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Hybrid Pines: Opportunities for Life on the Edge©

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The hybrid pine, *Pinus attenuata* Lemmon × *Pinus radiata* D. Don was successfully tissue cultured using protocols established for *P. radiata*. This is the first published report comparing production and performance of adventitious shoots from cryopreserved cotyledons, with axillary shoots from epicotyls of the same zygotic embryo of this hybrid. Genotypes (189) were tested for shoot formation, with 74% producing epicotyl-axillary shoots and 89% producing adventitious-axillary shoots. Following 6 weeks of culture, genotypes had an overall average of 2 shoots/genotype from the epicotyl-axillary treatment and 15 from the adventitious-axillary treatment. Genotypes were serially cultured until all genotypes had 20 shoot tips per treatment suitable for rooting treatments. At the time of this presentation, shoots were being set in a high-humidity environment for root initiation.

INTRODUCTION

Interest in growing hybrid pines for commercial forestry in New Zealand has been increasing (e.g., Dungey et al., 2003; Gea et al., 2006). Important factors, including climate change and land use economics, mean that where we grow trees now may not be where we grow them in the future. Different pine species, in comparison with radiata pine (*Pinus radiata* D.Don), possess different attributes, such as tolerance to subtropical climatic conditions, better growth at higher altitudes, susceptibility to disease, and, importantly, different wood quality characteristics (Dungey et al., 2003, CAMCORE 2000). Hybridizing between different pine species occurs infrequently in the wild, and is generally restricted to closely related species, within subsections (Little and Critchfield, 1969; Price et al., 1998). Even within a subsection, resultant seed is often produced in small quantities and is often of low vigour due to parental incompatibility. However, the combination of improved control-pollination methods and propagation techniques mean that more novel combinations are becoming available for field testing.

Useful tissue culture methods include early embryo rescue methods (soon after fertilisation) and use of different propagation methods simultaneously to provide material that is either juvenile or more mature. The former can be used for initial clonal field trials and the latter for earlier seed production of F2 generation hybrid seed. Second generation seed is often more vigorous and provides opportunity for more intensive breeding programs where particular characteristics of interest are actively selected.

Recent research evaluating field performance of the *P. attenuata* × *P. radiata* hybrid (syn. P. ×*attenuradiata*) for afforestation of New Zealand high country has been encouraging (Dungey, 2008). *Pinus attenuata* has potential to contribute drought resistance, cold resistance, and resistance to damage from wet snow to *P. radiata,* which does not have those attributes (Dungey et al., 2003). Field trial

results indicated that at ages 4 and 8 years, the hybrids were the tallest taxon at the two coldest trials and had the most acceptable crop tree when compared with the pure species. At the warmer site, *P. radiata* was the tallest and *P. attenuata* was the shortest. *Pinus radiata* had the greatest snow damage, *P. attenuata* the lowest, and the hybrids were intermediate (Dungey, 2008).

This paper details recent tissue culture work with the hybrid *P. attenuata* × *P. radiata,* testing methods developed for *P. radiata* (Hargreaves et al., 2004, 2005, 2007).

MATERIALS AND METHODS

Control-pollinated *P. attenuata* × *P. radiata* seed was supplied by Proseed NZ Ltd. from their Amberley seed orchard. The *P. attenuata* (999.014 × 101.001 ATT) mother trees were crossed with a *P. radiata* pollen mix (268.248, 268.262, 268.531, 268.556, 875.242, and 880.728). Seeds were sterilised in a solution of 50% chlorodux (calcium hypochorite, 5% v/v), plus a surfactant (0.1 ml Silwet L-77 per L) for 20 min, followed by rinsing under running water overnight to facilitate imbibition. Seeds were sterilised in four batches of 50 over a week to facilitate time management in the laboratory. Following imbibition, seeds were re-sterilised using 6% hydrogen peroxide solution with 0.1 ml Silwet L-77 per L for 10 min and then rinsed three times in sterile water.

Embryos (50 per batch) were dissected onto a modified Quoirin and Lepoivre medium (LP) (Quoirin and Lepoivre, 1977; modification of Aitken-Christie et al.,1988) including 3.5 $g \cdot L^{-1}$ activated charcoal (Merck) (LPch) in petri dishes. The petri dishes with embryos were wrapped in foil and placed in a domestic refrigerator at 4 °C overnight. The following day, the cotyledons were removed from the embryo by gently snapping them off with a scalpel at the zone of abscission. After removal, the cotyledons were put onto 10 × 5 mm foil strips (1 strip per genotype) and then into 90 × 10 mm petri dishes (10 genotypes per dish) and lidded. When all 50 sets of cotyledons were prepared, the lids were taken off the petri dishes, and the cotyledons were left to dry on the laminar flow bench until the cotyledons reached a moisture content of approximately 7%. The airflow was 0.46 $m \cdot s^{-1}$, relative humidity 40% +/- 5%, and air temperature 22 +/- 2 °C.

Following drying, the foil strips with cotyledons were transferred to Nunc cryovials, with one genotype per vial, and plunged directly into liquid nitrogen (LN), where they were stored for 3 to 4 weeks. The embryos, now without cotyledons, were put onto LPch medium in 90 × 25 mm petri dishes with the radical end of the embryo embedded in the medium. All cultures were placed in a light incubator, with a photoperiod of 16 h light (photosynthetic photon flux density 80 $\mu E\ m^{-2} \cdot s^{-1}$) at 24 °C and 8 h dark at 18 °C. The petri dishes were covered with four layers of shade cloth (three layers removed after 1 week). Following storage in LN, the cryovials were thawed in 40 °C sterile water for 2 min. Care was taken to hold the vials in the vapour state of the LN storage prior to thawing to reduce the risk of the vials exploding. No cotyledons were kept in LN in this experiment. Thawed cotyledons were placed on half-strength LP medium (including microelements, iron stock, and vitamins) containing 5 mg benzylaminopurine (BA) per L ($2.22 \times 10\text{-}5\ mol \cdot L^{-1}$) and 3% sucrose (½ strength LP5) and placed in the light incubator with four layers of shade cloth covering the petri dishes. Shade cloth was reduced to two layers after 1 week and to one layer after 3 weeks. Following 28 days on half LP5 medium, cotyledons were transferred to LPch medium in petri dishes and returned to the light rig.

After 4 to 5 weeks in the light rig, the noncryopreserved embryos had their roots and part of their hypocotyls trimmed off, and were moved onto a fresh position in the petri dish where the epicotyls continued outgrowth.

All cultures were observed regularly, and cotyledons on LPch were cut to improve medium contact as adventitious shoots began to elongate. After 4 to 8 weeks culture (4 weeks cotyledon explants, 8 weeks epicotyl explants) on LPch medium, the treatments were assessed (epicotyl-axillary = Treatment 1; adventitious-axillary = Treatment 2) for survival and shoot formation. A schematic representation of treatments is given in Fig. 1 (modified from Hargreaves et al., 2005).

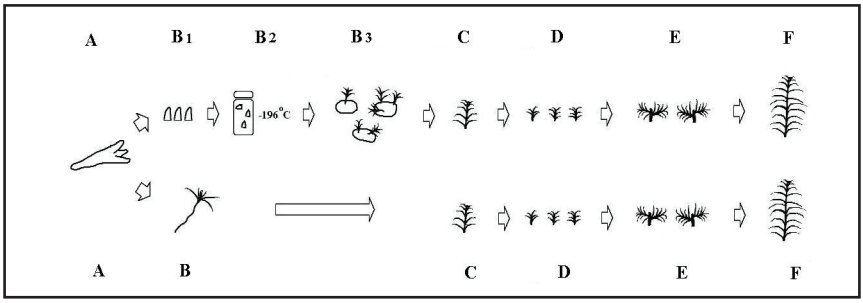

Figure 1. Schematic representation of the adventitious-axillary and epicotyl-axillary methodologies. Epicotyl-axillary (Treatment 1) lower row. A; as above. B; germination zygotic embryo minus cotyledons. C; elongating epicotyl shoot. D; subcultured shoots (shoot tips and stem segments). E; stem segments with axillary shoot formation. F; elongated shoot prior to root initiation treatment.

Adventitious-axillary (Treatment 2) top row. A; zygotic embryo. B1; cotyledons removed from zygotic embryo. B2; frozen in liquid nitrogen. B3; adventitious shoot formation from the cotyledons. C; elongated adventitious. D; subcultured shoots (shoot tips and stem segments). E; stem segments with axillary shoot formation. F; elongated shoot prior to root initiation treatment. Reproduced by kind permission from the Canadian Journal of Forestry Science (Hargreaves et al., 2005).

Following cycles of shoot amplification, 82 genotypes with 20 shoots per treatment per genotype were placed onto root initiation medium and set in potting mix (methodology fully described in Hargreaves et al., 2005). Rooting results were not available at the time of this conference.

RESULTS AND DISCUSSION

The *P. attenuata* × *P. radiata* seed used was healthy. Of the 200 seed dissected for this research, only eight were considered unsuitable for culture (Table 1). Of these

Table 1. Summary of genotype health following seed imbibition, seed sterilisation, embryo dissection, cotyledon removal, and cryopreservation.

Seeds imbibed and dissected (no.)	Sound embryos (no.)[1]	Cotyledons per sound embryo (avg. no.)[2]
200	189	6.8

[1] Some embryos were deformed (6), discoloured/rotten (2) and damaged by operator (3).
[2] The cotyledon number per embryo ranged from 4–12.

"unsuitable" seeds, two were deemed unhealthy. The remaining six had cotyledon deformity, consisting of small or bent cotyledons as if the corrosion cavity in the seed had been too small for the hybrid embryo. Cotyledon numbers ranged from 4–12 per embryo, although 12 was unusual, with only one genotype (203; data not detailed here) of the 189 put into culture having cotyledons in excess of nine per embryo. Six embryos had four cotyledons and 22 embryos had five. The rest of the embryos sampled (160) had between six to nine cotyledons.

Shade cloth was left longer on both the epicotyls and cotyledons during post-initiation/thawing than is usual in *P. radiata* (4 weeks instead of one). Work with other pine species (*P. pinaster*, *P. elliottii* × *P. caribaea;* scion, unpublished data) has indicated that more shade is necessary at culture initiation for species other than *P. radiata*.

Embryos were assessed for response after 6 to 8 weeks post-treatment growth (Table 2). Only six of the 189 embryos deemed sound at dissection failed to respond to either treatment. Almost 74% of the epicotyls had elongated forming an average of two shoots per genotype (Table 2). The number of shoots formed from the epicotyl ranged from one to five. In a normal embryo, the epicotyl usually forms the apical meristem and is a single shoot elongating in the centre of the whorl of cotyledons. The removal of the cotyledons probably works in several ways to stimulate the extra shoot formation, allowing axillary meristems that surround the epicotyl meristem to elongate. Another possibility is that the primary epicotyl meristem is damaged by the scalpel blade in the process of cotyledon removal. There was some concern that the removal of all cotyledons impaired the epicotyl by removing photosynthetic/nutritional support. However, the average response of 74% shown by the *Pinus attenuata* × *P. radiata* hybrid is bet-

Table 2. Summary of genotype response to Treatment 1; Epicotyl-axillary shoots (formed from embryo epicotyl) and Treatment 2; adventitious-axillary shoots (formed from cryopreserved cotyledons).

	Treatment [1]		Treatment [2]	
Number of dead dissected embryos[1]	Genotypes forming epicotyl-axillary shoots (no.)	Epicotyl-axillary shoots surviving/epicotyl (avg. no.)	Genotypes forming adventitious-axillary shoots (no.)	Adventitious-axillary shoots/ cotyledon (ave. no.)
6	135 (73.8)[2]	2.0	162 (88.5)[2]	14.5

[1] Embryo and cotyledons white and failed to respond to either treatment.
[2] Genotype capture (%), excluding the dead embryos.

ter than a 59% average response shown by *P. radiata* in similar work, when three or more cotyledons were left on the embryos (Hargreaves et al., 2005).

The cryopreserved cotyledons (Treatment 2) gave a higher genotype capture (89%). This is not surprising, as there is more opportunity for shoots to form adventitiously over the surface of the average of 6.8 cotyledons than from what is essentially a single shoot meristem, the epicotyl (Tables 1 and 2). This is further illustrated by the initial shoot count average of 14.5 per "group" of cotyledons versus two per epicotyl (Table 2). Shoot numbers formed from the cotyledons ranged from 1–44 (detailed data not given here).The hybrid required 7 days longer exposure to half LP5 medium to form meristematic tissue on the cotyledons than *P. radiata*. If the adventitious shoots perform well and show no adverse maturation effects after field establishment, the cotyledon cryopreservation could be a very useful method for maintaining juvenility while field testing takes place. However, for this preliminary investigation, no cotyledons were retained in liquid nitrogen.

Another point to note with regard to shoot formation is that not all genotypes automatically formed adventitious-axillary shoots if the epicotyl treatment did so. Of those forming epicotyl-axillary shoots, 15 failed to produce adventitious shoots when exposed to half LP5, indicating that this treatment is selective (data not detailed here). The hybrid also performed better (89%) on the adventitious-axillary treatment than *P. radiata* (80% — Hargreaves et al., 2005), though not as markedly as in the epicotyl-axillary treatment described earlier (The *P. radiata* results were from 10 control-pollinated families and a total of 170 seeds; Hargreaves et al., 2005).

Following assessment (Table 2), the best 100 genotypes represented by both treatments were selected for ongoing in vitro observation, multiplication, and rooting (Table 3). Epicotyl-axillary shoot numbers ranged from one to five per genotype and adventitious-axillary from 2 to 42 (data not given here). General observations indicated that the hybrid shoots tended to have finer foliage in-vitro than *P. radiata* shoots. The hybrid also seemed to be showing improved in-vitro elongation, although this was difficult to determine without a direct comparison. A point to note was that epicotyl-axillary shoots had more needles per cm of stem and therefore more potential axillary meristems for shoot amplification at a future date. Spontaneous rooting was noted on some of the adventitious-axillary shoots in a number of hybrid genotypes, which is uncommon in *P. radiata* adventitious-axillary shoots.

Table 3. Summary of shoot numbers per genotype of the top 100 genotypes selected for ongoing multiplication and in-vitro performance observations.

	Treatment 1	Treatment 2
Genotypes	Epicotyl-axillary shoots per genotype (no.)	Adventitious-axillary shoots per genotype (no.)
100	2.0	16.6

CONCLUSIONS

- *Pinus attenuata* × *P. radiata* seed used in this work was healthy and compares favourably with similar *P. radiata* control-pollinated seed.
- The hybrid was slower to form meristematic tissue on the cotyledons than *P. radiata*, although the response was variable and may be indicative of the influence of *P. attenuata*.
- Overall, the hybrid was highly amenable to organogenesis tissue-culture techniques optimised for *P. radiata*, including cryopreservation.

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