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# Ageing delays the cellular stages of adventitious root formation in pine

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## Summary

Vegetative propagation programs internationally are affected by the significant decline of rooting success as trees mature. This study compared the cellular stages of root formation in stem cuttings from 15-week-old (juvenile) and 9-y-old (mature) stock plants of the slash × Caribbean pine hybrid (*Pinus elliottii* var. *elliottii* × *P. caribaea* var. *hondurensis*). The cellular stages of root formation were the same in both juvenile and mature cuttings, beginning with cell divisions of the vascular cambium forming callus tissue. Within the callus, tracheids differentiated and elongated to form root primordia. Roots in juvenile cuttings developed faster than those in mature cuttings and the juvenile cuttings had a much higher rooting percent at the end of the study (92% and 26% respectively). Cuttings of the two juvenile genotypes had more primary roots (5.5 and 3.3) than the three mature genotypes (0.96, 0.18 and 0.07). The roots of juvenile cuttings were more evenly distributed around the basal circumference when compared with those on cuttings from the mature genotypes. Further work is needed to improve understanding of physiological changes with maturation so that the rooting success and the speed of development in cuttings from mature stock plants can be optimised, hence improving genetic gain.

*Keywords*: vegetative propagation; juvenility; maturation; rooting capacity; adventitious roots; histology; *Pinus*

## Introduction

As plants age they pass through different ontogenetic phases including embryonic, juvenile and adult vegetative to adult reproductive phases (Greenwood 1987; Diaz-Sala *et al.* 1996). There is some discussion as to whether the adult vegetative phase is indeed separate to the mature reproductive phase (Leakey 2004) as the adult vegetative tissues are capable of switching to flowering. We argue, however, that the physiology of trees and branches during reproductive phases is quite different from the physiology of vegetatively growing adult branches. Regardless of these views, within each of these phases physiological aging also occurs, characterised by a slowing of growth processes. In this study we tested plants with different physiological ages within the vegetative stage(s). Many changes occur as trees age, but the most economically significant is a loss in rooting success of cuttings taken from increasingly mature stock plants (Diaz-Sala *et al.* 1996; Greenwood *et al.* 2001; Dick and Leakey 2006). This affects many enterprises, from the propagation of rare or endangered species to production forestry. In forestry, clonal pine production systems have long been recognised for the marked decrease in rooting between seedlings or hedged stock plants and large mature trees (Hackett 1985; Greenwood 1987; Diaz-Sala *et al.* 1996; Horgan *et al.* 1997).

The hybrid between slash pine (*Pinus elliottii* Engelm. var. *elliottii* Little & Dorman) and Caribbean pine (*P. caribaea* Mor. var. *hondurensis* (Seneclauze) Barrett & Golfari) is the most important softwood plantation taxon in subtropical Australia (Nikles 1996, 2000; Dungey 2001). Unfortunately, as production hedges age cuttings taken from hedges exhibit lower rooting percentages and inferior root systems. To overcome this issue and maintain high strike rates, the goal is to maintain a juvenile physiology in cuttings and their stock plants. Various maintenance systems and treatments have been suggested to improve the strike rate, including hedging and serial propagation (Leakey 1983; Howard 1994; Hamann 1998; Mitchell *et al.* 2004). There appears to be confusion, however, whether the processes lead to a reversion to juvenility or to a temporary reinvigoration (Hackett 1985). This has implications for other characteristics including spiral grain, wood density and growth rates.

Before treatment or managerial suggestions can be identified as rejuvenation or reinvigoration, an understanding of how juvenility is expressed in the several stages of root developmental is essential. Some studies suggest easy-to-root species form roots directly from the stem (direct root formation), while in difficult-to-root species a callus first develops and roots arise within the callus tissue (indirect root formation) (Goldfarb *et al.* 1998; Hamann 1998; Ballester *et al.* 1999). However, some studies have found no difference in the stages of root formation (Diaz-Sala *et al.* 1996; Ballester *et al.* 1999 — both direct; and Cameron and Thomson 1969; Greenwood *et al.* 2001 — both indirect) but instead, the speed of root development (Goldfarb *et al.* 1998) and final rooting percent were altered (Ballester *et al.* 1999; Greenwood *et al.* 2001; Dick and Leakey 2006). Previous work by the authors determined that in mature cuttings a callus stage is required in the basal end of cuttings prior to root formation (Rasmussen *et al.* 2009).

Very few studies have investigated differences in rooting between cuttings of the same taxa from stock plants in the juvenile and mature vegetative states. The few studies that have compared juvenile and mature rooting stages have either compared epicotyl and hypocotyl root development (Diaz-Sala *et al.* 1996) or compared different locations on a single mature tree (Ballester *et al.* 1999) or hedges (Tchoundjeu and Leakey 1996). The trouble with hypocotyl–epicotyl comparisons is that the hypocotyls contains pericycle cells that also provide the founder cells for lateral root development while epicotyls do not (Goldfarb *et al.* 1998) and the process of lateral root formation is different from that of adventitious root formation specifically because of the lack of this tissue. Comparing cuttings from locations on the same tree is confounded by different chronological ages. That is, apical shoots are often more recently formed than basal shoots but also form from ontogenetically older tissues and so can be interpreted as more mature. Dick and Leakey (2006) compared cuttings of suckers from two seasons, and cuttings from two different ages of lateral shoots in the sexually-active canopy. This study was very detailed in its characterisation of the different cutting tissues or materials, but it still compared cuttings from different locations within a tree and which had experienced different environments.

This study aimed to fill the gap in knowledge on differences in root formation between physiologically juvenile and mature cuttings (within a single ontogenetic phase) of the one taxon and from the same tissue or organ of stock plants of similar size or height.

## Methods

### Genetic material

Five genotypes were used. For mature genotypes, two clones were selected based on availability of seed stock (used for juvenile genotypes), availability from hedges (in this case archive hedges) and putative differing strike rates (clones $A_M$ and $B_M$). The hedge plants from which this material was collected were planted in 2000 (from seed originally sown in 1998). In addition, cuttings from a clone that has demonstrated excellent rooting for 23 y from seed was included in the study (clone $C_M$). The clone $C_M$ hedge plants were planted in 2001 (seed originally sown in 1984). These three genotypes were all considered mature ($_M$). The two juvenile genotypes (Genotype $D_J$ and Genotype $E_J$) were the parental material for clones $A_M$ and $B_M$ respectively. These were family (rather than clonal) material and were derived from the same parents as $A_m$ and $B_m$. Seed from the two families were sown in potting media in cells 4.5 × 4.5 × 7.5 cm of Queensland Native Tube (QNT) trays and placed in a glasshouse at Gympie, Queensland, on 27 October 2006. On 22 December 2006 the trays with the seedlings were transferred to a nursery where they received 90 seconds of water from aerial sprinklers (Nelson™ rotators, Nelson, Darra, Queensland), every hour between 6 am and 6 pm — about 6 mm day$^{-1}$ in total. When the seedlings were about 10 cm tall they were topped to induce branching.

The seedling stock plants were kept in full sunlight after topping but remained in pots while the mature stock plants used were in-ground operational hedges kept at 30 cm height in full light (in south-eastern Queensland photosynthetic photon flux density (PPFD) during summer months exceeds 2000 $\mu$mol m$^{-2}$ s$^{-1}$ and conditions were mostly fine for the length of the experiment). The hedges were watered at least once every week; the application varied in response to demand in order to prevent wilting or soil drying and averaged 4–6 mm day$^{-1}$ during summer.

### Rooting trials

Lateral tip cuttings were harvested from both the cuttings hedges and seedlings at Toolara Nursery (25°58′47″S, 152°53′52″E) on 12 February 2007. The cuttings were 10 cm long, at least 3 mm in diameter and had well-developed primary needles and some secondary needle development. In the case of the juvenile cuttings, no secondary needles were present. All shoots, from which the cuttings were taken, were about 5–6 weeks old (the juvenile stock plants having been topped 5 weeks previously and mature hedges 6 weeks previously). The cuttings were stored in cooler bags or insulated boxes and then set in QNT trays in pasteurised coarse perlite:pine bark peat 1:1 (v/v) potting media in the setting area at Toolara. The media also had 0.5 kg Micromax® m$^{-3}$ and 2.5 kg Low Start Osmocote® m$^{-3}$.

Each tray contained two clones (2 blocks) with 25 cuttings from each clone and there were 10 trays in total in the propagating area (4 blocks of each genotype) resulting in 500 cuttings. Genotypes were randomly allocated to trays and the trays were randomly arranged on the benches. All cuttings were under 50% shade-cloth and received water for five minutes every hour between 6 am and 6 pm.

Samples were collected daily from day 0 (day of setting) for 5 days and then weekly from day 7 for 7 weeks. Samples were also collected at week 9 and then at the final harvest in week 14. One cutting was taken from each half-tray each sampling session (4 replicates × 5 genotypes = 20 samples). A final harvest was conducted at week 14 (22 May 2007) on all remaining material (12 in each block of each genotype = 48 for each genotype in total). At this final harvest the number of roots was recorded along with the number of quadrants around the base from which roots emerged. Roots were defined as protrusions larger than 2 mm and primary roots were those growing directly from the callus at the base of the cutting.

### Microscopy

The basal end of each cutting was removed and sliced longitudinally before being placed in labelled microscopy cassettes (Cat. no. RCH30, ProSciTech, Thuringowa, Australia). The cassettes were placed in water and microwaved (900 W, 2450 MHz, NN-7855, Panasonic) on high for 8 minutes. The samples were then left in the hot water for an hour. The water was replaced with formaldehyde acetic acid (FAA) and left for 24 h.

The samples were dehydrated using an ascending series of tert-butyl alcohol (TBA), from 70% to 100%, and the cassettes left in each concentration for 1 h. After 6 h the existing 100% TBA was refreshed and left overnight. The TBA was replaced with xylene and left for 1 h under vacuum in an oven (800 W, Vacuum Oven, Thermoline Scientific Equipment Pty Ltd, Sydney, Australia). Xylene was replaced by liquid paraffin oil and after 2 h this was replaced with melted Shandon Histoplast Wax (B1002490,

Thermo, Noble Park, Australia). The wax was refreshed after 6 h and left for 5 days to infiltrate under a vacuum.

Samples were then placed in disposable base moulds (RM475-4, ProSciTech, Thuringowa, Australia) and sectioned on a Shandon Finesse 325 microtome (Thermo Electron Corporation, Noble Park, Australia). The slides were stained with Safranin O and Fast Green and coverslips were mounted and left to dry. Slides were examined for different stages of root development using a Nikon Eclipse e600 microscope and the $RT_{KE}$ SPOT (Diagnostic Instruments Inc.) camera.

### Definitions of categories

Each sample was allocated to a subcategory in each of the categories callus, tracheids and roots. The callus category was first divided according to the presence or absence (rating 0) of callus cells. These appear as non-differentiated parenchyma cells. If callus cells were present then where division had only just begun and only a very few callus cells were present these were allocated to the beginning subcategory and allocated a rating of 1. If more cells than this were present but the callus was not continuous around the base, this was allocated a rating of 2, while continuous callus around the base of the cutting was rated 3. Similarly for tracheids, no tracheids led to a rating of 0; if only a small number had begun to form in patches they were called tracheid nests, rating 1. If trails of tracheids were present but not yet obviously connected from a primordia to the main tracheids of the stem they were rated 2 and if they did connect primordia to stem they were defined as continuous and rated 3. Roots were scored according to whether they were absent (0) or present (1).

### Data analysis

GenStat Ninth Edition (Version 9.2.0.153, 2007, Lawes Agricultural Trust) was used to analyse all results. An unbalanced anova was applied to all sets of data as one of the clones was present only as mature material. Where time was a factor, the model fitted was: feature = age × time × genotype. In the final harvest data, time was not a factor and so the model was: feature = age × genotype. In the tests where clone was not significant, a second analysis was run excluding genotype. For the tests where genotype was examined, $A_M$ and $D_J$ were considered one treatment and $B_M$ and $E_J$ were considered another treatment.

## Results

### Anatomical development

The anatomy of the juvenile cuttings at the time of severance from stock plants was similar to that of the mature cuttings, with closed rings of xylem and phloem. The main difference was in the thickness of each ring, with the more mature cuttings having more cell layers of xylem and phloem.

The cellular stages and cell sizes in root formation did not differ between juvenile and mature cuttings. Cell divisions occurred in the cambial region and often (but not always) proximal to ray parenchyma. Cell division continued as the callus increased in size. Tracheids were then seen to differentiate within the callus before elongating towards existing vascular tissue and in the opposite direction towards the outer cell layers. Lastly, a root could be observed outside the base of the cutting for all five types of cutting.

### Callus and tracheid formation

There was no difference in the extent of callus formation between the two juvenile genotypes or between the three mature genotypes ($P = 0.46$) so the data was pooled to form Figure 1a. Similarly there was no difference in the extent of tracheid formation between the juvenile genotypes or between the mature genotypes ($P = 0.87$) and so data were pooled to form Figure 1b.

After seven days the juvenile genotypes already exhibited more extensive callus (higher average ratings) than the mature genotypes ($P < 0.001$), and by week 4 complete callus had formed around the basal end of the juvenile cuttings while the mature cuttings were just beginning to form callus (average rating <1).

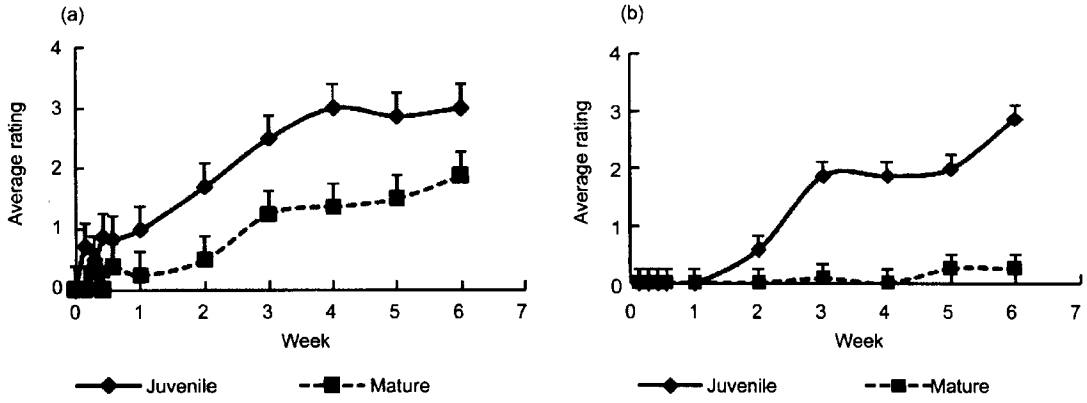


**Figure 1.** Callus growth (a) and tracheid formation (b) of cuttings from juvenile and mature stock over time. (a) If a few callus cells were present this was rated 1, many but not complete basal coverage by callus cells was rated 2, continuous callus was rated 3. (b) Similarly the presence of tracheid nests was rated 1, elongated but non-continuous tracheids rated 2 and continuous tracheary elements rated 3. Error bars are LSD bars. *n* for juvenile cuttings = 8 and *n* for mature cuttings = 12 for each point.

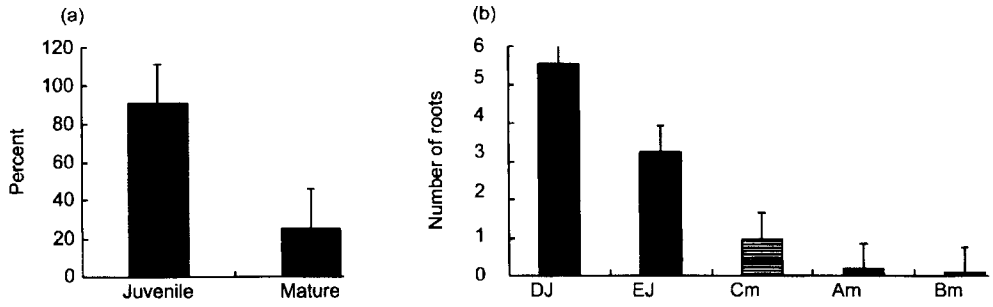


**Figure 2.** (a) Rooting percent of cuttings from juvenile and mature stock at week 14. (b) Number of primary roots produced at week 14 by cuttings of different clones. DJ and EJ are from juvenile families DJ and EJ while Cm, Am and Bm are from mature clones CM, AM and BM. Error bars are LSD bars (SE = 11.6).

The mature cuttings had not formed callus completely around the base by week 6 and had an average rating of only 1.75.

Cell division in the juvenile callus occurred at a constant rate from initiation of division within the first week up to the maximum size of the callus at week 4. For the mature cuttings, callus enlargement followed a more step-wise trend with initiation not occurring until around week 1–2. After this delay callus tissue grew until about week 3 when growth slowed until week 5 when it again increased in size.

Over time the average rating for tracheid development increased for all cuttings ($P < 0.001$) and for each age group ($P < 0.001$).

There were significant differences in the extent of tracheid formation between juvenile and mature cuttings ($P < 0.001$). This difference was visible from week 2 when the two juvenile genotypes began developing tracheid nests (average rating about 0.5). By week 3 the two juvenile genotypes had formed well-developed tracheid networks (average rating nearly 2) and by week 6 those networks were continuous from roots to the existing tracheary system (average rating of 3). The mature genotypes began to form tracheid nests only around week 5 (average rating <0.5).

### Root formation

At week 6 within maturation groupings (juvenile or mature) there were no significant differences between genotypes in the extent of root formation ($P = 0.39$) (data not shown). However, comparing maturation groupings, no roots were observed in the mature cuttings by week 6 while all the juvenile genotypes had roots at this time ($P < 0.001$) (data not shown). The interaction between genotype and age was not significant ($P = 0.82$). At the final harvest the juvenile cuttings had a significantly higher rooting percentage (92%) than the mature cuttings (26%) ($P < 0.001$) (Fig. 2a).

### Root system quality

At week 14 the juvenile genotype $D_J$ cuttings produced significantly more primary roots than the other genotypes or ages ($P < 0.001$) (Fig. 2b). The juvenile genotype $E_J$ plants produced the second-most abundant roots ($P < 0.001$) followed by $C_m$ cuttings ($P < 0.001$). Lastly $A_M$ and $B_M$ mature cuttings had produced the least number of primary roots by week 16 ($P < 0.001$).

There were no significant differences between genotypes within maturation groups in spatial distribution of roots around the base at week 14 ($P = 0.17$). However, the juvenile cuttings produced roots most evenly distributed around the base (87%), with the distribution of roots on mature cuttings averaging only 47% around the circumference ($P < 0.001$) (Fig. 3).

## Discussion

This study has shown that cuttings from juvenile stock plants progressed through the same cellular stages of root formation as did mature cuttings, but faster. By the final harvest at week 14, the cuttings from juvenile plants had a considerable start in development over those from mature plants with respect to rooting percentage, number of primary roots, and tertiary, secondary and primary root systems. Hamann (1998) also found that changes in rooting ability were not related to changes in cellular stages. In a previous study (Rasmussen *et al.* 2009) the process observed was the same as in the current study, but the coarser time scale of the earlier work meant that the cambial divisions were missed and cell divisions were observed only once in the cortex. Clone $C_M$ tended to perform better than the other two mature clones but not

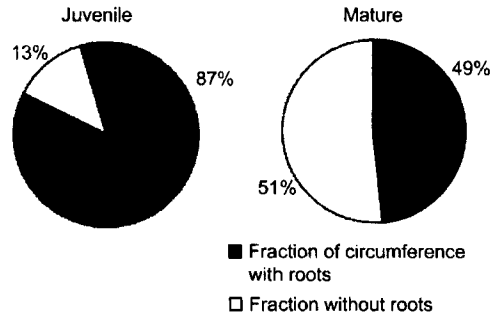


**Figure 3.** Fraction of circumference of cuttings from juvenile and mature stock exhibiting root development. LSD = 7.06.

as well as the two juvenile genotypes. This was not unexpected, as these plants have demonstrated good rooting success over 23 y. Cuttings from 1-y-old mahogany stock plants had higher rooting percentage and number of roots than cuttings from 3-y-old stock plants (Opuni-Frimpong *et al.* 2008). Unfortunately, in that study, all cuttings were treated with IBA, leaving us with no data on the 'natural' rooting ability of these materials of different ages. Some studies have shown that auxin has a greater inductive effect on juvenile than on mature cuttings, an effect that may have confounded the differences observed between the 1- and 3-y-old cuttings. As in our study, Dick and Leakey (2006) also found that rooting percentage decreased in older cuttings of *Prunus* taken from different locations within the tree.

Maturation is suggested to increase within a plant with increasing distance from the roots, as branches formed later in time (more recently) are of later developmental stages while those formed earlier were of a more juvenile stage (Leakey 2004; Dick and Leakey 2006). Based on this premise, many studies have investigated maturation effects on cuttings using shoots from different locations within a plant. For example, after comparing hypocotyl ('younger') to epicotyl ('more mature') cuttings it has been suggested that cuttings from juvenile stock plants undergo direct root formation without first forming a callus (Smith and Thorpe 1975; Goldfarb *et al.* 1998; Greenwood *et al.* 2001). However, hypocotyl anatomy is similar to root anatomy with pericycle, xylem poles and endodermis (Goldfarb *et al.* 1998). The pericycle provides the founder cells for lateral roots and nodule formation and is absent in all aerial tissues. The present study compared epicotyl cuttings of both juvenile and mature cuttings.

Other authors have compared epicotyl cuttings taken from more apical with those from more basal branches and have had very varied results. *Khaya ivorensis,* (Tchoundjeu and Leakey 1996) was found to root better from basal cuttings than from apical cuttings, while *Triplochiton scleroxylon* (Leakey and Mohammed 1985) and *Milicia excelsa* (Ofori *et al.* 1996) was found to root better from apical shoots. Even more confusingly, Husen and Pal (2007) found that cuttings taken from middle branches rooted best. Many factors change with height, including the amount of light that branches are exposed to. Leakey (1983) found lower branches rooted better under natural lighting, but when he illuminated all branches evenly all cuttings rooted equally.

Another difficulty in addressing maturational differences in rooting ability is that when comparing cuttings from mature trees with those from juvenile trees there is inevitably a size difference between the different stages of maturity, making it difficult to differentiate between the two processes. Onate and Munne-Bosch (2008) addressed this by taking cuttings of 9-y-old *Cistus clusii* plants and then using these new, smaller plants as stock plants to compare rooting between 9-y-old plants of different size. They found that rooting was better in the cuttings from the smaller plants than in those from the larger plants, demonstrating that size can interfere with maturational comparisons. Matsuzaki *et al.* (2005) addressed the same problem, not by creating small plants but by grafting shoots into the crowns of mature Japanese cedar. They compared the water relations and gas exchange of these grafted shoots with those of ungrafted intact shoots (in the same canopies) and to rooted cuttings of the shoot material. They found that the grafted (younger) shoots behaved very similarly to the intact older shoots, further supporting the theory that differences in height are important. This study did not however eliminate the possibility that hormones produced in the older material were transferred to the grafted material and hence caused 'maturation', but it does suggest that the change is signal-based and not a genetic or static state of the meristem cells.

The current study tried to avoid differences in height and tissue type by comparing rooting ability of cuttings from stock plants of different age but similar heights in order to determine maturational differences in rooting ability and features. As the same process is occurring in both juvenile and mature material, it is clearly a rate limitation that brings about the difference in performance between juvenile and mature cuttings, and this is very helpful in identifying probable mechanisms. It is now necessary to determine what is driving the change in these with age.

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