-1}$ sucrose; M3 = 0.5 mg $L^{-1}$ BAP, 0.2 mg $L^{-1}$ NAA, and 20 g $L^{-1}$ sucrose; M4 = 0.1 mg $L^{-1}$ BAP, 0.01 mg $L^{-1}$ NAA, 0.2 mg $L^{-1}$ kinetin, and 25 g $L^{-1}$ sucrose.

| Hybrid | Clone | No. shoots/buds | | | |
|---|---|---|---|---|---|
| | | M1 | M2 | M3 | M4 |
| *grandis* × *nitens* | GN1 | $8 \pm 1.1^{ab,*}$ | $4 \pm 1.0^{a}$ | $2 \pm 0.5^{a}$ | $3 \pm 0.7^{ab}$ |
| | GN9 | $12 \pm 2.1^{b,*}$ | $11 \pm 1.5^{b,*}$ | $4 \pm 0.2^{ab}$ | $6 \pm 0.6^{b}$ |
| | GN15 | $8 \pm 1.0^{ab}$ | $13 \pm 1.5^{b,*}$ | $4 \pm 0.2^{ab}$ | $6 \pm 0.8^{ab}$ |
| | GN108 | $6 \pm 1.3^{ab}$ | $11 \pm 0.5^{b,*}$ | $5 \pm 0.6^{b}$ | $5 \pm 0.4^{ab}$ |
| | GN121 | $4 \pm 0.6^{a}$ | $8 \pm 0.3^{ab,*}$ | $2 \pm 0.3^{a}$ | $2 \pm 0.4^{a}$ |
| Average | | $7.6 \pm 1.8$ | $9.4 \pm 2.6$ | $3.4 \pm 0.9$ | $4.4 \pm 1.2$ |
| *grandis* × *nitens* (natural hybrid) | NH0 | $21 \pm 3.8^{b,*}$ | $4 \pm 0.3^{a}$ | $5 \pm 0.5^{b}$ | $6 \pm 0.8^{b}$ |
| | NH58 | $4 \pm 0.4^{a}$ | $9 \pm 1.4^{a,*}$ | $2 \pm 0.1^{ab}$ | $3 \pm 0.3^{a}$ |
| | NH70 | $20 \pm 1.3^{b,*}$ | $9 \pm 2.5^{a}$ | $2 \pm 0.2^{b}$ | $7 \pm 1.4^{b}$ |
| Average | | $9 \pm 6.7$ | $7.3 \pm 2.1$ | $3 \pm 0.9$ | $5.3 \pm 1.3$ |
| *grandis* × *urophylla* | GU21 | $2 \pm 0.3^{a}$ | $3 \pm 0.1^{a}$ | $3 \pm 0.8^{a}$ | $4 \pm 0.3^{a}$ |
| | GU151 | $4 \pm 0.5^{ab}$ | $5 \pm 0.4^{b}$ | $4 \pm 0.2^{a}$ | $3 \pm 0.2^{a}$ |
| | GU244 | $5 \pm 0.8^{b}$ | $3 \pm 0.1^{a}$ | $5 \pm 0.3^{a}$ | $5 \pm 0.5^{ab}$ |
| | GU297 | $3 \pm 0.3^{ab,*}$ | $6 \pm 0.4^{b}$ | $5 \pm 1.3^{a}$ | $7 \pm 0.9^{b}$ |
| Average | | $3.5 \pm 0.8$ | $4.3 \pm 1.1$ | $4.3 \pm 0.6$ | $4.8 \pm 1.2$ |

[a, b]: Values represent mean ± standard error; mean values followed by different superscript letters within the same column within the same hybrid indicate significant differences amongst clones ($P \leq 0.05$).
*: Significant differences for the parameter amongst treatments for each clone (rows).

**Table 3.** Effect of light on shoot elongation from different clones of *E. grandis* × *nitens* (GN and NH) and *E. grandis* × *urophylla* (GU). Shoots were placed in constant dark or on a 16 h light/8 h dark photoperiod.

| Hybrid | Clone | Shoot length (mm) | | |
|---|---|---|---|---|
| | | Initial | Dark (3 weeks) | Photoperiod (3 weeks) |
| *grandis* ×*nitens* | GN1 | $4.9 \pm 0.1^{a}$ | $5.1 \pm 0.2^{a}$ | $40.3 \pm 1.5^{b,*}$ |
| | GN9 | $4.4 \pm 0.2^{a}$ | $4.7 \pm 0.2^{a}$ | $32.3 \pm 1.5^{b,*}$ |
| | GN15 | $5.6 \pm 0.3^{a}$ | $5.7 \pm 0.3^{a}$ | $28.6 \pm 1.4^{b,*}$ |
| | GN108 | $8.1 \pm 0.5^{a}$ | $8.5 \pm 0.2^{a}$ | $33.0 \pm 0.8^{b,*}$ |
| | GN121 | $8.1 \pm 0.6^{a}$ | $8.2 \pm 0.6^{a}$ | $35.1 \pm 326^{b,*}$ |
| Average | | $6.2 \pm 1.2$ | $6.4 \pm 1.2$ | $33.9 \pm 3.2$ |
| *grandis* × *nitens* (natural hybrid) | NH0 | $5.6 \pm 0.2^{a}$ | $7.7 \pm 0.3^{a}$ | $33.0 \pm 1.3^{b,*}$ |
| | NH58 | $5.3 \pm 0.2^{a}$ | $5.7 \pm 0.7^{a}$ | $7.3 \pm 0.3^{a}$ |
| | NH70 | $5.7 \pm 0.1^{a}$ | $6.0 \pm 0.6^{a}$ | $35.1 \pm 1.0^{b,*}$ |
| Average | | $5.5 \pm 0.1$ | $6.5 \pm 0.6$ | $25.1 \pm 9.8$ |
| *grandis* × *urophylla* | GU21 | $5.3 \pm 0.1^{a}$ | $6.4 \pm 0.5^{a}$ | $28.7 \pm 1.9^{b,*}$ |
| | GU151 | $5.4 \pm 0.2^{a}$ | $6.5 \pm 0.8^{a}$ | $30.8 \pm 0.8^{b,*}$ |
| | GU244 | $5.9 \pm 0.2^{a}$ | $6.4 \pm 0.7^{a}$ | $27.7 \pm 2.0^{b,*}$ |
| | GU297 | $5.8 \pm 0.03^{a}$ | $6.4 \pm 0.8^{a}$ | $35.3 \pm 2.3^{b,*}$ |
| Average | | $5.6 \pm 0.16$ | $6.4 \pm 0.02$ | $30.6 \pm 2.3$ |

[a, b]: Values represent mean ± standard error; mean values followed by different superscript letters within the same column within the same hybrid indicate significant differences amongst clones ($P \leq 0.05$).
*: Significant differences for the parameter amongst treatments for each clone (rows).

**Table 4.** Effect of rooting media composition on % rooting from different clones of *E. grandis* × *nitens* (NH and GN) and *E. grandis* × *urophylla* (GU). R1 = ½ MS salts and vitamins, 1 mg $L^{-1}$ IBA; R2 = ½ MS salts and vitamins, 0.1 mg $L^{-1}$ IBA; R3 = ½ MS salts and vitamins, 0.1 mg $L^{-1}$ IBA, 0.5 mg $L^{-1}$ NAA; R4 = ¼ MS, 0.1 mg $L^{-1}$ IBA, 0.22 g $L^{-1}$ $CaCl_2.2H_2O$, 0.185 g $L^{-1}$ $MgSO_4.7H_2O$.

| Hybrid | Clone | % Rooting | | | |
|---|---|---|---|---|---|
| | | R1 | R2 | R3 | R4 |
| *grandis* × *nitens* | GN1 | 66.7 ± 14.5$^{a}$ | 40.0 ± 5.8$^{a}$ | 43.3 ± 8.8$^{ab}$ | 63.3 ± 3.3$^{a}$ |
| | GN9 | 63.3 ± 8.8$^{a}$ | 60.0 ± 5.8$^{ab}$ | 16.7 ± 8.8$^{a,*}$ | 56.7 ± 12.0$^{a}$ |
| | GN15 | 86.7 ± 6.0$^{a}$ | 40.0 ± 5.8$^{a}$ | 6.7 ± 6.7$^{a,*}$ | 60.0 ± 10.0$^{a}$ |
| | GN108 | 70.0 ± 10.0$^{a}$ | 73.3 ± 8.8$^{b}$ | 80.3 ± 15.3$^{b}$ | 93.3 ± 6.7$^{a}$ |
| | GN121 | 76.7 ± 6.7$^{a}$ | 73.3 ± 8.8$^{b}$ | 76.7 ± 8.8$^{b}$ | 86.7 ± 6.7$^{a}$ |
| Average | | 72.3 ± 7.2 | 57.3 ± 11.7 | 44.7 ± 23.9 | 72 ± 10.9 |
| *grandis* × *nitens* (natural hybrid) | NH0 | 60.0 ± 15.3$^{b}$ | 46.7 ± 12.0$^{b}$ | 90.0 ± 5.7$^{b}$ | 73.3 ± 3.3$^{b}$ |
| | NH58 | 10.0 ± 5.8$^{a}$ | 13.3 ± 6.7$^{a}$ | 26.7 ± 8.8$^{a}$ | 36.7 ± 3.3$^{a,*}$ |
| | NH70 | 46.7 ± 3.3$^{ab}$ | 43.3 ± 3.3$^{b}$ | 60.0 ± 5.8$^{b}$ | 76.7 ± 3.0$^{b}$ |
| Average | | 38.9 ± 18.8 | 34.4 ± 13 | 58.9 ± 21.8 | 62.2 ± 16 |
| *grandis* × *urophylla* | GU21 | 13.3 ± 3.3$^{a}$ | 3.3 ± 3.3$^{a}$ | 23.3 ± 3.3$^{a}$ | 46.7 ± 8.8$^{b,*}$ |
| | GU151 | 63.3 ± 6.7$^{b}$ | 46.7 ± 3.3$^{b}$ | 36.7 ± 13.3$^{a}$ | 60.0 ± 20.8$^{a,b}$ |
| | GU244 | 76.7 ± 3.3$^{b}$ | 66.7 ± 12.0$^{bc}$ | 56.7 ± 3.3$^{a}$ | 76.7 ± 3.3$^{a}$ |
| | GU297 | 70.0 ± 5.8$^{b}$ | 90.0 ± 5.8$^{c,*}$ | 63.3 ± 8.8$^{a}$ | 96.7 ± 3.3$^{a,*}$ |
| Average | | 55.8 ± 21 | 51.7 ± 28.8 | 45 ± 11.8 | 70 ± 16.7 |

$^{a, b}$: Values represent mean ± standard error; mean values followed by different superscript letters within the same column within the same hybrid indicate significant differences amongst clones ($P \leq 0.05$).
*: Significant differences for the parameter amongst treatments for each clone (rows).

**Table 5.** Approximate estimated yields of viable propagules per 100 nodal explants after each culture and acclimatisation based on the recommended protocol for all clones (Figure).

| Hybrids and clones | No. propagules after: | | | | |
|---|---|---|---|---|---|
| | Bud break | Multiplication 1 | Multiplication 2 | Rooting | Acclimatisation |
| GN1 | 90 | 1080 | 4320 | 2722 | 2449 |
| GN9 | 90 | 990 | 10,890 | 6207 | 5587 |
| GN15 | 87 | 1131 | 14,703 | 8822 | 7940 |
| GN108 | 88 | 979 | 10,769 | 10,015 | 9014 |
| GN121 | 90 | 1440 | 11,520 | 10,022 | 9020 |
| | | | | | |
| NH0 | 75 | 600 | 2400 | 1752 | 1577 |
| NH58 | 33 | 297 | 2673 | 989 | 890 |
| NH70 | 20 | 180 | 1620 | 1247 | 1123 |
| | | | | | |
| GU21 | 92 | 819 | 2457 | 1155 | 1039 |
| GU151 | 80 | 800 | 4000 | 2400 | 2160 |
| GU244 | 91 | 819 | 2457 | 1892 | 1703 |
| GU297 | 86 | 516 | 3096 | 3003 | 2703 |

| | | |
|---|---|---|
| **Bud break**<br><br>1–2 weeks | **Method 1:** MS, 0.1 mg $l^{-1}$ biotin, 0.1 mg $l^{-1}$ calcium panthotenate, 0.04 mg $l^{-1}$ NAA, 0.11 mg $l^{-1}$ BAP, 0.05 mg $l^{-1}$ kinetin, 20 g $l^{-1}$ sucrose, 3.5 g $l^{-1}$ Gelrite<br><br>25 ± 2°C day / 21°C night, 16 h light / 8 h dark, 66 µmol $m^{-1}$ $sec^{-1}$ PPFD |  |
| **Multiplication**<br>6 weeks | **Medium 2:** MS, 0.1 mg $l^{-1}$ biotin, 0.1 mg $l^{-1}$ calcium panthotenate, 0.01 mg $l^{-1}$ NAA, 0.2 mg $l^{-1}$ BAP, 25 g $l^{-1}$ sucrose, 3.5 g $l^{-1}$ Gelrite<br><br>25 ± 2°C day / 21°C night, 16 h light / 8 h dark, 66 µmol $m^{-1}$ $sec^{-1}$ PPFD |  |
| **Elongation**<br>3 weeks | MS, 0.1 mg $l^{-1}$ biotin, 0.1 mg $l^{-1}$ calcium panthotenate, 0.35 mg $l^{-1}$ NAA, 0.1 mg $l^{-1}$ IBA, 0.1 mg $l^{-1}$ kinetin, 20 g $l^{-1}$ sucrose, 3.5 g $l^{-1}$ Gelrite<br><br>25 ± 2°C day / 21°C night, 16 h light / 8 h dark, 66 µmol $m^{-1}$ $sec^{-1}$ PPFD |  |
| **Rooting**<br>31 days | **Medium R4:** ¼ MS, 0.1 mg $l^{-1}$ biotin, 0.1 mg $l^{-1}$ calcium panthotenate, 0.1 mg $l^{-1}$ IBA, 0.22 g $l^{-1}$ $CaCl_2.2H_2O$, 0.185 g $l^{-1}$ $MgSO_4.7H_2O$, 15 g $l^{-1}$ sucrose, 3.5 g $l^{-1}$ Gelrite<br><br>72h dark (25°C), 7 days at 25 ± 2 °C day/21°C night, 16 h light / 8 h dark, 37 µM $m^{-1}$ $sec^{-1}$ PPFD, followed by 21 days at 25 ± 2°C day / 21°C night, 16 h light / 8 h dark, 66 µM $m^{-1}$ $sec^{-1}$ PPFD |  |

**Figure.** Suggested protocol for clones of *E. grandis* × *nitens* and *E. grandis* × *urophylla*.

it is possible to reduce costs and time associated with 2 distinct stages by using Method 2 (one stage for both bud break and multiplication). Such an approach has been successful with *E. regnans* (Donald and Newton, 1991) and *E. radiata* (Blomstedt et al., 1998). In addition to the observed low yields for certain clones, Method 3 was deemed not practical for a commercial setting due to the labour involved in excising buds. Bud-break yields similar to those achieved in this study with Method 1, within the range of 84%–97% for GN and GU, compare favourably to those reported in the reviews for a variety of *Eucalyptus* spp. and hybrids (reviews by Le Roux and van Staden, 1991; Watt et al., 2003b), and to the 83% bud break reported more recently for 2 hybrids of *E. camaldulensis* × *tereticornis* (Arya et al., 2009). The poor results with 2 of the NH clones may be due to the fact that they are the progeny of 'natural' hybrids and, unlike the GN and GU clones, did not go through clonal selection.

The tested multiplication media did not bring about significant differences amongst the clones, indicating that this is not the limiting stage in the establishment of a common protocol. However, at the end of this stage not all shoots elongated sufficiently to allow rooting. In contrast to reports by various authors working with tree species (Puddephat et al., 1997), including eucalyptus (Fantini and Cortezzi-Graca, 1989), shoots did not elongate in response to etiolation under dark conditions. They did, however, elongate adequately under the photoperiod and, except for

NH58, there were no genotypic differences amongst the clones. The recalcitrance exhibited by this clone to culture manipulations was also observed during the rooting stage. For all other tested clones, rooting results compare favourably to those reported for *E. grandis* × *urophylla* (Jones and van Staden, 1994) and *E. nitens* × *nitens* clones (Mokotedi et al., 2000).

It is clear from the results that a common protocol cannot be used for all clones of the tested hybrids: clone NH58 showed throughout that it is recalcitrant to all of the tested culture conditions, whereas clones GN1 and GU21 seem to require a clone-specific multiplication and rooting medium, respectively. In all other cases, the estimated yields of the clones for each hybrid are comparable, but they are not sufficiently high for the protocol to be used for the production of planting units for commercial plantations. For such an application, the use of a temporary immersion system, which results in 4.9 to 7.3 times (depending on hybrid) the yield obtained with a semisolid substrate, is advised (MacAllister et al., 2005). However, managers of commercial tissue culture laboratories have to make decisions as to costs and time in order to meet the demands of their customers. In the case of eucalyptus, when new clones are brought into production, it is suggested that they could be first tested on a common protocol, such as that presented in the Figure. The managers can then decide the strategy to be employed, whether to initiate the necessary number of explants to meet the demand or to embark on lengthy research to optimise the culture stage(s).

Many commercial forestry enterprises use micropropagation primarily for the establishment of macro- and hydroponic hedges to supply macro- and mini-cuttings, respectively; this information is based on personal knowledge as it is not often reported in the scientific literature, one exception being the review by de Assis et al. (2004). That choice is based on the observations that cuttings from micropropagated eucalyptus exhibit higher percent rooting and, in some cases, produce more shoots than conventional adult sources (Watt et al., 1997; de Assis et al., 2004). As for the purpose of clonal hedges, it is not necessary to produce very large number of plants; it is more practical to employ the same, rather than specific, protocols for a variety of clones. For the same reason, another potential application for the nonclone-specific plant regeneration protocol is for the bulking-up of vegetative germplasm material (in this case axillary buds) stored under minimal growth conditions or cryopreserved.

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