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In vitro propagation of *Corymbia torelliana* × *C. citriodora* (Myrtaceae) via cytokinin-free node culture

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Abstract. Hybrids between *Corymbia torelliana* (F.Muell.) K.D.Hill & L.A.S.Johnson and *C. citriodora* subsp. *variegata* (F.Muell.) A.R.Bean & M.W.McDonald are used extensively to establish forestry plantations in subtropical Australia. Methods were developed for *in vitro* seed germination, shoot multiplication and plantlet formation that could be used to establish *in vitro* and *ex vitro* clone banks of juvenile *Corymbia* hybrids. Effects of sodium hypochlorite concentration and exposure time on seed contamination and germination, and effects of cytokinin and auxin concentrations on shoot multiplication and subsequent rooting, were assessed. A two-step surface sterilisation procedure, involving 70% ethanol followed by 1% sodium hypochlorite, provided almost no contamination and at least 88% germination. A novel method of cytokinin-free node culture proved most effective for *in vitro* propagation. Lateral bud break of primary shoots was difficult to induce by using cytokinin, but primary shoots rooted prolifically, elongated rapidly and produced multiple nodes in the absence of exogenous cytokinin. Further multiplication was obtained either by elongating lateral shoots of nodal explants in cytokinin-free medium or by inducing organogenic callus and axillary shoot proliferation with 2.2 μM benzyladenine. Plantlets were produced using an *in vitro* soil-less method that provided extensive rooting in sterile propagation mixture. These methods provide a means for simultaneous laboratory storage and field-testing of clones before selection and multiplication of desired genotypes.

Introduction

Eucalypts are the world's most widely planted hardwood trees because of their large number of species, wide adaptability to soils and climate, fast growth rates and variety of wood and non-wood products (Campinhos 1999; Turnbull 1999). Eucalypts include three genera, *Eucalyptus*, *Corymbia* and *Angophora* (Hill and Johnson 1995; Ladiges and Udovicic 2000), of which the first two provide important species for forestry and oil production. *Corymbia* species form a major component of the flora of tropical northern Australia and are a significant component in parts of subtropical eastern Australia. Species such as *C. torelliana* and *C. citriodora* are increasingly important sources of germplasm as plantation forestry expands into marginal production regions of the tropics and subtropics (Campinhos 1999; Lee *et al*. 2001; Dickinson *et al*. 2004). Hybrids of these two species are used extensively for plantation establishment in subtropical Australia because they combine frost and disease tolerance from *C. torelliana* with the excellent wood properties of *C. citriodora* (Lee 2007).

Clonal propagation is used to maximise growth and decrease variability of plantation trees, following selection of individual genotypes in field tests. Clonal propagation can also overcome seed supply limitations for species such as *C. citriodora* that flower irregularly or for hybrids such as *C. torelliana* × *C. citriodora* that are produced by hand-pollination. A major disadvantage of clonal propagation is that the effects of tree maturation can erode genetic gains made by clonal selection. By the time that elite individuals have been selected in field tests, their cuttings may demonstrate diminished rooting ability and field performance compared with cuttings from seedlings of the same pedigree (Greenwood and Hutchison 1993). A clonal selection program ideally incorporates a method for long-term storage of clones in their juvenile state while clonal testing and selection occurs in the field (Aimers-Halliday and Burdon 2003; Mitchell *et al*. 2004; Trueman 2006).

This study aimed to develop methods for (1) *in vitro* germination and shoot multiplication to establish a laboratory archive of juvenile *C. torelliana* × *C. citriodora* clones, and (2) root induction to generate plantlets of the same clones. Very high proliferation rates are not necessary to establish an *ex vitro* clone bank of *Corymbia* hybrids because only one nursery mother plant of each clone is needed to produce sufficient rooted cuttings for field tests. Therefore, the objective was to develop methods for shoot culture and axillary shoot production (Blomstedt *et al*. 1991; Niccol *et al*. 1994; Gomes and Canhoto 2003) rather than shoot regeneration via an intervening callus phase. Indirect organogenesis via callus also poses a greater risk for releasing or inducing somaclonal variation (George 1993), which is highly undesirable in a clonal selection program.

Establishment of *C. citriodora* seedling cultures has been described briefly by Lakshmi Sita and Vaidyanathan (1979), Gupta *et al*. (1981) and Rojas Caballero *et al*. (1993), but only Koriesh *et al*. (2003) provided data comparing a range of culture media. Methods for culture establishment of *C. torelliana* or its hybrids with *C. citriodora* have only been described for explants from adult trees (Gupta *et al*. 1983; Kapoor and Chauhan 1992; Bisht *et al*. 2002). The current study presents the results of large factorial experiments assessing surface-sterilisation methods for

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C. torelliana × *C. citriodora* seed, and effects of benzyladenine (BA) and naphthalene acetic acid (NAA) concentrations on subsequent shoot multiplication. Rooting of these shoots was then assessed in an *in vitro* soil-less (IVS) system developed by Newell *et al*. (2003, 2005).

Materials and methods

Plant material

Full-sibling seeds of *Corymbia torelliana* (F.Muell.) K.D.Hill & L.A.S.Johnson × *C. citriodora* subsp. *variegata* (F.Muell.) A.R.Bean & M.W.McDonald were obtained from the Hardwood Tree Improvement Group, Department of Primary Industries and Fisheries, Queensland. Three hundred seeds were counted from each of three families that were produced by controlled pollination of individual trees: 1CT2–013 × 1CV2–109 ('13'), 1CT2–016 × 1CV2–105 ('16') and 1CT2–019 × 1CV2–114 ('19'). Mean weights per seed were 1.72, 1.74 and 1.91 mg for families 13, 16 and 19, respectively.

Sterilisation method

Batches of 60 seeds were washed in 70% ethanol (v/v) for 1 min in 70-mL vials containing one drop of Tween 20, rinsed in sterile distilled water for 1 min, and then separated into groups of five seeds that were transferred to 12 separate vials containing one of the sterilisation treatment solutions. Seeds were placed on sterile paper to remove excess liquid between solutions. Twelve treatments were arranged in a factorial combination, with three levels of sodium hypochlorite (1, 3 or 5% w/v) and four exposure times (5, 10, 20 or 30 min). Treatments were applied by swirling the vials, each containing one drop of Tween 20, on an orbital shaker at 110 rpm. After three subsequent rinses in sterile distilled water, seeds were plated (5 seeds per 90-mm petri dish) onto germination medium consisting of half-strength MS basal salts (Murashige and Skoog 1962) and 20 g L^{-1} sucrose, solidified with 8 g L^{-1} agar, with pH adjusted to 5.8 before autoclaving (121°C, 20 min). This procedure was repeated four times for each family to obtain five replicates per family.

Seeds were germinated under continuous light (approximately 30 μmol m^{-2} s^{-1} by fluorescent tubes) at 25°C (Boland *et al*. 1980; Wallace and Trueman 1995). Seed contamination was assessed daily, and germination and shoot length (root collar to shoot apex) were measured after 2 weeks. At this stage, all upright shoots >5-mm length were transferred to shoot induction media.

Shoot induction (first passage)

Shoots were dissected just above the root collar and transferred to 375-mL glass jars containing 50 mL of shoot induction medium consisting of half-strength MS medium with 30 g L^{-1} sucrose, solidified with 8 g L^{-1} agar, and pH adjusted to 5.8 before autoclaving (121°C, 20 min). Shoots from within each of the three families were transferred randomly across the twelve shoot induction treatments. Treatments contained one of four levels of BA (0, 0.9, 2.2 or 4.4 μM) and one of three levels of NAA (0, 0.05 or 0.27 μM). The number of replicate shoots per treatment was 16–17, 23–24 and 22–23 for families 13, 16 and 19, respectively.

Glass jars were placed under continuous light (approx. 70 μmol m^{-2} s^{-1}) at 25°C. Length of the main shoot, the numbers of nodes, macroscopic lateral shoots and primary roots (i.e. roots arising directly from the shoot) were recorded after 4 weeks. Leaves were usually arranged in an opposite fashion on the main shoot but, where leaf pairs were slightly alternate, they were still counted as a single node. The enclosed shoot apex with several young leaves was also counted as a single node. Each node, and any lateral shoot >1-cm length, was dissected for transfer to multiplication medium.

Shoot multiplication (second and third passages)

Dissected shoots were transferred to new glass jars containing the same medium used for shoot induction, but with one of seven different hormone combinations (0.4, 0.9 or 2.2 μM BA × 0 or 0.05 μM NAA, plus a hormone-free medium). All dissected shoots from the one seedling (i.e. one clone) were placed together in one jar. The 16–24 clones per family from within each of the shoot induction treatments were randomly transferred across the seven multiplication treatments. The number of clones (i.e. number of jars) for each of the 84 induction × multiplication treatment combinations was therefore, 2–3, 3–4 and 3–4 for families 13, 16 and 19, respectively. Length of the longest shoot, number of shoots with roots and number of macroscopic lateral shoots per jar were recorded after 4 weeks (second passage).

At this stage, the shoots in each jar (i.e. all the shoots of one clone) were dissected and transferred to fresh multiplication medium containing the same concentration of hormones. In addition a random subsample of shoots from each clone was transferred to a long-term storage experiment, and so final values for shoot production for the current experiment were extrapolated using the formula

$$S = S_A \times (S_S + S_R)/S_R,$$

where S is the final number of shoots that would have been produced for that clone, S_A was the actual number of shoots produced, S_S was the number of shoots subsampled for transfer to the other experiment, and S_R was the number of shoots retained for the current experiment. After 5 weeks in the second jar of multiplication medium (third passage), length of the longest shoot in each jar and the number of shoots with roots were recorded. At this stage, shoots were only dissected for root induction by removing roots or basal callus, except that long shoots (>50-mm length) were also divided into segments of at least 25-mm length. The number of shoots available for transfer to root induction medium was then counted (corresponding to S_A, above).

Root induction

Shoots were transferred to new jars containing half-strength MS shoot induction medium supplemented with 4.9 μM indolebutyric acid (IBA). Jars were placed in darkness for 3 days at 20°C. Shoots were then transferred to a sterile propagation mixture based upon the IVS method of Newell *et al*. (2003, 2005). The current IVS mixture comprised equal volumes of perlite and shredded pine bark, which was watered to capacity and dispensed into propagation trays containing fifteen 12-mL propagation tubes. Propagation trays were placed in covered 1-L plastic take-away food containers and autoclaved at 121°C for 40 min. Upon transfer of shoots to IVS medium, the container lid was replaced with another container and sealed with plastic film, creating a volume of approximately 2 L. Containers were

placed under continuous light (approximately 70 μmol m^{-2} s^{-1}) at 25°C for 4 weeks, at which time all shoots were extracted to determine the number of shoots from each clone that had formed roots. Because a subsample of shoots from each clone had been transferred to a separate experiment, final values for number of shoots with roots for each clone were extrapolated using the formula outlined above for shoot multiplication.

Statistical analyses

Germination, transfer, and mean shoot length at 2 weeks after plating were analysed by 2-way ANOVA (concentration × exposure time) for each family because significant family × treatment interactions were detected by 3-way ANOVA (family × concentration × exposure time). Proportions were arcsine square root transformed when data was not normally distributed.

Shoot length, and numbers of nodes, lateral shoots, primary roots and transferred shoots after 4 weeks on shoot induction medium were analysed by 1-way ANOVA for each family because significant family × medium interactions were detected by 2-way ANOVA. Length of the longest shoot, number of lateral shoots, and number of shoots transferred after 4 weeks or 9 weeks on multiplication medium, as well as the proportion and number of shoots with roots for each clone after 4 weeks in IVS mixture, were analysed by 2-way ANOVA (shoot induction treatment × multiplication treatment) for each family because significant family × treatment interactions were detected by 3-way ANOVA. One-way ANOVAs were conducted where significant induction × multiplication treatment interactions were identified. Square root or log-transformations were applied when data was not normally distributed.

Post hoc least significant difference (l.s.d.) tests (for 3 means) or Duncan's multiple range tests (for 7 or 12 means) were applied if significant differences were detected by ANOVA. Means are reported with standard errors, and treatment differences or interactions were regarded as significant at $P < 0.05$.

Results

Sterilisation method

All sterilisation treatments were highly effective, with only 1 of 900 seeds contaminated during 2 weeks on germination medium. The contaminated seed had been treated with the lowest concentration of sodium hypochlorite (1%) for the shortest exposure period (5 min). Seed germination, percentage of seeds with shoots transferred to shoot induction medium, and shoot length were not significantly affected by period of exposure to sodium hypochlorite (5–30 min), nor was there a significant exposure period × concentration effect, for any family (data not shown). However, in family 13, increasing the sodium hypochlorite concentration from 1 to 3 or 5% significantly decreased germination from 88 ± 3% to 74 ± 4% and 64 ± 5%, and the percentage of seeds with shoots transferred to induction medium from 78 ± 4% to 65 ± 5% and 52 ± 5%, respectively (Fig. 1). For the other two families, the percentage of seeds with shoots transferred to the first passage was at least 86 ± 4% and 83 ± 6%. Shoot length was not affected by sodium hypochlorite concentration in any family (Fig. 1).

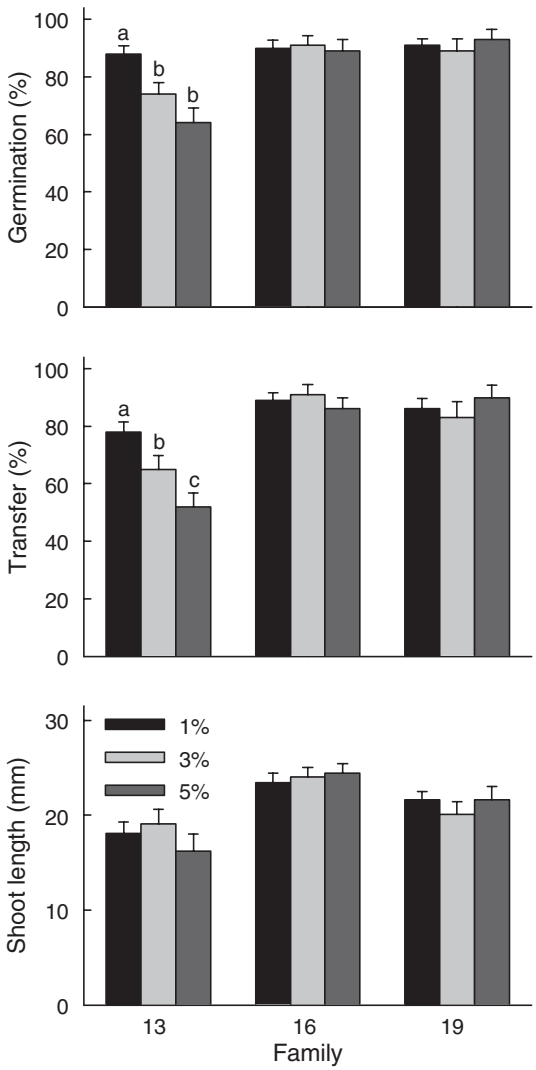

Fig. 1. Effect of sodium hypochlorite concentration (1, 3 or 5%) on seed germination, percentage of plated seeds with shoots transferred to shoot induction medium, and shoot length after 2 weeks on germination medium for three families of *Corymbia torelliana* × *C. citriodora*. Means (± s.e.) with different letters are significantly different ($P < 0.05$, ANOVA and least significant difference test, $n = 20$ dishes).

Shoot induction (first passage)

Shoots placed for 4 weeks on induction media devoid of BA were approximately twice as long and with twice the number of nodes as shoots on induction media containing BA (Table 1). Addition of BA, in most cases, stimulated production of lateral shoots, but BA completely suppressed root formation (Fig. 2*A*). Rooting occurred on at least 87% of shoots in BA-free media, except that only 57% of family 19 shoots formed roots when the medium was also devoid of NAA (data not presented). Addition of NAA to BA-free medium significantly increased the number of primary roots in families 16 and 19 (Table 1).

Lateral shoots were generally small (<1-cm length), and so they were usually not separated from the main shoot when seedlings were dissected for transfer. As a result, the number of shoots transferred to multiplication medium was closely related

Table 1. Shoot length, and numbers of nodes, lateral shoots and primary roots following a 4-week exposure of *Corymbia torelliana* × *C. citriodora* seedling shoots to 1 of 12 shoot induction media

For each family, means (± s.e.) within columns followed by different letters are significantly different ($P < 0.05$, ANOVA and Duncan's multiple range test (shoot length, number of nodes, number of lateral shoots), ANOVA and least significant difference test (number of roots)). '–' indicates roots did not form in these treatments

BA (μM)	NAA (μM)	Shoot length (mm)	Number of nodes	Number of lateral shoots	Number of primary roots
Family 13 (n = 16–17 shoots)					
0	0	55.4 ± 4.6a	4.7 ± 0.3a	0.6 ± 0.3ab	2.4 ± 0.2a
0	0.05	54.6 ± 3.4a	5.1 ± 0.2a	0.2 ± 0.1a	2.6 ± 0.2a
0	0.27	57.5 ± 4.6a	5.1 ± 0.4a	0.6 ± 0.2abc	2.5 ± 0.2a
0.9	0	28.8 ± 2.2b	2.4 ± 0.2b	1.4 ± 0.3cde	–
0.9	0.05	30.4 ± 2.1b	2.6 ± 0.2b	1.4 ± 0.2de	–
0.9	0.27	27.8 ± 2.0b	2.4 ± 0.2b	1.1 ± 0.3bcd	–
2.2	0	29.3 ± 2.2b	2.4 ± 0.2b	3.1 ± 0.5h	–
2.2	0.05	30.3 ± 2.3b	2.0 ± 0.2b	2.5 ± 0.5gh	–
2.2	0.27	25.9 ± 1.7bc	1.9 ± 0.2b	2.3 ± 0.3fg	–
4.4	0	25.5 ± 1.7bc	2.0 ± 0.1b	1.7 ± 0.2def	–
4.4	0.05	27.4 ± 2.0bc	1.6 ± 0.1b	1.9 ± 0.4efg	–
4.4	0.27	20.8 ± 2.0c	1.9 ± 0.2b	1.6 ± 0.3def	–
Family 16 (n = 23–24 shoots)					
0	0	45.7 ± 3.2a	4.1 ± 0.2a	0.2 ± 0.1a	1.3 ± 0.2a
0	0.05	54.0 ± 3.7b	4.1 ± 0.3a	0.2 ± 0.1a	1.8 ± 0.2ab
0	0.27	47.3 ± 2.9a	4.2 ± 0.2a	0.3 ± 0.1a	2.1 ± 0.2b
0.9	0	28.5 ± 1.6c	1.7 ± 0.1b	1.5 ± 0.3b	–
0.9	0.05	27.3 ± 1.6c	2.5 ± 0.1d	1.4 ± 0.2b	–
0.9	0.27	25.6 ± 1.5c	2.4 ± 0.2d	0.7 ± 0.2a	–
2.2	0	28.3 ± 1.7c	2.3 ± 0.2d	1.7 ± 0.3b	–
2.2	0.05	28.5 ± 2.3c	2.2 ± 0.1cd	1.5 ± 0.2b	–
2.2	0.27	25.7 ± 1.1c	1.7 ± 0.1b	1.3 ± 0.2b	–
4.4	0	29.8 ± 1.4c	2.4 ± 0.1d	1.6 ± 0.3b	–
4.4	0.05	31.2 ± 2.0c	1.7 ± 0.1b	1.4 ± 0.2b	–
4.4	0.27	31.7 ± 1.6c	1.8 ± 0.1bc	1.8 ± 0.2b	–
Family 19 (n = 22–23 shoots)					
0	0	52.1 ± 4.6a	4.1 ± 0.4a	1.0 ± 0.5a	0.9 ± 0.2a
0	0.05	70.5 ± 4.3b	5.6 ± 0.3b	0.3 ± 0.2a	2.1 ± 0.2b
0	0.27	65.8 ± 3.7b	5.6 ± 0.2b	0.4 ± 0.2a	2.1 ± 0.2b
0.9	0	33.7 ± 2.0d	2.7 ± 0.2cd	2.1 ± 0.2b	–
0.9	0.05	42.8 ± 1.4c	3.2 ± 0.2c	2.0 ± 0.3b	–
0.9	0.27	29.0 ± 1.5d	2.6 ± 0.2cd	2.9 ± 0.4b	–
2.2	0	33.2 ± 1.6d	2.4 ± 0.1d	2.4 ± 0.2b	–
2.2	0.05	35.5 ± 1.9d	2.9 ± 0.2cd	2.1 ± 0.1b	–
2.2	0.27	34.7 ± 2.0d	2.8 ± 0.2cd	2.9 ± 0.4b	–
4.4	0	33.0 ± 1.3d	2.5 ± 0.1d	2.7 ± 0.3b	–
4.4	0.05	32.6 ± 1.8d	2.4 ± 0.1d	2.1 ± 0.2b	–
4.4	0.27	35.0 ± 2.2d	2.6 ± 0.2cd	2.6 ± 0.3b	–

to the number of nodes per seedling rather than the sum of nodes plus lateral shoots. Significantly more shoots were transferred from media devoid of BA than from any medium containing BA (Fig. 3). Addition of NAA did not significantly affect shoot number, except when it was added to BA-free medium for family 19 (Fig. 3). In this family, NAA had significantly increased root formation and node production in BA-free medium (Table 1).

Shoot multiplication: second passage

Shoots placed for 4 weeks on BA-free multiplication medium were much longer, but with fewer lateral shoots, than shoots cultured in the presence of BA (Table 2). Shoot induction × multiplication treatment interactions were not significant, and so means for each multiplication medium were pooled across the 12 induction media. Root formation only occurred when shoots were cultured in BA-free multiplication medium, and the percentage of these shoots forming roots was independent of their induction treatment. The number of shoots available for transfer to the third passage was also highest when shoots were cultured on BA-free multiplication medium (Fig. 4). There was no significant difference in shoot numbers among other treatments, except that 0.4 μM BA provided higher shoot numbers than 2.2 μM BA in the absence of NAA for family 19.

Positive carry-over effects from BA-free induction media (first passage) were observed on length of the longest shoot and number of shoots transferred to the third passage. For example,

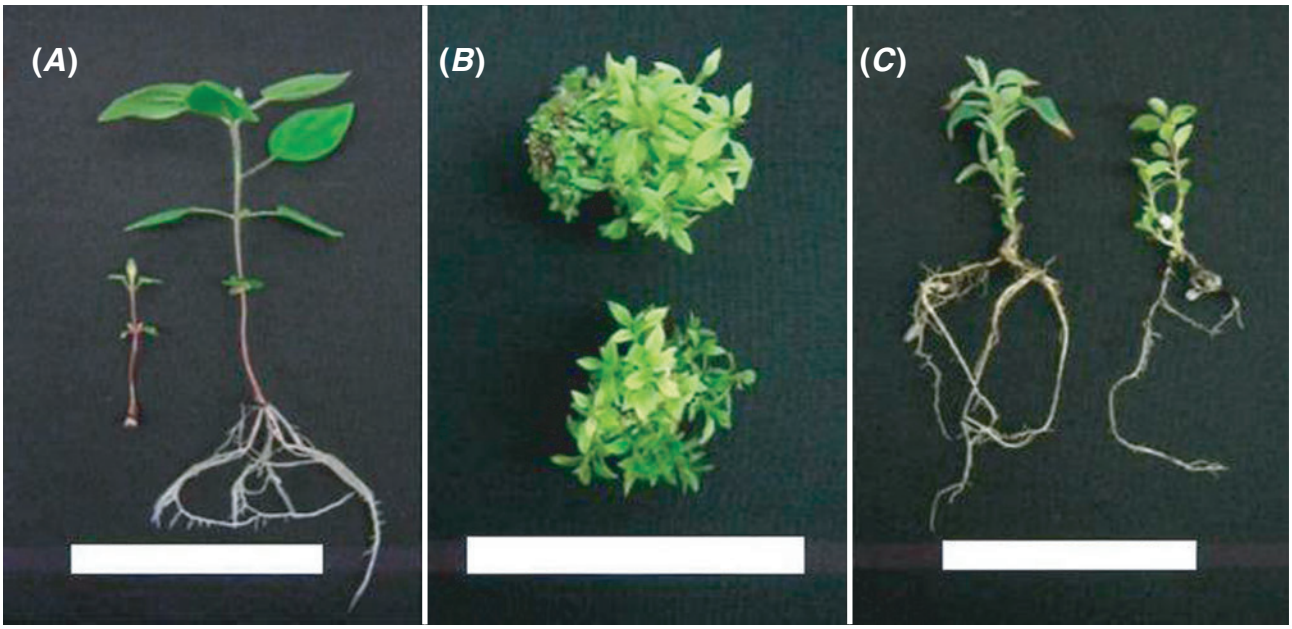

Fig. 2. (*A*) Seedling shoots of *Corymbia torelliana* × *C. citriodora* after 4 weeks in induction medium containing 2.2 μM BA (left) or nil BA (right). (*B*) Shoot clumps arising by organogenesis and axillary shoot proliferation after 9 weeks in multiplication medium containing 2.2 μM BA. (*C*) Root production on shoots obtained from multiplication medium containing 0.9 μM BA (left) or 2.2 μM BA (right) after 4 weeks in *in vitro* soil-less (IVS) mixture. Scale bars = 50 mm.

longest shoots for induction media containing 0 *v.* 4.4 μM BA with 0.27 μM NAA were 31.5 ± 5.4 *v.* 16.6 ± 2.7 mm, 23.6 ± 3.1 *v.* 14.9 ± 3.3 mm, and 24.8 ± 3.1 *v.* 16.7 ± 2.5 mm for families 13, 16 and 19, respectively (other data not presented). Consequently, shoot numbers for the same induction media were 15.0 ± 1.8 *v.* 2.9 ± 0.4, 6.6 ± 0.9 *v.* 2.5 ± 0.5, and 12.0 ± 1.3 *v.* 4.2 ± 0.7, respectively.

Shoot multiplication: third passage

After 9 weeks on multiplication medium, longest shoot lengths from families 13 and 16 remained much greater in the absence of BA than in media containing BA (Table 3). Induction × multiplication treatment interactions were not significant for these two families, and so means for each multiplication medium were pooled across the 12 induction media. For family 19, longest shoot lengths from multiplication medium lacking BA were not significantly greater than those from media containing BA, except in the case of shoots emanating from the 0.9 μM BA + 0.05 μM NAA shoot induction treatment. Root formation continued to occur when shoots were cultured without BA (Table 3) but the percentage forming roots had declined since the second passage (cf. Table 2).

Shoots were only divided at this stage if all subsequent shoots would be >25-mm length or to remove basal callus, which meant that only the very longest shoots were divided into two or more segments. The number of shoots transferred to root induction medium was therefore, in many cases, only slightly higher than the number transferred 5 weeks earlier (Fig. 5 cf. Fig. 4). Induction × multiplication treatment interactions were not significant for shoot number in any family.

There remained a positive carry-over effect from the BA-free induction media (first passage) on length of the longest shoot for families 13 and 16, and on shoot number in all families, at the end of the third passage. For example, mean longest shoots for induction media containing 0 *v.* 4.4 μM BA with 0.27 μM NAA were 23.7 ± 4.7 *v.* 12.0 ± 2.5 mm, and 17.7 ± 3.4 *v.* 12.3 ± 4.6 mm, for families 13 and 16, respectively. Shoot numbers per clone for the same induction media were 23.1 ± 5.0 *v.* 6.1 ± 1.9, 7.7 ± 1.1 *v.* 2.4 ± 0.6, and 22.8 ± 3.6 *v.* 7.5 ± 1.6 for families 13, 16 and 19, respectively (other data not shown).

Organogenic callus developed, usually in combination with axillary shoot proliferation, at the base of some shoots in families 13 and 19 particularly in the presence of 2.2 μM BA (Fig. 2*B*). Removal of callus provided large numbers of new shoots for these clones. The highest number of shoots for a clone produced on BA-free multiplication medium was 27, but some clones that formed organogenic callus had higher shoot numbers. Percentages of clones that formed >27 shoots in 2.2 μM BA were 15% and 13% (without NAA) and 7% and 32% (0.05 μM NAA) for families 13 and 19, respectively. The majority (61%) of these clones had emanated from shoot induction media devoid of BA. The highest number of shoots produced from a clone that formed organogenic callus was 168 but, because of high variability in the organogenic response, only the medium containing 0.05 μM NAA and 2.2 μM BA for family 19 provided a significantly higher shoot number per clone than hormone-free medium (Fig. 5). The highest mean differed significantly from the lowest 4, 3 and 3 means for families 13, 16 and 19, respectively, but other means did not differ significantly (Fig. 5).

Root induction

Plantlets arising from the IVS system possessed extensive root systems that often protruded well beyond the base of the 12-mL propagation tubes (Fig. 2*C*). This was particularly

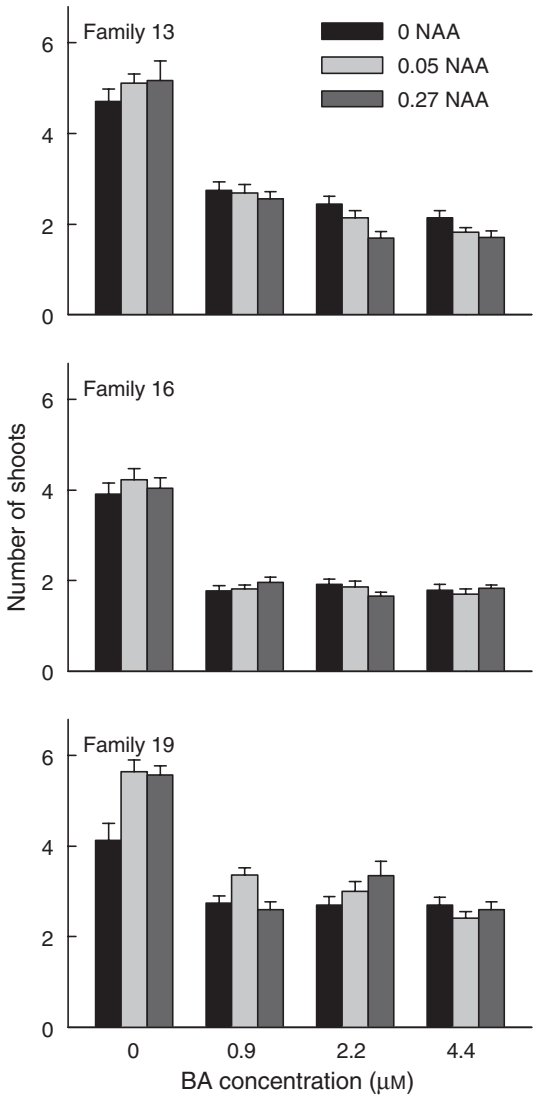

Fig. 3. Effect of BA and NAA concentrations on number of shoots transferred per *Corymbia torelliana* × *C. citriodora* seedling after 4 weeks on shoot induction medium. Means are provided with s.e. ($n = 16$–24 seedlings).

Table 2. Length of the longest shoot, number of lateral shoots per plantlet, and percentage of shoots with roots following a 4-week exposure of *Corymbia torelliana* × *C. citriodora* seedling shoots to one of seven shoot multiplication media

For each family, means (± s.e.) within columns followed by different letters are significantly different ($P < 0.05$, ANOVA and Duncan's multiple range test). '–' indicates roots did not form in these treatments

BA (μM)	NAA (μM)	Length of longest shoot (mm)	Number of lateral shoots per plantlet	Shoots with roots (%)
Family 13 (n = 27–28 shoots)				
0	0	52.0 ± 3.5a	0.8 ± 0.1a	70.2 ± 5.5
0.4	0	19.4 ± 1.1b	1.6 ± 0.2b	–
0.4	0.05	18.5 ± 1.2bc	1.7 ± 0.3b	–
0.9	0	14.8 ± 0.8cd	2.1 ± 0.3b	–
0.9	0.05	17.8 ± 1.1bc	1.8 ± 0.2b	–
2.2	0	13.5 ± 0.9d	3.3 ± 0.6c	–
2.2	0.05	13.5 ± 1.0d	2.1 ± 0.2bc	–
Family 16 (n = 38–39 shoots)				
0	0	41.6 ± 4.2a	0.6 ± 0.1a	46.3 ± 6.5
0.4	0	13.9 ± 1.1b	1.4 ± 0.2bc	–
0.4	0.05	15.5 ± 1.1b	1.3 ± 0.2b	–
0.9	0	13.6 ± 0.8b	1.6 ± 0.2bcd	–
0.9	0.05	14.9 ± 0.8b	1.7 ± 0.2bcd	–
2.2	0	12.6 ± 1.0b	1.9 ± 0.2cd	–
2.2	0.05	12.4 ± 0.9b	2.3 ± 0.3d	–
Family 19 (n = 38–39 shoots)				
0	0	46.8 ± 4.2a	1.1 ± 0.2a	35.8 ± 5.5
0.4	0	18.0 ± 1.0b	1.9 ± 0.2b	–
0.4	0.05	17.6 ± 0.9b	1.8 ± 0.1b	–
0.9	0	15.4 ± 1.1bc	2.2 ± 0.1bc	–
0.9	0.05	15.7 ± 1.0bc	2.2 ± 0.2bc	–
2.2	0	13.4 ± 0.6c	2.5 ± 0.2c	–
2.2	0.05	13.7 ± 0.6c	3.0 ± 0.5c	–

the case for the longest shoots, many of which had been raised in BA-free multiplication medium. Significant shoot induction × multiplication treatment interactions were found for percentage of shoots per clone from families 13 and 16 that formed roots. Multiplication treatment means are therefore, provided only as a general indication of rooting success for these families (Table 4). For family 19, induction × multiplication treatment interactions were not significant, and there was no carry-over effect from the induction medium on rooting percentages. Relatively few shoots of this family produced roots in IVS medium after being cultured in BA-free multiplication medium (Table 4).

The final number of shoots that formed roots in each clone was affected significantly by both the shoot induction treatment and the multiplication treatment in each family (Fig. 6). Shoot induction × multiplication treatment interactions were not significant for number of shoots that formed roots (cf. percentage of shoots that formed roots, above). Shoot induction media lacking BA provided the highest numbers of plantlets per clone, with the mean for hormone-free induction medium significantly higher than the lowest 8, 3 and 7 means for families 13, 16 and 19, respectively (Fig. 6). NAA only affected plantlet number when applied at 0.27 μM in the presence of 2.2 μM BA for family 16.

The most effective multiplication medium for producing plantlets varied between families, with hormone-free medium providing significantly more plantlets per clone than any other treatment in family 16, but providing the lowest number in family 19 (Fig. 6). Highest numbers of plantlets for families 13 and 19 were provided by multiplication medium containing 2.2 μM BA in the absence of NAA, but only in family 19 was this mean significantly higher than the mean for hormone-free medium. The highest 1, 1 and 2 means were significantly higher than the lowest 5, 6 and 2 means for families 13, 16 and 19, respectively. Other means did not differ significantly except that hormone-free medium provided more shoots with roots than the medium containing 2.2 μM BA and 0.05 μM NAA for family 13 (Fig. 6).

The actual numbers of plantlets per clone using a combination of BA-free induction medium (first passage) followed by BA-free multiplication medium (second and third passages) were 9.5 ± 2.5, 3.2 ± 1.1 and 1.7 ± 0.6 ($n = 8$–10) for families

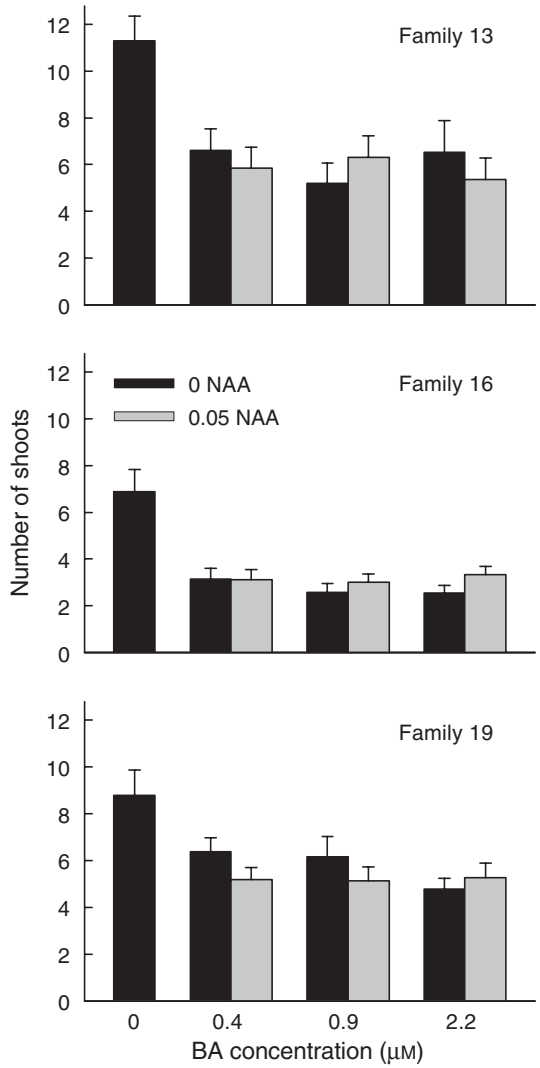

Fig. 4. Effect of BA and NAA concentrations on number of shoots transferred per *Corymbia torelliana* × *C. citriodora* clone after 4 weeks on shoot multiplication medium. Means are provided with s.e. ($n = 27–39$ clones).

13, 16 and 19, respectively. Corresponding plantlet numbers using the same shoot induction medium, but followed by multiplication medium with 2.2 μM BA in the absence of NAA, were 35.6 ± 19.0, 0.6 ± 0.3 and 15.8 ± 12.0 ($n = 7–10$). Percentages of clones that survived and formed at least one plantlet using the former treatment combination (lacking BA) were 88, 70, and 70% for the three respective families, compared with 71, 30 and 50% of clones surviving to form a plantlet using the latter combination (2.2 μM BA in the multiplication medium).

Discussion

Seedlings of *C. torelliana* × *C. citriodora* were established in a laboratory clone bank using simple surface sterilisation and *in vitro* germination procedures, followed by primary shoot elongation and multiple node production in medium devoid of cytokinin. Further multiplication was obtained either by elongating the lateral shoots of nodal explants in cytokinin-free medium or by inducing a combination of organogenic callus and axillary shoot proliferation in the presence of 2.2 μM BA. Plantlets were then produced using an IVS method that provided extensive rooting in sterile propagation mixture. These methods provide a means for simultaneous field-testing and laboratory storage of clones before selection and multiplication of desired genotypes. They could also form the basis for long-term storage of germplasm under minimal growth conditions or by cryopreservation (e.g. Watt *et al*. 2000; Blakesley and Kiernan 2001).

The two-step surface sterilisation procedure for seeds, involving a 70% ethanol pre-treatment followed by treatment with sodium hypochlorite, was highly effective with almost no contamination during two weeks on germination medium. Seeds were not affected by the duration of immersion in sodium hypochlorite (5–30 min), but germination of one family was adversely affected by raising the sterilant concentration above 1%. *Corymbia* seeds are large compared with most eucalypts (Boland *et al*. 1980), and so the effectiveness of 5–10 min immersion in 1% sodium hypochlorite suggests that the longer immersion times (20–30 min) and higher concentrations (3–30%) used for other eucalypts (Blomstedt *et al*. 1991; Cheng *et al*. 1992; Cid *et al*. 1999; Arruda *et al*. 2000; Nugent *et al*. 2001; Glocke *et al*. 2005, 2006) might

Table 3. Length of the longest shoot and percentage of shoots with roots following a 9-week exposure of *Corymbia torelliana* × *C. citriodora* seedling shoots to one of seven shoot multiplication media

For families 13 and 16, means followed by different letters are significantly different ($P < 0.05$, 2-way ANOVA and Duncan's multiple range test). '–' indicates roots did not form in these treatments

BA (μM)	NAA (μM)	Length of longest shoot (mm)	Shoots with roots (%)
Family 13 (n = 27–28 shoots)			
0	0	55.0 ± 4.0a	35.2 ± 5.4
0.4	0	12.7 ± 1.0b	–
0.4	0.05	13.6 ± 1.2b	–
0.9	0	12.8 ± 1.0b	–
0.9	0.05	13.0 ± 0.9b	–
2.2	0	9.7 ± 1.5c	–
2.2	0.05	10.8 ± 1.0bc	–
Family 16 (n = 27–31 shoots)			
0	0	46.7 ± 4.4a	18.9 ± 3.8
0.4	0	11.7 ± 1.1b	–
0.4	0.05	10.8 ± 1.1b	–
0.9	0	11.2 ± 0.9b	–
0.9	0.05	10.0 ± 0.9b	–
2.2	0	9.5 ± 1.2b	–
2.2	0.05	10.1 ± 0.9b	–
Family 19 (n = 35–36 shoots)			
0	0	29.4 ± 5.3	5.6 ± 1.7
0.4	0	19.1 ± 1.5	–
0.4	0.05	16.0 ± 1.8	–
0.9	0	15.3 ± 1.9	–
0.9	0.05	15.7 ± 1.4	–
2.2	0	12.8 ± 0.9	–
2.2	0.05	14.9 ± 1.3	–

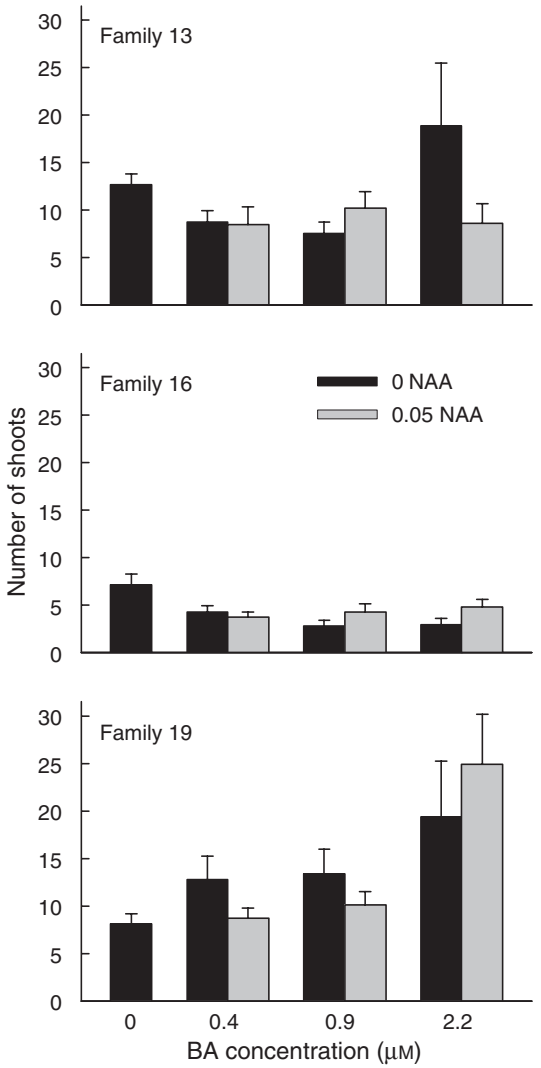

Fig. 5. Effect of BA and NAA concentrations on number of shoots transferred per *Corymbia torelliana* × *C. citriodora* clone after 9 weeks on shoot multiplication medium. Means are provided with s.e. ($n = 27$–39 clones).

be unnecessary or detrimental. Immersion in 1% sodium hypochlorite for 3 min (Niccol *et al*. 1994) or 0.12% for 15 min (Termignoni *et al*. 1996) without ethanol pre-treatment has been used to establish seedling cultures of *Eucalyptus microcorys* F.Muell. and *E. dunnii* Maiden. Immersion in 1.5% sodium hypochlorite for 15 min following ethanol pre-treatment is used routinely for surface sterilisation of *E. globulus* Labill. and *E. saligna* Sm. seeds (Fett-Neto *et al*. 2001; Fogaça and Fett-Neto 2005; Schwambach *et al*. 2005).

Following germination, the most effective method for establishing *C. torelliana* × *C. citriodora* cultures was to place shoots initially onto cytokinin-free shoot induction medium. In this medium, they formed roots spontaneously, elongated rapidly, and produced approximately twice as many nodes as shoots on media containing cytokinin. BA stimulated production of lateral shoots but, typically, only 1–3 shoots were produced,

Table 4. Percentage of *Corymbia torelliana* × *C. citriodora* seedling shoots (derived from one of seven shoot multiplication media) that had formed roots after 4 weeks in IVS mixture

For family 19, means (± s.e.) followed by different letters are significantly different ($P < 0.05$, 2-way ANOVA and Duncan's multiple range test)

BA (μM)	NAA (μM)	Shoots with roots (%)
Family 13 (n = 27–28 shoots)		
0	0	57.4 ± 4.1
0.4	0	35.8 ± 7.1
0.4	0.05	48.0 ± 7.6
0.9	0	39.2 ± 7.6
0.9	0.05	35.6 ± 7.0
2.2	0	39.7 ± 8.2
2.2	0.05	23.1 ± 6.2
Family 16 (n = 24–28 shoots)		
0	0	36.3 ± 6.0
0.4	0	17.2 ± 5.7
0.4	0.05	10.6 ± 3.8
0.9	0	25.4 ± 8.3
0.9	0.05	6.8 ± 3.2
2.2	0	10.0 ± 4.7
2.2	0.05	4.1 ± 1.9
Family 19 (n = 35–36 shoots)		
0	0	15.2 ± 3.8a
0.4	0	47.2 ± 5.9b
0.4	0.05	39.8 ± 5.2bc
0.9	0	29.1 ± 5.4c
0.9	0.05	41.9 ± 6.6b
2.2	0	28.5 ± 5.0c
2.2	0.05	34.3 ± 4.7bc

they were <1-cm length, and they were not separated from the main axis when shoots were dissected. The number of explants transferred to fresh medium was closely related to the number of nodes per seedling, and cytokinin-free media therefore, provided approximately twice the number of secondary explants. This method is best described as a node culture rather than a shoot culture because nodal explants from an unbranched stem were used, instead of dissected axillary shoots, for establishment of the multiplication phase (George 1993).

Shoots from the nodal explants produced in cytokinin-free induction medium were longer than other shoots 4 weeks later (for all families) and 9 weeks later (for two of the three families). The final carry-over effect of the induction medium was very strong, such that final numbers of shoots and plantlets per clone (15 and 19 weeks, respectively, after plating of seeds) remained greatest when shoots had initially been cultured in the absence of cytokinin. Addition of NAA to cytokinin-free induction medium increased the number of nodes for the family with relatively poor rooting in hormone-free medium, but this effect was weaker and did not carry through to final shoot or plantlet numbers.

The optimum medium for further shoot multiplication depended on the family of seedlings and its inherent rooting capacity in the absence of exogenous hormones. For the two families with highest spontaneous rooting, an effective option was to continue culturing secondary explants in the absence of cytokinin, and allow root formation and rapid stem elongation to occur. These shoots were 3–4 times the length of shoots cultured

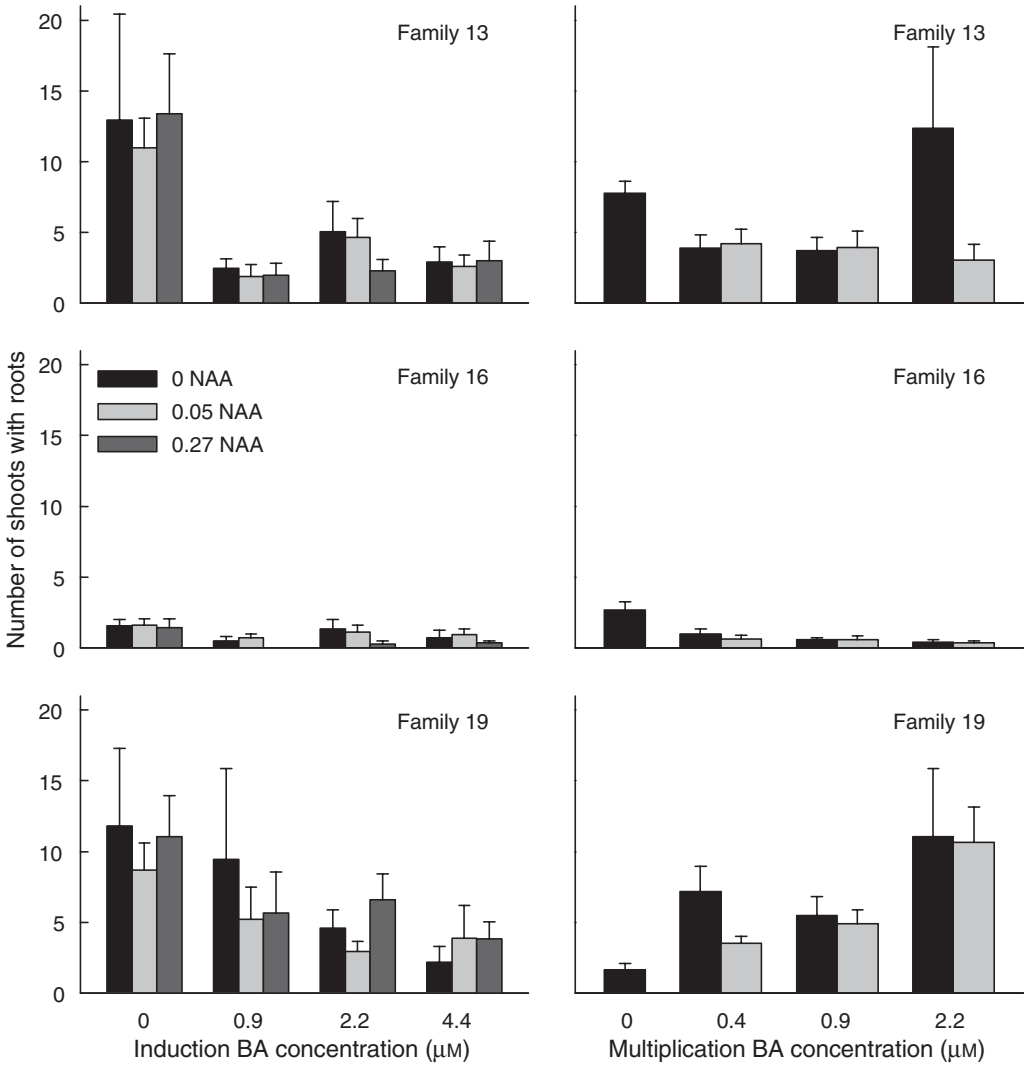

Fig. 6. Effect of BA and NAA concentrations in shoot induction medium (left) and shoot multiplication medium (right) on number of *Corymbia torelliana* × *C. citriodora* shoots with roots after 4 weeks in *in vitro* soil-less (IVS) mixture. Means are provided with s.e. ($n = 14$–23 clones for induction media; $n = 27$–39 clones for multiplication media).

in the presence of BA, they provided higher numbers of explants when dissected for transfer, they rooted well in IVS mixture, and so they formed relatively high numbers of plantlets. Their final shoot numbers (Fig. 5) would have been much greater relative to other treatments if moderately long shoots (between 20-mm and 50-mm length) had been dissected for further multiplication or long-term storage rather than kept intact for root induction.

This method of multiplying seedling shoots in node culture, by allowing spontaneous root formation and rapid shoot elongation in the absence of exogenous cytokinin, is novel for eucalypts. A wide range of species produce roots prolifically *in vitro* in the absence of hormones; e.g. *Verbena tenera* Spreng. (Hosoki and Katahira 1994), *Acmella oppositifolia* (Lamarck)R.K.Jansen (Salgado-Garciglia *et al*. 1996), *Scaevola* spp. (Bhalla and Sweeney 1998), *E. saligna* (Corrêa *et al*. 2005) and *Yucca valida* Brandegee (Arce-Montoya *et al*. 2006). Little or no spontaneous rooting occurs on seedling shoots of many eucalypts (Blomstedt *et al*. 1991; Niccol *et al*. 1994; Koriesh *et al*. 2003; Schwambach *et al*. 2005). Eucalypt multiplication is instead obtained in media containing cytokinin, either by inducing axillary shoot proliferation in shoot cultures or by inducing organogenic callus (Lakshmi Sita and Vaidyanathan 1979; Gupta *et al*. 1983; Blomstedt *et al*. 1991; Niccol *et al*. 1994; Gomes and Canhoto 2003). None of these eucalypt studies reported a hormone-free or cytokinin-free control medium. Koriesh *et al*. (2003) incorporated a hormone-free control in their study of *C. citriodora*, but little spontaneous rooting occurred and shoot numbers were low compared with media containing 2.2–8.8 μM kinetin. Cuttings of *C. torelliana* × *C. citriodora* are easier to root than *C. citriodora* cuttings (Potts and Dungey 2004; Lee 2007), and this higher rooting capacity *in vivo* may explain the *in vitro* rooting capacity that makes them suited to node culture.

An alternative multiplication method for the *C. torelliana* × *C. citriodora* family with consistently lowest rooting and relatively poor proliferation in the absence of

hormones (family 19) was to stimulate organogenesis and axillary shoot proliferation with 2.2 μM BA. This method was also effective for family 13. Only some clones were responsive, with 7–32% producing clumps of more than 27 shoots by 15 weeks after plating of seeds. This approach reduced the percentage of clones that survived and formed a plantlet, which could be a major limitation for establishment of an *in vitro* and *ex vitro* clone bank. Our aim was to develop culture methods that did not involve a callus phase. However, an optimised organogenesis approach, possibly using smaller explants such as hypocotyls, cotyledons, apices, stem segments or leaves (Azmi *et al*. 1997; Bandyopadhyay *et al*. 1999; Dibax *et al*. 2005; Glocke *et al*. 2005, 2006), has great potential when very high rates of clonal multiplication are required, but from only a minority of the plated seeds.

The IVS system proved a reasonable method for producing plantlets of *C. torelliana* × *C. citriodora*, with highest rooting percentages for each family associated with optimum multiplication treatments. IVS rooting percentages were low compared with most species reported by Newell *et al*. (2003, 2005), although they compare favourably with some clones and species of *Pimelea* and *Conospermum*. A notable difference between the current IVS method and that of Newell *et al*. (2003, 2005) was the lower IBA concentration (4.9 μM *v.* 40 μM) and shorter pulse (3 days *v.* 7 days), which was derived on the basis of previous studies of eucalypt rooting *in vitro* (Cheng *et al*. 1992; Niccol *et al*. 1994; Cid *et al*. 1999; Fett-Neto *et al*. 2001; Gomes and Canhoto 2003; Koriesh *et al*. 2003) and the spontaneous rooting ability of *C. torelliana* × *C. citriodora* shoots in hormone-free media. Further work will focus on higher IBA concentrations and longer pulse durations, particularly for families that have little spontaneous rooting in media lacking auxin.

The current methods were effective, but not necessarily optimal, for establishing *C. torelliana* × *C. citriodora* clones *in vitro* and *ex vitro*. Half-strength MS formulations have been used for micropropagation of many eucalypts (Jones and van Staden 1994; Niccol *et al*. 1994; Cid *et al*. 1999; Jasrai *et al*. 1999; Fett-Neto *et al*. 2001; Bunn 2005; Dibax *et al*. 2005), but symptoms of micronutrient deficiency appeared in *C. torelliana* × *C. citriodora* shoots in this medium. Axillary bud outgrowth was also very poor in the shoot induction media, and higher BA concentrations (>4.4 μM) or shoot decapitation of germinants might provide more lateral shoots, albeit at the expense of shoot elongation. Most importantly, this study has developed preliminary methods for node culture, axillary shoot proliferation and organogenesis, but there appears ample scope for optimising the callogenesis and organogenesis methods based upon current results using 2.2 μM BA and previous results from *C. citriodora* (Lakshmi Sita 1979; Muralidharan *et al*. 1989; Jasrai *et al*. 1999).

Node culture is considered of particular value when (1) a plant produces elongated shoots in culture, (2) lateral bud break is difficult to induce with cytokinins, and (3) it is important to reduce the risk of genetic irregularities arising from a callus phase (George 1993). All three conditions are met when establishing an *in vitro* clone bank of *C. torelliana* × *C. citriodora* seedling shoots for plantation forestry. An unusual feature of this eucalypt hybrid was its propensity for rooting and rapid shoot elongation in hormone-free medium. However, rooting of all families declined over several passages and so future clone banking might involve a dual approach, using node culture for culture establishment and plantlet production, and organogenesis for very rapid proliferation of desired clones.

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