Cryopreservation of *Pinus radiata* Embryogenic Tissue

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Five *Pinus radiata* embryogenic cell lines were successfully recovered following storage in liquid nitrogen for periods of up to nine weeks. Sorbitol and DMSO were used as the cryoprotectants, and a simple protocol using a -30°C freezer as an intermediate step was used. Nurse cultures using established embryogenic cell lines facilitated rapid post-thaw recovery. Cryopreservation of *P. radiata* offers a new tree-improvement tool, as clones from superior families may be maintained in a vigorous juvenile phase while clonal field trials are carried out.

INTRODUCTION

Plantation forestry covers 1.2 million hectares of land in New Zealand, 89% of which is *Pinus radiata* D. Don. Forestry products accounted for 9.5% of New Zealand's total exports in 1990 and this figure is predicted to increase to perhaps 30% by the year 2010 (Ministry of Forestry Official Statistics, 1990).

Annually, 40,500 ha of forest is established (Forestry Facts and Figures, 1992) and/or restocked from seedlings grown from seed in nurseries. A small portion of planting stock is from cutting material taken from trees less than 5 years old. Most seeds are produced from commercial seed-orchards. Orchard trees are selected for commercially desirable traits such as growth rate, stem straightness, freedom from major defects, and disease resistance. Radiata pine has many different end uses with traits such as those listed being required by most users. Tree breeders manipulate these and other traits, like wood density and branching habit, to produce seed lots that meet the specific requirements of their clients (What's New in Forest Research No. 182, 1990).

Pinus radiata exhibits maturation characteristics with age. The overall growth rate slows, dormant buds form at shoot tips, and male and female cones start to develop. Due to these characteristics, cuttings taken after about age four show significantly slower growth rates than seedlings, which reduces the potential advantages of using them (Menzies et al., 1991)

Various processes are available which enable scarce control-pollinated seed to be "vegetatively amplified" to make planting stock available over a larger area. A seed may be used to produce a stool bed from which cuttings may be taken for setting directly in the nursery bed (Menzies et al., 1985). This operation has a reasonably low cost, but the multiplication rates are low.

Commercial-scale micropropagation (tissue culture) technology has been developed to vegetatively amplify limited numbers of seeds (Nairn, 1992). The micropropagation option is more expensive to set up than a cutting option, and the planting stock produced is three to seven times as expensive as seedlings (Smith, 1986). However, some genotypes may be cool-stored at 4 to 10°C (Smith et al., 1982; Aitken-Christie and Singh, 1987) while clonal material is field tested. The stored

material may retain its juvenile state (this has yet to be fully tested), and would circumvent the problems of maturation in clones grown in the field. Thus it may be possible to return to a collection of stored juvenile material to provide a source of tested clones within superior seed families.

One potential problem with cool storage is that the tissue is still physiologically active at 4 to 10°C. It is necessary to return material to room temperature and to transfer to fresh medium at intervals of between six months and two years, following which the material is cool-stored once more. Not all genotypes tolerate this treatment, and some are lost during storage. Since the physiological processes in material stored at 4 to 10°C are not totally suspended, some physiological and maturational changes may be expected, especially when storage for up to eight years is required.

Cryopreservation, the storage of tissues in liquid nitrogen at -196°C, has been used for many years to preserve animal semen. In recent years, cryopreserved animal embryos have developed into viable animals when placed into a receptive womb. Plant tissue presents some difficulties as most plant cells contain small vacuoles that contribute to tissue damage during freezing and thawing. Whole plant organs are difficult to cryopreserve. However, embryogenic tissue is readily preserved due to the absence of vacuoles in the proliferative tissue.

Somatic embryogenesis in a conifer was first achieved with *Picea abies* (Hakman et al., 1985), and since then with other species including *Pinus radiata* (Jones, 1990; Smith et al., 1991). Successful plant formation after cryopreservation of coniferous embryogenic tissue has been reported with *Picea glauca* (Kartha et al., 1987), *Picea mariana* and *Larix* ×*eurolepis* [=*L.* ×*marschlinsii* Coaz.] (Klimaszewska et al., 1992), and *Pinus caribaea* (Laine et al., 1992).

As with other plant species, four components of the cryopreservation process contribute to success:

1) **State of the tissue:** Embryogenic tissue should be in a vigorous growth phase, with a high density of embryo initials that have no vacuoles.

2) **Freezing of tissue:** The non-permeating compound sorbitol and the permeating compound dimethylsulphoxide or similar "cryoprotectants" should be used to minimise tissue damage during the first freezing stage to -30°C. Although both of these have both proved to be cytotoxic at high concentrations (Chen and Kartha, 1988), they are useful for coniferous tissue.

3) **Thawing of tissue:** Embryogenic tissue should be thawed relatively quickly, effectively done by plunging cryopreservation vials into water at 40°C.

4) **Growing-on tissue:** After thawing, tissue should be rinsed to remove cryoprotectants, and then cultured on media suitable for cells at low density.

GENERAL METHODS FOR CRYOPRESERVATION OF *PINUS RADIATA* EMBRYOGENIC TISSUE

Embryogenic Tissue Pretreatment. Embryogenic tissue was removed from a maintenance medium and suspended in liquid embryogenesis medium (EM) with 73 g/l sorbitol at a ratio of 1 g fresh weight to 3 ml of EM (embryogenesis medium was developed at the Forest Research Institute). The suspension was pipetted into 25 ml flasks which were stoppered with a cotton bung and then covered by

aluminum foil to prevent contamination. Flasks were incubated at 24°C on an orbital shaker (50 rev/min) for 12 to 48 h.

Freezing. For cryopreservation, 0.5 ml of suspended tissue was transferred to 1.8 ml cryovials (Nunc). Cryovials and 20% dimethylsulphoxide (DMSO) were held on ice to equilibrate, and 0.5 ml of DMSO solution was added to the 0.5 ml of suspension to give a final concentration of 10% DMSO. In preliminary experiments, a final concentration of 5% DMSO was used. Vials were put into aluminum tubes called canes and transported in ice to a -30°C freezer. Canes were placed in the freezer for 2 h to freeze the suspension then put directly into liquid nitrogen (-196°C). The liquid nitrogen was held in a dewar and kept in a cool room (4°C). Regular checks were made of the liquid nitrogen levels to ensure samples remained fully immersed. Embryogenic tissue was held at -196°C for up to 9 weeks.

Thawing. To reinstate growth, vials (up to 5 at one time) were removed from a cane and immersed in 40°C water until the frozen suspension plug dissolved. This took no more than 2 minutes. Vial contents were then poured, one at a time, onto a nylon screen in a millipore filter unit (Fig. 1). Rinsing medium was poured over the sample which was centred in the nylon screen by the use of autoclavable plastic washers. The contents of each vial was rinsed with 100 ml of rinsing medium (EM + 30 g/l sucrose). One millipore unit was used for 15 vials before being replaced with

Figure 1. Thawed samples are poured onto a nylon screen in a millipore filter unit.

a new sterile unit. Washings were sucked into a receiver bottle on a vacuum line. The nylon screen with embryogenic tissue was put onto initiation medium for one week in experiment 1, and for 2 h in experiment 2 (Fig. 2). After 2 h or 1 week, the nylon screen and tissue were transferred to maintenance medium, embryo-development medium, or onto fresh embryogenic tissue that then served as a "nurse" culture. Nurse tissue was separated from the thawed embryogenic tissue by a 30-micrometer-pore nylon screen. A nurse culture consists of an actively growing mass of embryogenic tissue in direct contact with a nutrient medium.

Survival Assessment. The fresh weight of regrown tissues was recorded at completion of experiments.

Early observations of thawed embryogenic tissue were made using a stereo microscope. Embryogenic tissue was stained with acetocarmine, to determine cell survival.

RESULTS AND DISCUSSION

Experiment 1. The period of storage in liquid nitrogen was evaluated in this experiment. At -196°C, cells are metabolically inactive and duration of storage should not affect subsequent regrowth. Five cell lines were tested; two (M-31 and M-33) shared the same parent trees (full sibs) and the other three cell lines are

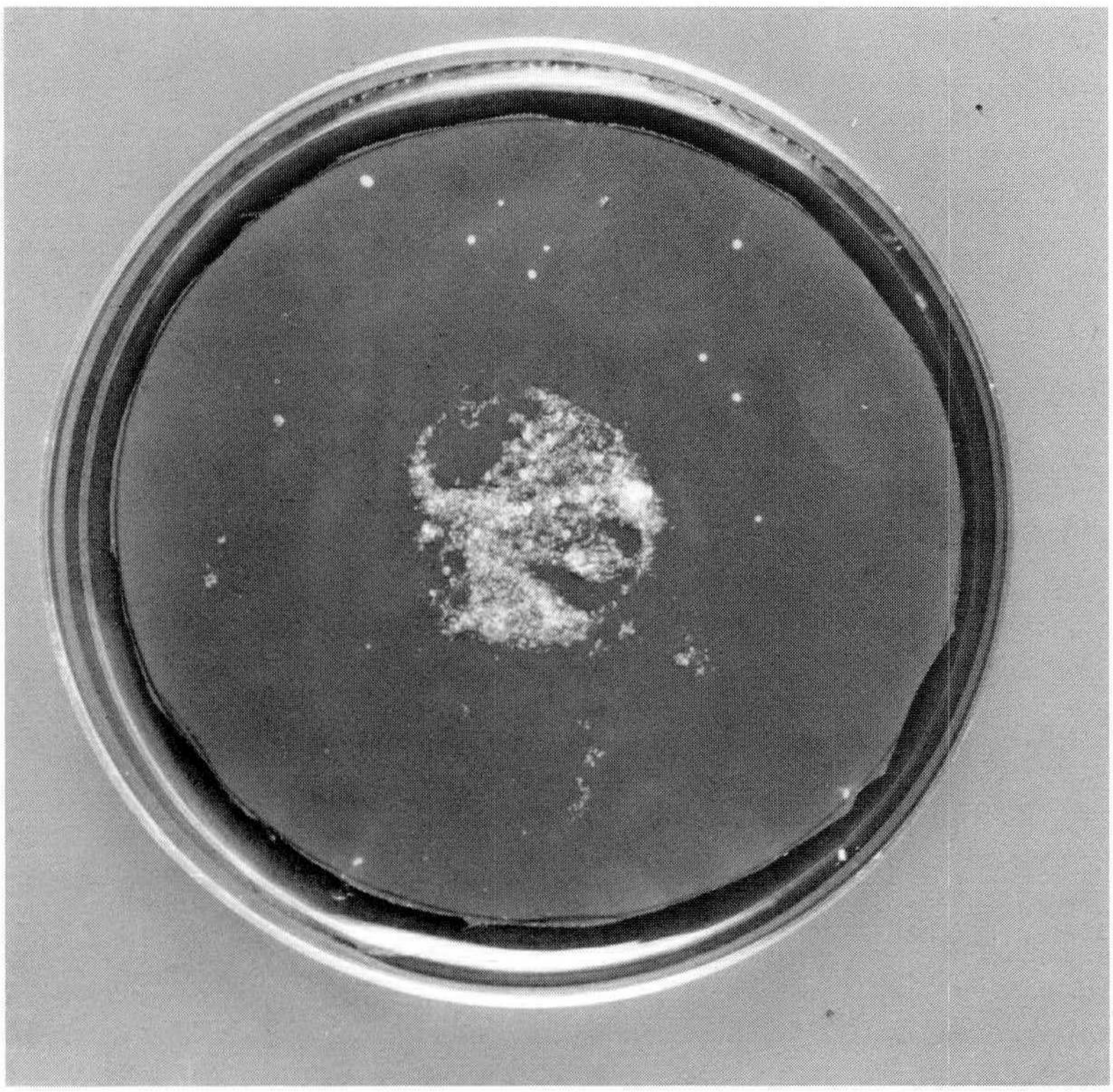

Figure 2. Nylon screen with rinsed embryogenic tissue on initiation medium.

unrelated. Three cell lines were white (76-2, 191-8, and M-33), while K-18 and M-31 were brown in colour, a characteristic of some embryogenic cell lines. Six vials of each cell line were frozen on day 0 of the experiment, and two vials were removed at each time period. Fresh weight of tissue per vial was 0.166 g.

Table 1 shows the fresh weight of embryogenic tissue 16 to 20 weeks after thawing from liquid nitrogen (LN). Each treatment had two replications of each cell line.

Table 1. Fresh weight (g) of tissue after regrowth on maintenance medium.

Time in liquid nitrogen	24 hours		1 week		1 month	
Weeks on maintenance culture	20		19		16	
Replication	1	2	1	2	1	2
Cell line						
76-2	6.87	1.79	0.05	0.03	1.52	0.04
M-31	0.06	0.04	0.07	0.06	0.07	0.08
191-8	9.73	6.97	5.64	9.36	3.85	6.00
M-33	2.31	3.14	0.06	11.73	0.05	0.06
K-18	0.03	0.05	0.05	0.06	0.05	0.04

Cell lines M-31 and K-18 did not survive immersion in liquid nitrogen. Cell line M-33 showed regrowth after storage in liquid nitrogen for 24 h and 1 week.

Cell line 76-2 showed no survival after 1 week in liquid nitrogen, but after 1 month in liquid nitrogen, replication one survived. Cell line 191-8 was unaffected by the storage period in liquid nitrogen and all replications grew well on maintenance medium.

The lack of survival in cell lines K-18 and M-31 may be attributed to their growth state before cryopreservation. They were both brown in colour, possibly indicating a lack of vigour. Laine et al. (1992) emphasised that for the optimal recovery of viable cells from *Pinus caribaea*, embryogenic cell suspensions must be vigorous. When they used suitable cell suspensions, it was possible to achieve 100% recovery of cryopreserved samples. If non- or poorly-embryogenic cultures were cryopreserved, it was difficult to obtain any regrowth after thawing.

Pinus radiata embryogenic tissue that did not grow after thawing showed loss of fresh weight. This weight loss was due largely to the collapse of highly vacuolated suspensor cells that make up most of the tissue bulk on maintenance medium, and had become plasmolysed during the pre-freezing incubation step.

Experiment 2. For Experiment 2, there were 10 vials each of unrelated cell lines F92-2 and Q92-1, which were frozen and subsequently thawed after 9 weeks storage in liquid nitrogen. Fresh weight of tissue per vial was 0.166 g. Two treatments were used; after 2 h on initiation medium, rinsed tissue was either placed directly onto embryo development (ED) medium or onto a nurse culture on ED medium. Tissue was weighed nine weeks after thawing.

Table 2. Fresh weight (g) of cryopreserved tissues directly on agar medium or on nurse tissue, after nine weeks of culture.

Cell line	F92-2		Q92-1	
Replication	Directly on medium	Nurse tissue	Directly on medium	Nurse tissue
1	0.04	contaminated	0.04	3.08
2	0.04	0.72	0.06	1.38
3	0.04	0.47	0.08	1.73
4	0.04	0.92	0.05	contaminated
5	0.04	0.61	0.04	4.73
Average	0.04	0.68	0.05	2.73

The nurse culture ensured survival and growth of all replications, while the tissue placed directly on embryo development medium did not grow. Cells on the nurse treatment when stained with acetocarmine were bright red and had dense cytoplasm. The tissue placed directly on embryo development medium showed extensive cytoplasmic damage.

The positive effect of the nurse culture may be attributed to the benefit of growth promoting substances released by the actively growing cells. It is also possible that the mass of nurse cells absorb cryoprotectants or toxic metabolic products from damaged cells after thawing.

CONCLUSIONS

In these two preliminary experiments, five unrelated *P. radiata* embryogenic cell lines have survived immersion in liquid nitrogen for periods of 1 day to 9 weeks. Post-thawing growth is enhanced by the use of a nurse-tissue treatment.

These preliminary experiments indicate that *P. radiata* embryogenic tissue can be cryopreserved without recourse to sophisticated programmable freezing equipment. It is reasonable to expect that storage for longer periods than 9 weeks in liquid nitrogen should be possible. Long-term storage of tissue should allow the field testing of clones within superior families. When phenotypes with useful industrial properties are identified, tissue from the same clone can be recovered from cryopreserved foundation stocks. Since the cryopreserved tissue will still be in the embryogenic state, it will serve as a source of vigorous, juvenile planting stock. Thus, truly juvenile donors of tested clones will be available, and it will finally be possible to circumvent the problem of maturation which has been identified as an impediment to clonal forestry (Sweet and Wells, 1974).

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