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Plant regeneration through indirect organogenesis and genetic transformation of *Eucalyptus polybractea* R.T. Baker

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ABSTRACT

Eucalyptus polybractea (blue mallee) is the most widely harvested species in Australia for the production of pharmaceutical grade eucalyptus oil. Its use for both oil and biomass production is set to expand because blue mallee grows vigorously on semi-arid land where agricultural crop production is unsustainable. One of the most important tools for improvement of blue mallee, and for dissection of its unique traits, is an efficient method of indirect organogenesis. Our aim was to develop such a method, which is efficient for all genotypes and applicable to adult-derived explants. We also aimed to test whether genetically modified plants could be produced using this protocol. *In vitro* cultures were developed from three field-grown adult clones and it was found that over 95% of the resultant leaf explants initiated callus in a medium supplemented with thidiazuron and 1-naphthaleneacetic acid. The effect of the cytokinins, 6-benzylaminopurine (BA) and N_6-(2-isopentenyl) adenine (2iP) on plant regeneration from the initiated callus was compared. Ninety percent of callus from all three clones regenerated shoots in a medium supplemented with BA, whereas only one clone regenerated shoots at high efficiency (93%) with 2iP. Roots were successfully developed on regenerated shoots with high efficiency (>60%) *via* exposure to indole-3-butyric acid, and hardened plants were successfully established in soil. Leaf explants from one clone were transformed with *Agrobacterium tumefaciens* T-DNA containing genes for hygromycin phosphotransferase (Hpt) resistance and green fluorescent protein (GFP), both constitutively expressed using the CaMV35S promoter. Transformants showed GFP fluorescence in calli and plantlet leaves and were successfully rooted and hardened into plants. This work will enable large scale cloning of adult blue mallee plants and, after optimization of the transformation protocol, provide an important platform for further research on the biosynthesis of eucalyptus oils with the potential to dramatically improve blue mallee as a crop.

1. Introduction

Blue mallee (*Eucalyptus polybractea*) is a member of a small group of mallee eucalypts (the oil mallees) that are both well adapted to semi-arid conditions (Wildy et al., 2004) and capable of producing and storing high levels of essential oil in their foliage (Goodger and Woodrow, 2012). The oil is a complex mixture of largely mono- and sesquiterpenes that is synthesized and stored in secretory cavity complexes embedded in the leaf mesophyll (King et al., 2006). Plantations of these mallees have enormous economic potential in semi-arid regions—both in Australia and worldwide—where crop production is marginal or unsustainable. Once established, short-rotation coppice plantations of mallee trees can yield an indefinite and regular supply of biomass, essential oil, and other valuable natural products (Goodger and Woodrow, 2011), as well as stored carbon—an increasingly attractive economic prospect (Bryan et al., 2010). Mallees produce these outputs at high water-use-efficiency and with consistency because of their long-lived nature and relatively unique structural and functional attributes. Their deep and resilient root system, and large nutrient and carbon-rich lignotuber, enable them to buffer erratic rainfall regimes (Noble, 2001; Wildy and Pate, 2002). Importantly, these attributes also enable mallees to be indefinitely harvested as coppice on an annual to bi-annual basis, depending on growth rate (Goodger et al., 2007; Bartle and Abadi, 2010).

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There are challenges to be overcome in order to maximize the profitability of oil mallees. Optimal growth rates require that plants invest photosynthate astutely between the root system, lignotuber, stems and leaves. This investment pattern will be different for different harvesting frequencies, given the need for sufficient stored carbon and nutrients to be present in the roots and the lignotuber to initiate rapid coppice regeneration following harvest (Noble, 2001). Essential oil yield in turn depends on the growth rate of the above ground parts, as well as the proportion of biomass allocated to leaves and the amount of oil in each leaf. Many of these attributes have been shown to be highly variable, even between closely related individuals (Davis, 2002; Doran, 2002). For example, King et al. (King et al., 2004) found that blue mallee plants within a single, naturally occurring population varied in foliar essential oil content from less than 1% to greater than 5% of fresh weight. Similarly, plants showed considerable quantitative variation in major oil constituents, growth rates, leaf morphology and water-use-efficiency (King et al., 2004).

This natural variation provides significant scope for developing improved mallee lines with regard to biomass and essential oil production. Some progress has been made in improving mallees through conventional selective breeding (Doran, 2002), but given that it typically takes several years for mallees to flower and that several generations are needed to realize significant selection gains, this approach will likely prove unsatisfactorily slow. Goodger et al. (Goodger et al., 2008; Goodger and Woodrow, 2008) adopted a more rapid approach involving seed production from a series of elite clones, which were selected for their exceptionally high foliar oil content, desirable oil quality and high growth rate. Their strategy was based on findings that oil and growth traits are highly heritable (Goodger and Woodrow, 2012; Barton et al., 1991; Doran and Matheson, 1994), and genetic correlations between oil and growth traits can be neutral or even slightly positive depending on the genotype (King et al., 2006). Clonal forestry approaches of this kind involving other species have produced good results. For example, in a related Myrtaceous species (*Melaleuca alternifolia*), clonal seed orchards yielded significant selection gains in terms of tea tree oil production at both the leaf and plantation level (Butcher et al., 1996; Doran et al., 2006). Preliminary analyses of large-scale plantations derived from seed from the elite mallee clones have shown a marked improvement in foliar oil yield and growth rates relative to natural populations (I. Woodrow, unpublished results).

Establishment of a clonal seed orchard by Goodger et al. (Goodger and Woodrow, 2009) relied on the development of an *in vitro* plant regeneration protocol for blue mallee, given that this species is not amenable to efficient propagation from potted stem cuttings (Slee, 2007). This method involves proliferation of axilliary buds from lignotuber-derived explants, with subsequent rooting of shoots (Goodger et al., 2008). Ramets developed using this protocol have shown very close similarity to the ortet in all important attributes, including foliar oil quality and quantity (Goodger and Woodrow, 2009). Nevertheless, because of the difficulty of genetic transformation, a protocol involving proliferation of axilliary buds is unlikely to be sufficient to underpin many future improvement strategies in blue mallee as a crop. Moreover, an efficient genetic transformation protocol will be required for investigations of the genetic and physiological processes underlying growth, development and secondary metabolism. Blue mallee is a useful target for these latter studies given that none of the model plant species share some of the most remarkable attributes of this species, including exceptionally large, sub-dermal foliar secretory cavities. The implementation of forward and reverse genetics approaches, such as transposon-insertional mutagenesis (Fladung and Polak, 2012), activation tagging (Busov et al., 2011), gene and enhancer trapping (Groover et al., 2004), and targeted genome engineering (Maggio and Goncalves, 2015), in blue mallees, will require an efficient system for proliferating cloned plants from genetically transformed cells.

Table 1
Composition of media used for *E. polybractea* organogenesis.

Components	Culture media			
	M1	M2	M3	M4
Woody plant basal salt mixture[a] ($g\,l^{-1}$)	2.3	2.3	2.3	
Murashige & Skoog modified vitamins ($\times 1000$) ($ml\,l^{-1}$)	1.0	2.0	1.0	1.0
Sucrose ($g\,l^{-1}$)	25	30	25	25
2iP (μM)	3.0			
IBA (μM)		100		
TDZ (μM)			3.0	
NAA (μM)			0.1	
BAP (μM)				4.4
Agar ($g\,l^{-1}$)	7.0	7.0		7.0
Gelrite ($g\,l^{-1}$)			2.5	

[a] Lloyd & McCown woody plant basal salt mixture (Austratec, Australia).

Here, we outline a highly efficient protocol for regeneration of blue mallee clones through indirect organogenesis and use it to develop the first genetically transformed plants of this species. Successful genetic transformation and regeneration, *via* indirect organogenesis or somatic embryogenesis, has been reported for a sizeable number of commercially important (largely for hardwood timber) *Eucalyptus* species (Chauhan et al., 2014). Nevertheless, several challenges need to be overcome before transformation and regeneration of healthy, stable transgenic plants can be undertaken routinely. First, because target trees are generally identified at the adult stage, protocols must work efficiently with adult explant material. It is noteworthy in this context that most of the research on eucalypt transformation and *in vitro* regeneration has involved seedling-derived explants, with some notable exceptions (Mullins et al., 1997; Chen et al., 1996, 2001; Spokevicius et al., 2005). Second, protocols need to function independently of genotype. It has generally been found that aspects of both the transformation and *in vitro* regeneration protocol need to be modified for each new genotype (Chauhan et al., 2014). In this paper, we outline a protocol for *in vitro* regeneration of blue mallee that is applicable to adult-derived explants and shows exceptionally high efficiency across several genotypes. We used one genotype to show that genetically transformed plants can be successfully regenerated using this protocol.

2. Materials and methods

2.1. Plant material

In vitro cultures of three clones (clones 1, 2 and 3) originating from nodal cultures of three separate adult trees were used for this study. These adult trees were from separate populations on private land (several kilometres apart) near Inglewood, Victoria, Australia, and each had relatively high amounts of foliar essential oil (>4% fresh weight). The nodal cultures were established according to Goodger et al. (Goodger et al., 2008) using explants taken from young (<20 cm in length) coppice shoots. Large numbers of ramets of each of other blue mallee clones have been produced from similar *in vitro* cultures, and these have been shown to have exceptional similarity with regard to essential oil quantity and quality as well as growth performance (Goodger and Woodrow, 2008). This indicates that, at least with regard to these traits, the occurrence of somaclonal variation is relatively improbable. Permission for the collection of plant material was granted by the land owner —FGB Natural Products. Cultures were maintained in the dark in a medium containing 2iP (M1) (Table 1) by sub-culturing at two monthly intervals.