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Research note:

Micropropagation of *Eucalyptus polybractea* selected for key essential oil traits

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Abstract. A protocol for the micropropagation of *Eucalyptus polybractea* R.T. Baker (blue mallee) using axillary bud proliferation from lignotuber-derived explants is described. Three different ages of plants were used as explant sources: glasshouse-grown seedlings, field-grown saplings, and coppice of field-grown mature lignotubers. Explants from each source initiated successfully and no significant difference was observed for shoot proliferation, rooting success or hardening success between explant sources. Leaf oil quantity and quality for hardened clones transplanted to a field plantation were assessed after 3 months of growth. Ramets of all clones contained high quality oil with over 80% 1,8-cineole. For seedling-derived clones, foliar oil concentrations of ramets were higher than those of the ortets from which they were derived. For sapling and mature lignotuber derived clones the opposite was the case. This suggests that ontogenetic and physiological constraints may be influencing yield in the young ramets. The age of the explant source did not appear to influence the success of micropropagation, and as a result older plants (for which key oil traits are known) can be selected as elite plants for multiplying selected genotypes via micropropagation.

Additional keywords: axillary bud proliferation, blue mallee, cineole, clone, eucalypt.

Introduction

Eucalyptus polybractea R.T. Baker (blue mallee) is a small tree restricted to two principal disjunct regions in Australia: Bendigo in central-northern Victoria and Wyalong in western New South Wales (Brooker and Kleinig 2006). Blue mallee is central to commercial production of eucalyptus oil in Australia because its oil generally contains between 80 and 88% (v/v) of the pharmaceutical 1,8-cineole (eucalyptol) together with very low concentrations of undesirable components (Goodger *et al*. 2007), and its foliage generally yields more oil than other commercial eucalyptus oil species. In addition, mallee species are characterised by multiple woody stems that arise from partially buried lignotubers (Wildy and Pate 2002): woody swellings that house a large store of potential bud-forming sites, enabling rapid regeneration of a canopy after decapitation (Noble 2001). Therefore, the species is amenable to large-scale mechanical harvesting when grown in short-rotation coppice cultivation (Davis 2002; Goodger *et al*. 2007). Traditional production of eucalyptus oil in Australia is primarily from natural stands in state forest on public land. The availability of such trees is diminishing, especially in Victoria where severe restrictions have been placed on harvesting of *Eucalyptus* leaves from public land (Parliament of Victoria 2002). In order to remain viable, the eucalyptus oil harvesting industry in Victoria must shift their operations to plantation grown trees.

Seedling-derived trial plantations have not resulted in oil production gains (Slee 2007), possibly as a result of the high variation observed for both oil quantity and quality in seedling families (Davis 2002; Doran 2002; King *et al*. 2006*a*). Establishing plantations or seed orchards based on clones of selected, elite oil producing plants is likely to be economically productive. To date, clones based on cuttings of blue mallee trees have been difficult to produce in large numbers (RM Gleadow, unpublished observations; Slee 2007). In addition, a micropropagation protocol has not been successfully developed for blue mallee, despite success for many other eucalypts in the last few decades such as *E. marginata* Smith (McComb and Bennett 1982), *E. camaldulensis* Dehnh and *E. torelliana* F. Muell. (Gupta *et al*. 1983), *E. grandis* Hill ex Maidenand *E. nitens* (Deane & Maiden) Maiden (Furze and Cresswell 1985), *E. tereticornis* Smith (Das and Mitra 1990), and more recently for *E. impensa* Brooker and Hopper, a mallee species from Western Australia (Bunn 2005).

This study aimed first to develop a successful micropropagation protocol for *E. polybractea* using axillary bud proliferation from lignotuber-derived explants, with the view to multiplying selected elite oil-yielding genotypes. Second, it aimed to assess at what life history stage (seedling, sapling or mature plant) lignotuber explants can be taken in order to maximise the chance of successful establishment of lines with desirable oil quality and quantity. Vegetative propagation of woody plants is often more successful if explants are taken during the juvenile phase (Giri *et al*. 2004), but the selection of elite genotypes is less certain at this stage as leaves of mallee

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seedlings typically have lower leaf oil concentrations compared with mature tree foliage (Milthorpe *et al*. 1998; Davis 2002; Doran 2002; King *et al*. 2004; Slee 2007). Therefore, it is preferable to vegetatively propagate blue mallee plants for which key traits such as oil quantity, quality and biomass yield are already known, such as the commercially harvested coppice of mature lignotubers or plantation grown saplings.

Materials and methods

Plant material

Eucalyptus polybractea R.T. Baker explants were collected from plants of three different ages representing distinct life history stages: glasshouse-grown seedlings, field-grown saplings, and field-grown coppice arising from commercially harvested, mature lignotubers. Seed for the glasshouse-grown seedlings was collected from 14 open-pollinated trees from various locations in the Bendigo and West Wyalong regions (12 and two trees, respectively) and germinated in soil (vermiculite : perlite : sand; 1 : 1 : 1, v/v/v) with Scotts Premix Fertiliser plus minor nutrients (The Scotts Co., Marysville, OH). Saplings had not yet reached reproductive maturity and were derived from seed collected from the Bendigo region that had been planted at a trial plantation in the region (see Goodger *et al*. 2007; for planting and location details). Mature lignotubers were of indeterminate age (>75 years) and naturally occurred in the Bendigo region. The coppice arising from the mature lignotubers had been mechanically harvested biannually for at least 50 years as part of a commercial eucalyptus oil harvesting operation.

Explant collection

Fourteen seedlings, nine saplings and fifteen mature lignotubers were selected on the basis of oil quantity and quality (1,8-cineole), and all above ground biomass was harvested 5 cm above soil level. Seedlings were harvested when 8 months old and saplings at 4 years of age, whereas the mature lignotuber coppice had last been harvested commercially 2 years prior. Coppice shoots arising from the lignotuber of all plants were harvested as explants 4–6 weeks later when 2 cm in length. Explants were immediately placed into sterile distilled water (SDW) and sterilised within 3 h of collection in a solution containing alkyldimethylbenzylammonium chloride (0.1%, w/v) and Tween-20 (0.1%, v/v) for 30 min with constant agitation, followed by rinsing in four changes of SDW under aseptic conditions. Damaged or dead tissue was excised from the sterilised explants and 5-mm sections encompassing axils were then plated onto initiation medium.

Tissue culture media

All powdered media and plant growth regulators (PGRs) were sourced from Sigma-Aldrich chemicals (St Louis, MO). The pH of all plant media was adjusted to 5.6 with 1 N KOH before adding agar (7 g L^{-1}) and autoclaving at 121°C for 20 min. Initiation medium plates consisted of half strength MS minerals (Murashige and Skoog 1962) with sucrose (30 g L^{-1}) and the PGRs NAA (5 μM) and zeatin (2.5 μM). Explants were incubated in darkness at 25 ± 1°C and deemed to have initiated successfully once shoot primordia began to appear from the axillary buds of uncontaminated explants (after 4–10 weeks). Initiated explants were transferred to plates containing multiplication medium consisting of a base medium (BM) of Lloyd and McGowan's woody plant basal salt mixture (2.3 g L^{-1}; Lloyd and McCown 1981), sucrose (30 g L^{-1}) and MS modified vitamin (1 mL L^{-1}). The PGRs NAA (16 μM) and BAP (4.5 μM) were added to BM to form multiplication medium. Explants on multiplication medium were placed in a low light growth cabinet at 25 ± 1°C with a 16 h photoperiod (50 μmol photons m^{-2} s^{-1}).

After 6–10 weeks, material had multiplied sufficiently for clumps of shoots to be placed onto elongation media. Elongation medium consisted of BM with the PGR 2iP (3 μM) in 250-mL tubs. An example of elongating plantlets is shown in Fig. 1*a*, and shoot proliferation was assessed at this stage as the number of elongating plantlets relative to the number of elongating plantlets of the most abundant clone of all explant sources (Table 1). After 4–6 weeks, single plantlets, 3 cm long, were dissected from clumps of elongated shoots and plated onto rooting media. Rooting medium consisted of BM with the PGR IBA (100 μM) in 250-mL tubs. Successfully rooted ramets were removed from media and planted in sterilised soil. Ramets were then hardened by slowly decreasing the humidity in a lighted growth chamber (25 ± 1°C, 16 h photoperiod, 250 μmol photons m^{-2} s^{-1}) over 2 weeks before further hardening and growth in a glasshouse for 4 weeks. Hardening success was appraised at this stage. Hardened ramets from a selection of clones were then planted in a trial plot at the aforementioned Bendigo region plantation in a block design where ramets of each clone were surrounded by a different clone at 2 × 2-m spacing.

Gland formation in ramets before hardening

Microscopic examination was used as a preliminary assessment of gland formation in a leaflet from a randomly selected ramet of each clone before hardening. After 5–6 weeks, a single leaflet was removed from each ramet and mounted in 0.1 mM tri-sodium citrate buffer (pH 5.6) and examined for oil gland presence using a stereomicroscope with transmitted lighting. Leaflets were then stained for 10 min using the specific neutral lipid dye Nile red (10 μg mL^{-1} in acetone; Sigma-Aldrich) and rinsed and mounted in the citrate buffer. Lipid staining was viewed as orange-yellow fluorescence using a stereomicroscope fitted with UV-light excitation and a blue excitation filter (360–500 nm).

Oil quantification in hardened ramets of selected clones

Six hardened clones were quantitatively assessed for oil yield and quality after 3 months in the field: two each of seedling, sapling and mature lignotuber-derived explants. Six fully expanded leaves were collected from seven ramets of each clone, stored on ice and immediately returned to the laboratory. Leaves from each ramet were ground to a fine powder under liquid nitrogen and extracted with hexane containing the internal standard tri-decane as described by King *et al*. (2006*b*). Samples were extracted for 5 days at 50°C with periodic agitation (see King *et al*. 2004). After this period, oil was prepared and analysed by GC-FID (see King *et al*. 2006*b*) and components identified as previously described (Goodger *et al*. 2007). After oil extraction, the remaining ground leaf material was oven-dried and weighed to obtain dry mass.

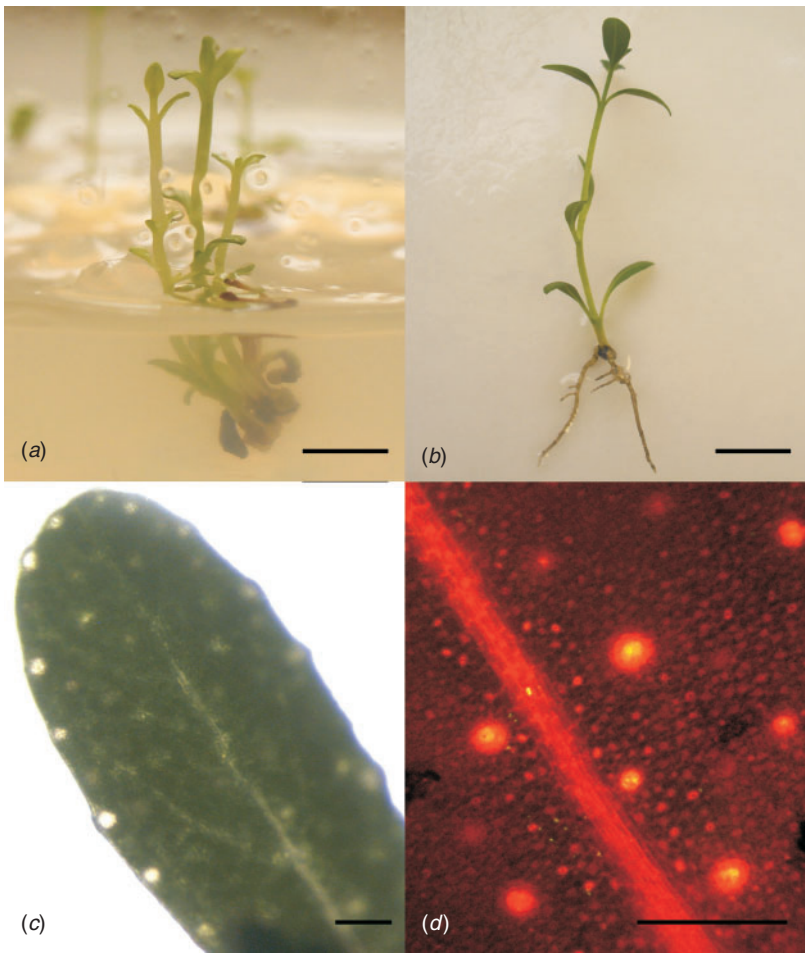

Fig. 1. Successful micropropagation of *Eucalyptus polybractea*. (*a*) Elongating plantlets derived from a seedling explant in an elongation medium tub, scale bar = 1 cm; (*b*) ramet with good root formation removed from rooting medium, scale bar = 1 cm; (*c*) leaflet removed from the ramet and viewed with transmitted light to show translucent oil glands, scale bar = 100 µm; (*d*) intense nile red lipid staining of oil glands in the ramet leaflet, scale bar = 100 µm.

Table 1. Assessment of *Eucalyptus polybractea* micropropagation

The number of clones initiated from seedling, sapling or mature lignotuber explants was 9, 5 and 8, respectively. Shoot proliferation was scored as the number of elongating shoots for each clone relative to the number from the most abundant clone of all explant sources. Root development was scored as the percentage of plantlets successfully forming roots for each clone. Hardening success was scored as the percentage of ramets successfully removed from tissue culture and propagated in pots in the glasshouse. The minimum and maximum values for the different clones for each explant source are presented along with the mean ± s.e. of all clones within each explant source. One-way ANOVA (after arcsin transformation) was performed between explant sources for each growth parameter

Growth parameter	Explant source	Min.	Max.	Mean	s.e. of mean	*F*	*P*
Shoot proliferation (%)	Seedling	40	90	62	6	–	–
	Sapling	30	100	61	13	–	–
	Mature	30	100	63	8	0.02	>0.05
Root development (%)	Seedling	38	95	78	6	–	–
	Sapling	29	95	61	12	–	–
	Mature	57	86	75	3	1.43	>0.05
Hardening success (%)	Seedling	51	94	82	5	–	–
	Sapling	60	96	81	7	–	–
	Mature	70	95	83	3	0.02	>0.05

Results and discussion

Micropropagation success

Axillary bud proliferation was used to successfully micropropagate *E. polybractea* for the first time. The use of axillary buds, rather than attempting to re-differentiate callus, aimed to avoid the potential introduction of somatic genetic variability in the resulting ramets (Giri *et al*. 2004). The source of explant material, whether coppice from harvested seedlings, saplings or mature trees, did not appear to influence the ability to micropropagate selected blue mallee individuals. The majority of plants selected for micropropagation were able to be introduced into culture (9/14 seedling, 5/9 sapling and 8/15 mature lignotuber-derived clones initiated successfully). Unsuccessful initiation was the result of contamination, explant death during sterilisation or the formation of callus that failed to produce differentiated shoots. The higher initiation success rate of seedling explants is likely due to the decreased fungal contamination observed from the glasshouse-grown material, compared with the sapling and mature tree material collected from the field. The collection of a greater number of explants from a given ortet is likely to increase the initiation success. For example, a previously recalcitrant mature lignotuber-derived clone was successfully initiated into culture when numerous (>50) explants were collected for sterilisation (data not shown).

No significant difference in shoot proliferation was detected between the three explant sources (Table 1). Within each explant source, however, some clones were very successful, whereas others multiplied and elongated more slowly. Each clone from all explant sources was capable of forming roots within 3 weeks of being placed on rooting medium, but the number of plantlets successfully developing roots within each clone varied. This variation appeared to be clone-specific, rather than related to explant source as no significant difference was found for rooting success between the clones produced from the different explant sources (Table 1). An example of a successfully rooted ramet from a seedling explant before hardening is shown in Fig. 1*b*. Hardening was generally very successful for all rooted ramets and no difference was detected between the explant sources (Table 1).

Woody trees are generally difficult to regenerate under *in vitro* conditions, and vegetative propagation, especially at the rooting stage, is often more successful if explants are taken during the juvenile phase (Giri *et al*. 2004). Nevertheless, the results of this study suggest that explants derived from the coppicing lignotubers of blue mallee seedlings, saplings, and mature plants can be successfully used as explant sources for micropropagation.

Oil quantity and quality of micropropagated clones

A preliminary microscopic assessment of early gland formation in ramets before hardening showed numerous glands as translucent spheres on immature leaflets (see example in Fig. 1*c*), and glands showed intense lipid staining, indicating the presence of oil (see example in Fig. 1*d*). After hardening, ramets of six clones were transplanted to the field and analysed for leaf oil quantity and quality after 3 months of growth. The oil extracted from all clones was of good quality, with 1,8-cineole comprising over 80% of the total monoterpenes in each ramet (Fig. 2). A one-way ANOVA was performed on the oil quantity data after pooling the two clones within each explant source (SPSS version 15.0, SPSS Inc. Chicago, IL). The results showed a significant difference in oil concentration between explant sources ($F = 18.794$, $P < 0.0001$), and a subsequent Tukey's post-hoc analysis determined the seedling-derived explant clones to have significantly lower oil than clones derived from the sapling and mature lignotuber explants ($P = 0.000$).

Despite displaying lower mean oil than the other explant sources, the seedling-derived ramets had higher oil concentrations than the ortets from which they were derived (Fig. 2). As leaves of blue mallee seedlings typically increase their leaf oil concentrations as they develop (Davis 2002; Doran 2002; King *et al*. 2004; Slee 2007), this result suggests that the ramets have continued to increase their monoterpene biosynthetic capacity and/or gland size and density during the 3 months in the field, and given the presence of copious oil glands in the pre-hardened ramets (Fig. 1*c*, *d*), perhaps even during their time in tissue culture. Seemingly contrary to this, the clones derived from the sapling and mature lignotubers contained oil at lower concentrations than their respective ortets (Fig. 2). This may reflect physiological constraints such as limited gland or leaf size in the relatively small ramets (~40 cm in height), rather than a biosynthetic limitation. It is expected that the seedling and mature lignotuber derived clones will attain the oil levels of their ortets as they develop. A detailed trajectory analysis is required to determine the temporal and ontogenetic controls on gland size and monoterpene biosynthetic capacity, research that is likely to be aided by the use of micropropagated blue mallee clones.

The successful micropropagation protocol described here will assist in establishing blue mallee plantations and elite seed orchards. The proliferation of selected genotypes will reduce the variability for key oil traits observed in naturally occurring populations, thus, providing a level of predictability in plantation establishment. The age of the explant source did not appear to influence the success of micropropagation. As a result, older

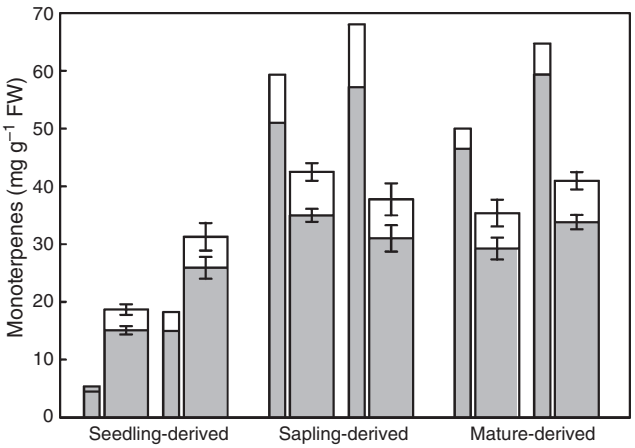

Fig. 2. Quantitative assessment of monoterpenes in micropropagated *Eucalyptus polybractea* clones after 3 months in the field. Each narrow bar represents the original ortet data and the adjacent wide bar represents the mean ± s.e. of seven ramets within the clone derived from that ortet. Entire bars represent the sum of all monoterpenes and the enclosed grey bars represent the 1,8-cineole proportion of the total monoterpenes.

plants (for which key oil traits are known) can be selected as elite plants for micropropagation and help establish commercially viable plantations more rapidly.

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