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Full Length Research Paper

Shoot and root morphogenesis from *Eucalyptus grandis* x *urophylla* callus

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***Eucalyptus grandis* x *urophylla* plantlets were regenerated via indirect organogenesis. Histological assessment of their development focused on identifying the calli, the differentiation of shoots from the calli and the shoot-root junction from the nascent shoots. Vascular tissue formation within the callus preceded that of organised nodular structures, from which adventitious shoots were ultimately formed. Vascular systems linked the nodular structures and their associated cambial meristems, observed at 15 to 22 days, were hypothesized as being the centres of differentiation for shoot formation. Roots developed from isolated adventitious shoots from either the callus created as a wounding response to isolation or from a region of the stem immediately above the callus. Roots developed from the latter had integrated root and shoot vascular systems, whereas those originating from the callus were not connected vascularly; however, this connection could be created during *ex vitro* acclimatization. The implications of the findings for increased plant yields through indirect morphogenesis were discussed.**

Key words: Eucalyptus, indirect organogenesis, microscopy.

INTRODUCTION

There is increasing interest and associated activities with respect to genetic improvement (via mutagenesis and genetic engineering) and conservation of germplasm of Eucalyptus species used in the forestry industry (Mycock et al., 2004; Harfouche et al., 2011). As previously discussed, such activities generally rely on high yield *in vitro* culture protocols for plant regeneration through a callus stage (Watt et al., 2003; Hajari et al., 2006). Although both the indirect regeneration routes of somatic embryogenesis and organogenesis have been attempted by numerous research groups, the achievable plant yields have been low (Le Roux and van Staden, 1991; Watt et al., 2003; Dibax et al., 2010), which may be ascribed to the small size of the callus produced. Reports from our laboratories and those of others (Tibok et al., 1995; Sartoretto et al., 2002) indicate that Eucalyptus callus cells appear to have a restricted number of cell divisions before differentiation is triggered. Efforts to prolong the period of active cell division and/or halt differentiation through repeated subcultures onto fresh auxin-enriched (amongst others) media have thus far been unsuccessful. This limit on callus size hinders the potential applications of indirect morphogenesis. For example, most of the attempts at genetic transformation of Eucalyptus have not been particularly successful with this being attributed mainly to low transformation efficiency (Quoirin and Quisen, 2006). An alternative or additional explanation is that the amount of callus production and the low yields of shoots (Nair et al., 2010) in the regeneration protocol are insufficient to allow for the recovery of transgenic plants. This is further exacerbated by the low percentage of transformation events and the stress of the transformation manipulations.

An indirect organogenic protocol was developed in our laboratories for improved commercially important *E. grandis and E. grandis* x *urophylla* clones (Hajari et al., 2006) and a similar protocol has been reported by Dibax et al. (2010). Whereas that of the latter used cotyledonous material (that is unknown genotypes), that of Hajari et al. (2006) makes use of *in vitro* shoots produced from clonal parent plants and thus the

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starting material is not only advantageously juvenile but can be taken from the well-defined and genotypically-superior material used in clonal production. Even though the yields of the Hajari et al. (2006) protocol are similar to those of others (Tibok et al., 1995; Watt et al., 2003; Dibax et al., 2010), they are still inadequate for effective use in genetic manipulations and conservation programmes. It is proposed that increased shoot regeneration is dependent on prolonging the period of active cell division and/or halting differentiation through strategies such as repeated subcultures onto fresh auxin-enriched (amongst others) media before cell differentiation is initiated. Towards this end, the present study aimed at investigating the morphogenic events and timing of shoot differentiation from callus cells of a highly selected and superior hybrid of importance to the South African forestry industry. Further, as the success of a micropropagation protocol is the attainment of fully functional plants, root ontogeny was also investigated to establish the level of integration and connection between the shoot and root systems.

MATERIALS AND METHODS

Plant material, callus induction, shoot and root initiation

In this study, the same clone (*Eucalyptus grandis urophylla* hybrid) was used and its growth conditions, production of *in vitro* shoots, indirect organogenesis, plant regeneration and acclimatisation were as described by Hajari et al. (2006). For plant regeneration via indirect organogenesis, the starting explants were fragmented *in vitro* shoots (1.5 to 4 cm), from which the axillary buds were removed. The conditions for the culture stages were: Stage 1, *(callus production and shoot regeneration)* - 5 shoots/40 ml of Murashige and Skoog (1962) (MS) salts and vitamins, 5 mg l^{-1} (28.54 µM) indole-3-acetic acid (IAA), 0.25 mg l^{-1} (1.11 µM) benzyl amino purine (BAP), 30 g l^{-1} sucrose and 4 g l^{-1} Gelrite™ for 4 weeks and sub cultured onto this medium for another 4 weeks; Stage 2, *(shoot growth)* - regenerated shoots were excised and individually cultured on 10 ml MS salts and vitamins, 15 g l^{-1} sucrose and 4 g l^{-1}Gelrite™ for one week; Stage 3, *(rooting)* - shoots were plated individually on ¼ MS salts and vitamins medium containing 15 g l^{-1} sucrose, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 4 g l^{-1} Gelrite™ and 0.5 mg l^{-1} (2.45 µM) IBA for four weeks. In addition to the protocol of Hajari (Protocol A) described above, two others were used. Protocol B involved a pre-treatment of 1 g of explant per RITA® vessel (Berthouly and Etienne, 2005) for 24 h after which the material was subjected to protocol A. In Protocol C, explants were subjected to protocol A up to the end of 4 weeks of stage 1 (that is up to subculture), after which the equivalent of 1 g explant was placed in RITA® vessels for 24 h and then re-planted on the solid medium of Protocol A. Each RITA® vessel contained 200 ml medium which comprised MS nutrients, 30 g l^{-1} sucrose and 5 mg l^{-1} (22.2 µM) BAP; explants were flushed for 30 s every 10 min. RITA® culturing took place in the dark and all other growth conditions, as well as acclimatisation procedures as described by Hajari et al. (2006).

Histological studies

Light microscopy was used to assess callus, shoot organogenesis, callus-shoot connection and shoot-root vascular connections of material produced using Protocol A. To follow shoot organogenesis, samples of callus were initially taken after 15 days and thereafter every seven days for the 8 weeks of stage 1. Shoot-root vascular connections were assessed from material taken 24 and 72 h into stage 3. All samples were viewed and photographed with a stereo microscope (Nikon SMZ660) prior to the embedding procedure. The samples were fixed in 3% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 12 h at room temperature, rinsed in phosphate buffer (3 x 10 min) and then post fixed in 1% (w/v) osmium tetroxide for 1 h. After dehydration through an ethanol series (10, 25 and 50%), the samples were placed in 1% (w/v) uranyl acetate in 70% ethanol for 1 h and then in 100% ethanol. The material was infiltrated and then polymerised in epoxy resin (Spurr, 1969). Sections (1 µm) were stained with Toluidine blue (1% [w/v] Toluidine blue and 1% glycerol (v/v) in 1% [w/v] Na bicarbonate) and mounted on glass slides using DPX (BDH). The sections were viewed and photographed with a light microscope (Zeiss Axiophot). At least ten samples from each sampling point were sectioned and viewed.

RESULTS AND DISCUSSION

Since the work reported by Hajari et al. (2006), we have tested various media to increase the cycles of cell division and/or delay (prior to) the onset of cellular differentiation to increase the percentage plantlet regeneration without any success (results not shown). The presently tested strategies attempted to improve regeneration efficiency; an example can be seen from Table 1 which tested treatment with a cytokinin (BAP) for 24 h; however, there were no significant differences in organogenic regeneration amongst treatments. These results are in agreement with those of Arora et al. (2009). Nevertheless, when explants were pre-treated with BAP prior to callus induction (stage 1), as predicted, the callus mass decreased. However, the final yield of shoots was numerically higher, thereby indicating that BAP increased the conversion efficiency of the undifferentiated cells into regenerated shoots. It is proposed that the continually observed high variability in the regenerative potential is the result of the heterogeneity of the callus. As described by Blakeway et al. (1993), *E. grandis* and *E. grandis* x *camaldulensis* callus, albeit small, usually comprised three different types of cellular masses (Type I, II and III) the proportion of which is unpredictable. Hence, in order to improve shoot yields, it is not only necessary to increase callus cell numbers but also to produce the type of callus with the highest regenerative potential.

At day 15, the calli of *E. grandis* x *urphylla* exhibited morphology identical to that described by Blakeway et al. (1993) for leaf explants of another hybrid, viz. three clearly discernible cell masses, nodular shiny/glassy that ranged in colour from white through yellow to green (Type I); brown and mottled (Type II) and white, crystalline and reflective (Type III) (Figure 1; Plate 1). This indicated that such callus morphology is a representative of *Eucalyptus per se* rather than being representative of the species/hybrid and cell/tissue from which they were derived.

Table 1. The effect of an acute benzyl amino purine (BAP) pretreatment (5 mg l^{-1} = 22.2 µM) on the production of callus and shoots from 1 g of *in vitro E. grandis* x *urphylla* starting material.

Protocol	Average per 1 g of starting explants		
	Callus fresh mass (g)		Number of regenerated shoots
	4 (weeks)	8 (weeks)	8 (weeks)
A (control)	7.21 ± 2.88[a]	34.39 ± 19.17[a]	372 ± 212[a]
B	2.78 ± 0.78[b]	35.08 ± 34.11[a]	1521 ± 1434[a]
C	5.57 ±1.2[ab]	65.52 ± 36.89[a]	418 ± 430[a]

The material was exposed to the cytokinin either prior to the organogenic procedure (Protocol B) or at subculture during the procedure (Protocol C). Protocol A was the standard procedure of Hajari et al. (2006). (n = > 9 and p < 0.05, a – b comparisons per column).

Histologically, the 15 day old callus comprised several discrete areas of tightly packed and cytoplasmically rich meristematic-like cells which were surrounded by larger, more highly vacuolated cells (Figure 1; Plate 2). Such a composition, which relates to the gross morphology of the calli, is common to calli of many species (George, 1993), including other Eucalyptus (Blakeway et al., 1993) and all subsequent studies in our laboratories). Generally, Type I callus consisted mainly of meristematic cells, Type III contained mainly large, highly vacuolated cells, whilst Type II had a mixture of both. According to the study of Cassels (1979), the meristematic regions contribute to callus expansion or cells that differentiate into xylem and phloem elements. It is also generally accepted that Type III callus does not have regenerative capacity (George, 1993; Vasil and Thorpe, 1994). It was also noteworthy that at this early stage (15 days) there were distinct areas of cellular differentiation/organisation (Figure 1; Plate 2). Furthermore, such areas were juxtaposed to well- defined vascular tissue elements and when these were observed in longitudinal section they appeared to be linked (Figure 1; Plate 3). It is possible that these meristematic cells were acting in a procambium/cambial-like manner, as seen in *Populus euphratica* (Ferreira et al., 2009).

At day 22, the vascular strands were surrounded by loosely packed parenchyma cells (Figure 1; Plate 4). At the surface of the calli (Figure 1; Plates 4 and 5), there were clearly visible nodular type structures which comprised small tightly packed meristematic like cells with distinct nuclei. However, within the nodules there was no clear layer/tissue organisation reminiscent of a shoot axillary meristem (Figure 1; Plate 5). These results indicate that shoot cellular differentiation is initiated between 15 and 22 days in culture. Hence, it is suggested that repeated subcultures on high auxin medium should be done at this stage, in order to stimulate further callus proliferation.

At the end of the first culture of stage 1 (28 days) the nodules seen on the surface of the callus were apparently no larger than the previous week, although the vascular network underlying these structures was far more developed (Figure 1; Plate 6). The overall histology of regions of the callus (Figure 1; Plate 6) at this stage was reminiscent of a radial longitudinal section through a bud. Working with Pelargonium, Chen and Galtson (1967) described a similar early development of vascular strands and they linked these with shoot formation, whilst Arvore et al. (2004) found that *in vitro*-regenerated adventitious buds of *E. grandis* x *urophylla* had vascular connections with the vascular system of the original stem explant. Further, Gaba et al. (1999) showed a similar histology to that shown in Figure 1; Plate 6 in portions of callus derived from cotyledonary tissues of melon. Since similar development was also observed in *E. camaldulensis* (Dibax et al., 2010) it could be hypothesized that in the Eucalyptus callus the outer 'meristematic like' regions (nodules that protrude from the callus, Figure 1; Plates 4, 5 and 6) were acting in a pre-corpus and pre-tunica manner and differentiating into adventitious buds.

By day 36, the non-morphogenic crystalline callus had increased in size (Figure 2; Plate 7). The rest of the callus appeared nodular and took on a greener hue and in some cases turned red; such pigmentation has been recorded in the callus of *Eucalyptus* spp., examples are *E. globulus* and *E. nitens* (Bandyopadhay et al., 1999) and *E. camaldulensis* (Dibax et al*.,* 2005).

The nodular surface layers of the callus were more prominent (Figure 2; Plate 7) and were covered with a darkly stained epidermal like layer (Figure 2; Plate 8). Deeper within the callus, the vascular tissue had differentiated further and cambium was clearly seen parallel to the vascular elements (Figure 2; Plate 9), which again were subtending and connecting the nodules. Ultimately the surface nodules differentiated into classic adventitious buds (Figure 2; Plate 10) with distinct tunica and corpus layers with associated leaf and bud primordia (Figure 2; Plate 9). Subsequent development appeared similar to that of a normal shoot bud. A similar pattern of shoot development was reported in *E. globulus* (Azmi et al., 1997).

Throughout stage 1 at each sampling time there was a high degree of variation amongst individual calli regarding the proportion of crystalline and nodular masses (compare Plates 1 (day 15); 5 (day 22); (Figure 1)); 7 (day 28) and 10 (day 50); (Figure 2). The general trend

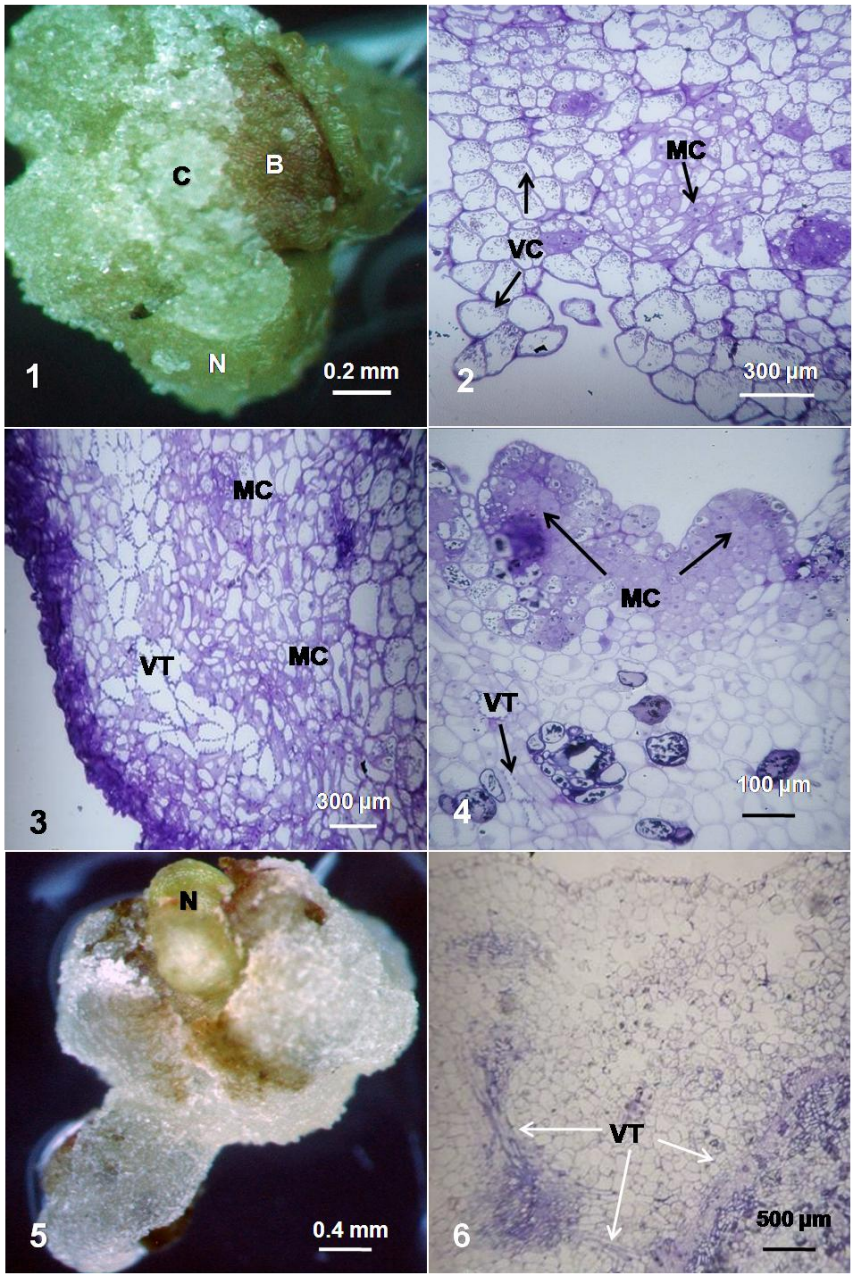

Figure 1. (Plates 1 – 6) Callus developed rapidly from the fragmented shoot material and three distinct types were discernible (Plate 1). The callus comprised both tightly packed meristematic like cells and larger highly vacuolated cells (Plate 2). Vascular elements were the first discernible tissues to be created in the callus (Plate. 3) these subtended the developing nodular callus regions (Plates 4 and 5) and with further development were seen to link the meristematic regions (Plate 6). B – Brown callus; C – crystalline callus; N – nodular callus; MC – meristematic like cells; VC – vacuolated cells; VT – vascular tissues.

appeared to be an increasing production of crystalline callus over time. This was also reflected at the histological level where there were relatively few areas of cellular organisation scattered in much larger areas of non-morphogenic type III callus. Collectively, this explains the repeatedly observed high variation amongst calli with respect to shoot yield. The data shown in Table 1 are such an example. In *Digitaria eriantha*, the increased proportion in the number of large, highly vacuolated cells over time was prevented by replacing half of the sucrose supply in the culture media with simple sugars (fructose, glucose, ribose) (Watt et al., 1989); however, this strategy has not been successful with Eucalyptus embryogenic and organogenic calli

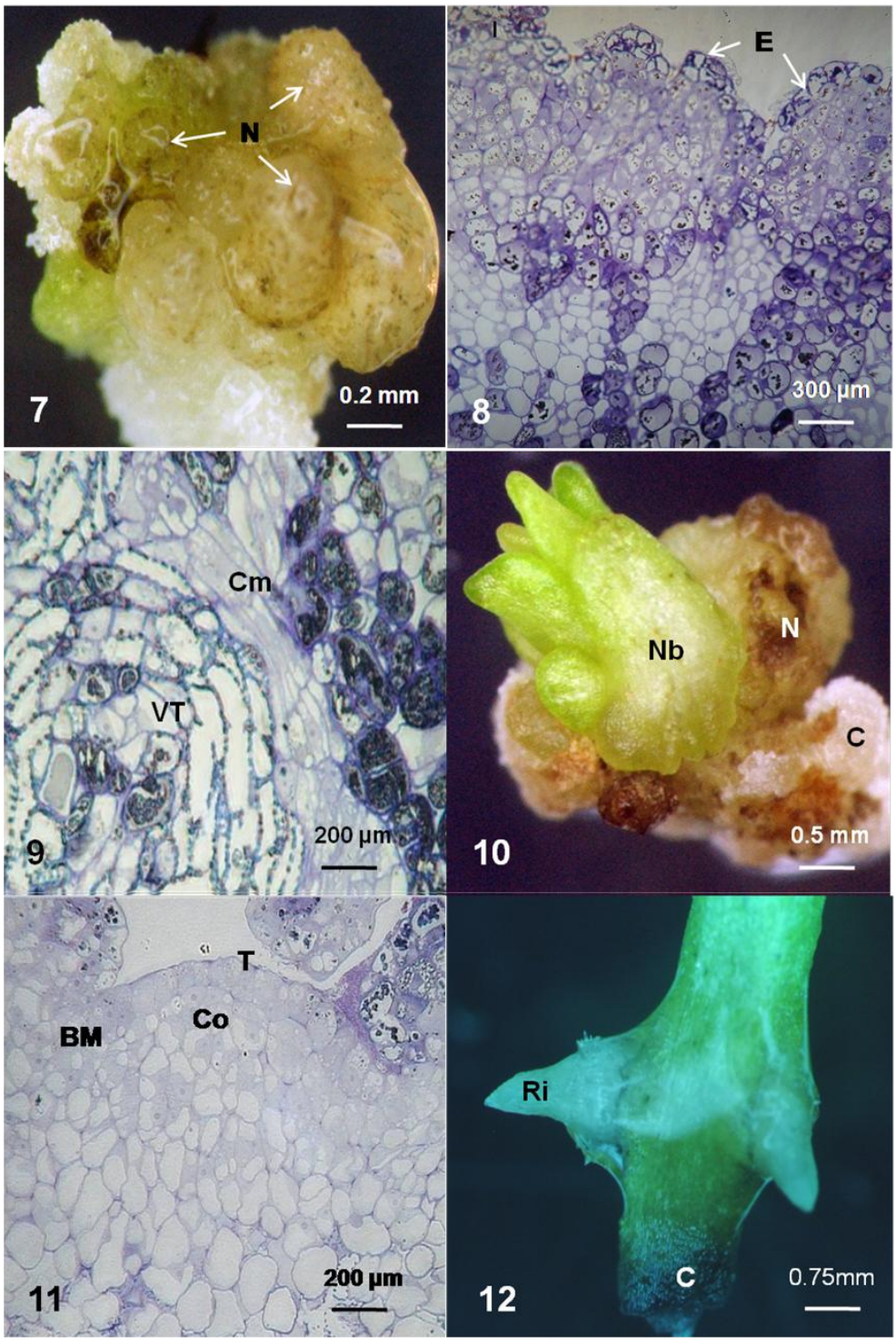

Figure 2. (Plates 7 – 12) After the first sub-culture (28 days) the callus was predominantly nodular (Plate 7) and tissue development was more pronounced with clearly discernible epidermal layers (Plate 8) and presumably functional and growing vascular systems. The latter were derived from well organised and defined cambium (Plate 9). Several adventitious buds developed per plated explant (Plate 10). These exhibited typical bud histology with distinguishable corpus and tunica layers and associated bud meristems (Plate 11). When the adventitious shoots were allowed to grow on plant growth regulator free medium roots were generated from the shoot region above the cut caused during isolation (Plate 12). BM – bud meristem; C – crystalline callus; Cm – cambium; Co – corpus layer; E – epidermal layer; N – nodular callus; Ri – root initial; T – tunica layer.

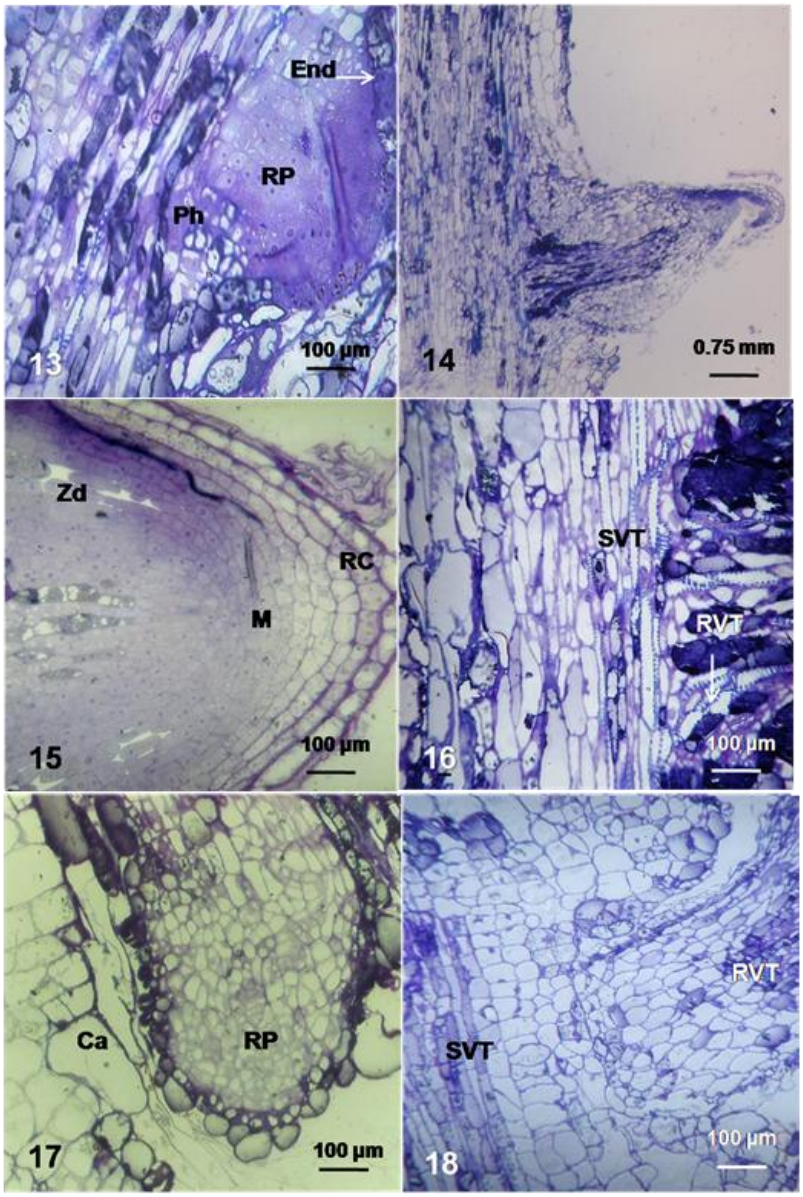

Figure 3. (Plates 13 – 18) The root primordia were generated from the pericycle between the phloem of the vascular elements and the endodermis (Plate 13). As the primordia grew they ruptured through the stem tissues (Plate 14) and were generated from typical root apical meristems (Plate 15). The vascular tissues of the nascent roots were fully integrated with the shoot vascular systems (Plate 16). In some cases roots developed from the callus at the base of the isolated stems (Plate 17) but the vascular systems of these roots were not connected to the stem (Plate 18). Ca – Callus; End – endodermis; M – meristem; Ph – phloem; RC – root cap; RP – root primordium; RVT – root vascular tissue; SVT – stem vascular tissue; Zd – zone of differentiation.

(results not shown).

At the end of the eight week culture period the shoots were transferred to rooting medium. Within three days of planting root initials were visible at the base of the stems and after 4 weeks 74% of the material had rooted. In all cases callus formed at the cut surface of the stem, presumably in response to the wounding excision during shoot isolation. The roots developed from both the region of the stem immediately above the callus and from the callus itself (Figure 2; Plate 12). Woody plant adventitious roots can be formed either directly from vascular cambium or indirectly via differentiation from callus (Altamura, 1996) and in the present investigation both developmental pathways were observed (Figure 3; Plates

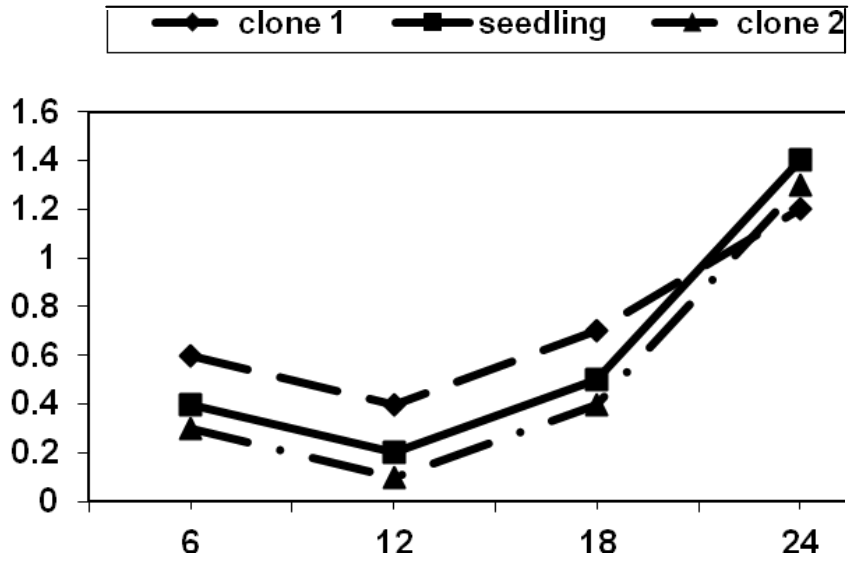

Figure 4. Change in root number per plantlet during acclimatisation of two micropropagated clones and seedlings. n = 120; * = significantly different values at days 12 and 24 ($p < 0.05$, ANOVA).

13 to 18).

Of the twenty samples sectioned, only half showed true vascular connection between the shoot and nascent adventitious root. It is important to note that this is based on observations from serial sections across the entire width of each sample. In the majority of such cases, the root had developed from the region of shoot immediately above the callus layer (Figure 2; Plate 12). The root primordia meristems appeared to be derived from the stem pericycle; that is the parenchyma layer between the endodermis and the phloem (Figure 3; Plate 13) as proposed by Soh and Bhojwani (1999). It is therefore suggested that meristematic primordia were formed through division and differentiation of these pericycle/cambium derivatives and they gave rise to root initials that ultimately ruptured through the endodermal layer (Figure 3; Plate 13), the covering cortex and emerged as root initials (Figure 3; Plate 14). A similar process of adventitious root formation has been recorded in *E. globulus* (Baltierra et al., 2004). The root initials exhibited classic tissue organisation of root cap subtended by a tightly packed meristem from which differentiation was occurring (Figure 3; Plate 15). The vascular tissue of the nascent root initial was integrated with and connected to the stem vascular cylinder (Figure 3; Plate 16).

Roots also developed from the callus at the base of the stem (Figure 3; Plate 17). These roots, although containing their own vascular tissues were not connected with the vascular systems of the stem (Figure 3; Plate 18).

All the rooted shoots were acclimatised and 70% of the plants survived this process even though based on the histological investigation, it was estimated that approximately 50% of the material had vascular connections between the shoot and root. Two possibilities exist to account for this difference: 1) During the acclimatisation process, the connections between shoot and root were created; or 2) the 'unconnected' roots were shed and new roots were produced. Data from our laboratories support the latter option. Repeatedly, we have found primary root number to increase from both Eucalyptus seedlings and micro propagated plantlets after approximately 18 days of acclimatization (an example is shown in Figure 4).

Conclusion

This detailed histological study showed that: 1) Eucalyptus calli induced by 5 mg l^{-1} (28.54 µM) IAA and 0.25 mg l^{-1} (1.11 µM) BAP exhibit signs of shoot morphogenesis as early as 15 days after culture initiation; 2) The shoots regenerated from shoot- derived callus had no organised cellular connection with the original explant tissue. The former suggests that greater plant yields through increased callus cell proliferation may be achieved through subculture onto high auxin medium after 2 weeks in culture, a focus of planned investigations. The latter indicates an adventitious origin and hence, chances of genetic variability amongst the regenerants (somaclonal variation). This is an important factor that requires further investigation and needs to be taken into account in planned genetic manipulations and *in vitro* conservation.

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REFERENCES

Altamura MM (1996). Root histogenesis in herbaceous and woody explants cultured *in vitro*: A critical review. Agronomie 16:589-602.

Arora K, Sharma M, Sharma AK (2009). Control of pattern of regenerant differentiation and plantlet production from leaflet segments of *Azadirachta indica* A. Juss (neem). Acta Physiol. Plant. 31(2):371-378.

Azmi A Noin M, Landré Prouteau M, Boudet AM, Chriqui D (1997). High frequency plant regeneration from *Eucalyptus globulus* Labill. Hypocotyls: Ontogenesis and ploidy level of the regenerants. Plant Cell Tiss. Org. Cult. 51:9-16.

Baltierra XC, Montenegro G, De Garcia E (2004). Ontogeny of *in vitro* rooting processes in *E. globulus*. *In vitro* Cell Dev. Biol–Plant 40:499-503.

Bandyopadhay S, Cane K, Rasmussen G, Hamill JD (1999). Efficient plant regeneration from seedling explants of two commercially temperate Eucalypt species – *Eucalyptus nitens* and *Eucalyptus globulus*. Plant Sci. 140:189-198.

Berthouly M, Etienne, H (2005). Temporary immersion system: a new concept for use liquid medium in mass propagation. In: Hvoslef-Eide K, Preil W (Eds) Liquid Culture Systems for *in vitro* Plant Propagation. Springer, Dordrecht, pp. 165-195.

Blakeway FC, Herman B, Watt MP (1993). Establishment of cell suspension cultures of *Eucalyptus grandis* and *Eucalyptus grandis* x *camaldulensis*. S. Afr. For. J. 166: 17-25.

Chen HR, Galtson AW (1967). Growth and development of Pelargonium pith cells *in vitro*. II Initiation of organised growth. Physiol. Plant. 20:533-539.

Dibax R, De Loola Eisfeld C, Cuguel FL, Koehler H, Quoirin M, (2005). Plant regeneration from cotyledonary explants of *Eucalyptus camaldulensis*. Sci. Agricola 62(4):406-412.

Dibax R, Quisen RC, Bona C, Quoirin M (2010) Plant regeneration from cotyledonary explants of *Eucalyptus camaldulensis* Dehn and histological study of organogenesis *in vitro*. Braz. Arch. Biol. Technol. 53(2):311-318.

Ferreira S, Batista D, Serrazina S, Pais MS (2009) Morphogenesis induction and organogenic nodule differentiation in *Populus euphratica* Oliv. leaf explants. Plant Cell Tiss. Org. Cult. 96(1):35-43.

Gaba V, Schlarman E, Elman C, Sagee O, Watad AA, Gray DJ (1999). *In vitro* studies on the anatomy and morphology of bud regeneration in Melon cotyledons. *In vitro* Cell Dev. Biol. Plant 35:1-7.

George EF (1993). Plant propagation by tissue culture. Exegetics, England, pp. 3-36.

Hajari E, Watt MP, Mycock DJ, McAlister B (2006). Plant regeneration from induced callus of improved *Eucalyptus* clones. S. Afr. J. Bot. 72:195-201.

Harfouche A, Meilan R, Altman A (2011). Tree genetic engineering and applications to sustainable forestry biomass production. Trends Biotechnol. 29:9-17.

Le Roux JJ, van Staden J (1991). Micropropagation and tissue culture of *Eucalyptus* – A review. Tree Physiol. 9:435-477.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Mycock DJ, Blakeway FC, Watt MP (2004). General applicability of *in vitro* storage technology to the conservation and maintenance of plant germplasm. S. Afr. J. Bot. 70:31-36.

Nair SG, Vijayalakshmi C (2010). Indirect organogenesis of a superior clone, ITC3 of *Eucalyptus tereticornis* Res. Crops 11(2):532-535

Quoirin M, Quisen R (2006). Advances in genetic transformation of *Eucalyptus* species. Mol. Biol. Trop. Plant., pp. 41-56.

Sartoretto LM, Cid LPB, Brasileiro ACM (2002). Biolistic transformation of *Eucalyptus grandis x E. urophylla* callus. Funct. Plant Biol. 29:917-924.

Soh WJ, Bhojwani SS (1999). Development and structural aspects of root organogenesis. In: Soh WJ, Bhojwani SS (Eds) Morphogenesis in plant tissue cultures. Kluwer Academic Publishers, Dordrecht. pp. 133-147.

Spur AR (1969). A low viscosity epoxy resin embedding medium for electron microscopy. J. UltraStruct. R. 26:31-41.

Tibok A, Blackhall NW, Power JB, Davey MR (1995). Optimized plant regeneration from callus derived from seedling hypocotyls of *Eucalyptus urophylla*. Plant Sci. 110:139-145.

Vasil IK, Thorpe TA, (1994). Plant cell and tissue culture, Springer, pp. 293-312.

Watt MP, Blakeway FC, Mokotedi MEO Jain SM (2003). Micropropagation of *Eucalyptus*. In Jain SM, Ishii K (Eds), Micropropagation of Woody Trees and Fruits. Kluwer Academic Publishers, Dordrecht. 75(2):217-244.

Watt MP, Mycock DJ, Cresswell CF (1989). Plant regeneration by somatic embryogenesis in *Digitaria eriantha* (Steud) subsp. *eriantha*. Proceedings of the XVI International Grasslands Symposium, Nice, France. 1:421-422.