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# Cryopreservation of embryogenic culture of *Pinus roxburghii*

G. MATHUR, V.A. ALKUTKAR and R.S. NADGAUDA*

*Tissue Culture Pilot Plant, National Chemical Laboratory, Dr. Homi Bhabha Road, Pune-411008, India*

## Abstract

Embryogenic cultures of chir pine (*Pinus roxburghii* Sarg.) were cryopreserved successfully in liquid nitrogen. It was found that using sorbitol and dimethyl sulfoxide (DMSO) as cryoprotectants was essential for the survival of the tissue. Among the different concentrations of the cryoprotectants used, the most effective treatment was observed to be 0.3 M sorbitol and 5 % DMSO. On staining the cryopreserved tissue with fluorescein diacetate, it was observed that only a few meristematic embryo heads survived and resumed growth after a very short initial lag phase. The recovered cultures showed normal regrowth on proliferation medium and, it was also observed that washing off the cryoprotectants was necessary for the cultures to survive. The results indicate that cryopreservation can be used for conserving the germplasm, and in maintaining the embryogenic capacity of the tissue.

*Additional key words*: chir pine, cryo-storage, somatic embryogenesis.

## Introduction

*Pinus roxburghii* Sarg. is a very common and important pine species of the lower Shivalik ranges in western Himalayas. This pine is valued mainly for its resin like many other pines growing in warmer regions (Tewari 1994). Indiscriminate tapping for resin and clearing of forests for timber or other purposes, has attracted attention for newer propagation techniques to supplement the conventional practices. Keeping this in mind, somatic embryogenesis was induced in chir pine (Arya *et al.* 2000, Mathur *et al.* 2000). Maintaining embryogenic cultures of pines for long a period may cause the tissue to lose the embryogenic capacity and also induce somaclonal variations during subculturing. These problems can be easily overcome by cryopreservation of cultures in liquid nitrogen.

Progress in somatic embryogenesis in pines has been significant (Attree and Fowke 1993, Gupta and Grob 1995, Timmis 1998) and cryopreservation has also been successful and reported for the following pine species: *Pinus caribaea* (Laine *et al.* 1992), *P. patula* (Ford *et al.* 2000), *P. sylvestris* (Häggman *et al.* 1998) and *P. taeda* (Gupta *et al.* 1987). In all these reports, the regrowth capacity of the cryo-stored cultures and their morphology has been compared to the non-frozen cultures, except for the work by Häggman *et al.* (1998) who have also checked the genetic fidelity of the cryopreserved *P. sylvestris* embryogenic tissue. Cryopreservation and the use of biochemical and molecular markers for testing the genetic homogeneity has also been reported earlier in other conifer genera viz. *Picea* (Eastman *et al.* 1991, Fourré *et al.* 1997, Isabel *et al.* 1993) and *Larix* (DeVerno *et al.* 1999).

The present studies were undertaken with the aim of optimizing the cryoprotectant treatments necessary for the embryogenic chir pine cultures to survive the cryo-storage. These results are important for the planning of the long-term cryopreservation studies that are presently underway.

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*Abbreviations*: 2,4-D - 2,4-dichlorophenoxyacetic acid; BA - $N^6$-benzyladenine; DCR - Douglas fir cotyledon regeneration; DMSO - dimethyl sulfoxide; FDA - fluorescein diacetate; kin - kinetin.

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*Corresponding author; fax: (+91) 20 5893338, e-mail: rsn@ems.ncl.res.in

## Materials and methods

**Plant cultures:** Embryogenic cultures of *Pinus roxburghii* Sarg. line R-29, were initiated by culturing immature megagametophytes as described by Mathur *et al.* (2000). The extruded embryogenic mass was proliferated and maintained on Douglas fir cotyledon regeneration (DCR) basal medium (Gupta and Durzan 1985) containing 50 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 20 μM $N^6$-benzyladenine (BA), 20 μM kinetin (kin), 500 mg $dm^{-3}$ L-glutamine, 500 mg $dm^{-3}$ casein hydrolysate, 1000 mg $dm^{-3}$ myo-inisitol, 2 % sucrose, 0.18 % gellan gum. The pH was adjusted to 5.8 before sterilization of the media and the embryogenic cell lines were kept in dark at 22 ± 2 °C, maintained by subculturing after every three weeks.

**Pre-treatments:** The main aim of this experiment was to determine the optimum pre-treatment by cryoprotectants for the embryogenic cultures to survive the freezing in liquid nitrogen. Different concentrations of sorbitol and dimethyl sulfoxide (DMSO) were tested for this purpose. Three to four grams of freshly subcultured embryogenic tissue (two weeks after last subculture) was precultured in 40 $cm^3$ liquid proliferation medium (same as above) in culture bottles. These suspension cultures were placed on shaker (100 rpm) in dark for 48 h at 22 ± 2 °C before they were used for cryopreservation experiments.

In the present investigation, following pre-treatments (P-T) with cryoprotectants (different combinations of 0.3 and 0.5 M sorbitol and 5 or 10 % DMSO) were tested. In some cases the pre-treatments were followed by slow freezing (SF), and the samples were stored in liquid nitrogen (LN) for 72 h.

For the pre-treatment involving sorbitol and DMSO, cultures were first primed with sorbitol for 24 h before adding DMSO. From 4 M filter sterilized stock solution, sorbitol (*Hi-media*, Mumbai, India) was added ten times over a period of 30 min to give a final concentration of 0.3 M or 0.5 M. The cultures were continuously and gently agitated during the addition of cryoprotectants and they were then incubated on shaker. On day two, culture bottles were kept on ice throughout the treatment with DMSO (*Sigma*, St. Louis, USA), which was also added to the cultures ten times over a period of 30 min to give a final concentration of 5 % or 10 % (v/v) (Ford *et al.* 2000). All pretreatments were done in sterile conditions in laminar airflow.

*Cryomed*™ (*Thermo Forma*, Marietta, USA), a programmable controlled-temperature cooling chamber was used for slow freezing the tissues till -50 °C. The pre-treated tissues were dispensed (1.5 $cm^3$ each) in *Nalgene*™ (*Nalgene*, Rochester, USA) cryo-vials and were kept on ice for 2 h before slow freezing. Thereafter, the vials were transferred to cryo-cans just before slow freezing protocol, which was as follows: Hold at -1 °C for 5 min; slow freezing 0.3 °C per min to -16.5 °C; hold at -16.5 °C for 15 min; slow freezing 0.3 °C per min till -35 °C; rapid freezing 25 °C per min to -50 °C (personal communication with Prof. Sara von Arnold). The cans containing the vials were then plunged immediately in liquid nitrogen and removed after 72 h.

The experiments were conducted as follows: control, only SF, only LN, P-T + SF, P-T + LN, P-T + SF + LN. Also, embryogenic cultures that were not subcultured for more than 5 weeks, were used for P-T + SF + LN. Ten replicates were tested for each pre-treatment and five each for the controls and the experiment was conducted twice.

**Thawing and re-growth:** Thawing of the cryo-stored tissue was done after removing the cryo-vials and placing them for 2 - 3 min in *Magenta*™ (*Sigma*, St. Louis, USA) jars containing sterile water at 45 °C. The vials were then dipped in 70 % ethanol and transferred to jars containing sterile water kept on ice, till they were poured out for re-growth. The thawed tissue was poured on filter paper discs (*Whatman No. 1*, 45 mm diameter), and excess supernatant was filtered out using vacuum. The tissue was washed with liquid DCR medium before placing the discs on fresh solid proliferation medium in *Laxbro*® (*Laxbro*, Mumbai, India) sterile disposable Petri dishes (55 × 1.5 mm). The cultures were transferred to fresh medium after 24 h and then subcultured every week.

**Viability assay:** Fluorescent stain, fluorescein diacetate (FDA) (*Sigma*) was used to test the viability of the cryopreserved tissue (Häggman *et al.* 1998, Widholm 1972). Cryopreserved embryogenic culture (20 - 30 mg) was harvested immediately after thawing and dipped in a 1.5 $cm^3$ Eppendorf tube containing 0.8 $cm^3$ liquid medium, to which 0.02 $cm^3$ FDA was added from the stock solution (final concentration of 0.05 % FDA) and the stained tissue was observed under UV light using *Zeiss Axioplan2* (*Carl Zeiss*, Jena, Germany) microscope.

**Analysis of growth:** The data was collected as the increase in fresh mass of the cryopreserved tissue kept for re-growth on proliferation medium. The readings were taken, starting on day zero and then at weekly intervals for 5 weeks. The regrowth of the tissue was calculated as per cent increase in fresh mass from day zero (Laine *et al.* 1992). This was to avoid the discrepancies that would have surfaced if the fresh masses were directly taken before and after placing them for regrowth, as there was a loss of culture during cryopreservation as well as during washing. Standard deviation was calculated for the increase in fresh mass for each treatment and the controls.

## Results and discussion

The present results, the first for any Indian pine species, clearly demonstrates the feasibility of the cryo-preservation technique to store the embryogenic cultures. Considering the importance of propagating this pine species using somatic embryogenesis, developing a workable method of cryopreservation was essential. The long life cycle of trees delays the outcome of clonal propagation results. Embryogenic cell lines can thus be cryo-stored and used when the desired elite characters are observed during field trials. Cryo-storing is not only beneficial for preserving the germplasm, but it also prevents any somaclonal variation that may ensue during the long-term maintenance of the embryogenic tissue (Ford *et al.* 2000). The methods tested for cryo-preservation, including pre-treatment, slow freezing and fast thawing after cryo-storing in liquid nitrogen, proved successful for the *P. roxburghii* Sarg. embryogenic cultures. This is in concurrence with studies conducted with different tissues (Kartha *et al.* 1988, Find *et al.* 1993, Cyr *et al.* 1994, Aronen *et al.* 1999, Ford *et al.* 2000). In our attempts to determine an effective cryopreservation treatment to enable the embryogenic cultures to sustain their viability, we observed that the embryogenic cultures survived the liquid nitrogen treatment for 72 h and could successfully recover as evident from their regrowth. The cultures subjected to slow freezing and/or storage in liquid nitrogen without any pre-treatment with cryoprotectants did not show any regrowth (Fig. 1*A*). This emphasizes the necessity of treating the tissue with cryoprotectants.

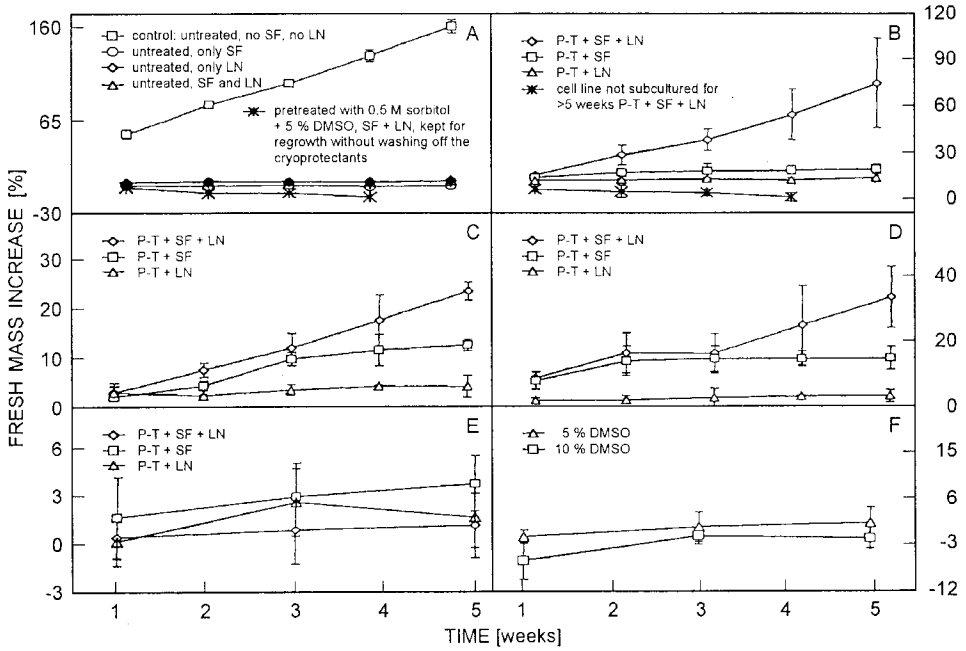


Fig. 1. Regrowth of cryopreserved *Pinus roxburghii* Sarg. embryogenic cultures: *A* - different controls, *B* - cultures pre-treated with 0.3 M sorbitol and 5 % DMSO and of a cryopreserved embryogenic culture that had not been subcultured for more than 5 weeks, before it was pre-treated with cryoprotectants, *C* - cultures pre-treated with 0.3 M sorbitol and 10 % DMSO, *D* - cultures pre-treated with 0.5 M sorbitol and 5 % DMSO, *E* - cultures pre-treated with 0.5 M sorbitol and 10 % DMSO, *F* - cultures pre-treated with 5 % and 10 % (v/v) DMSO (P-T - pre-treatment; SF - slow freezing; LN - liquid nitrogen).

The pre-treatment most conducive for survival of the embryogenic cultures of chir pine was observed to be a combination of 0.3 M sorbitol and 5 % DMSO. The cultures pre-treated with this combination of cryo-protectants showed good regrowth (69.94 % increase in fresh mass after 5 weeks) (Fig. 1*B*). The regrowth of the control (un-treated, un-stored) was 158.53 % after 5 weeks. There is an initial lag phase in the regrowth of the cryopreserved cultures, after which the growth is almost comparable to the normal embryogenic cultures. This is in agreement with observations made by Ford and co-workers (2000) in successfully cryopreserving the embryogenic cell lines of *P. patula*. Gupta *et al.* (1987) have reported a lag phase of 5 weeks before the regrowth of cryopreserved cultures of *Picea abies* and *Pinus taeda*, after thawing. However, in our studies the lag phase was very short, of 5 - 7 d, which is similar to observations of Find *et al.* (1993).

The cultures that were pre-treated with 0.5 M sorbitol and 5 % DMSO, also have survived and recovered (33.1 % average increase in fresh mass after 5 weeks). It may be possible that embryogenic cultures are unable to

tolerate DMSO at a concentration of 10 % and, 5 % DMSO is suitable for the survival of the tissue. Treatment with DMSO without priming with sorbitol, was also found to be lethal for tissues (Fig. 1*F*), since there was no regrowth observed in the cultures.

A pre-culture or priming of the cells in a medium enriched with an osmoticum like sorbitol, has been shown to enhance the tolerance to freezing (Kartha *et al.* 1988, Klimaszewska *et al.* 1992). The cytotoxicity induced by DMSO is reversed by the priming of the cells with sorbitol, prior to their exposure to DMSO (Kartha *et al.* 1988). But there are reports that suggest that the DMSO alone, and even at a concentration as high as 20 % (v/v), is not cytotoxic to the cells and recovery of

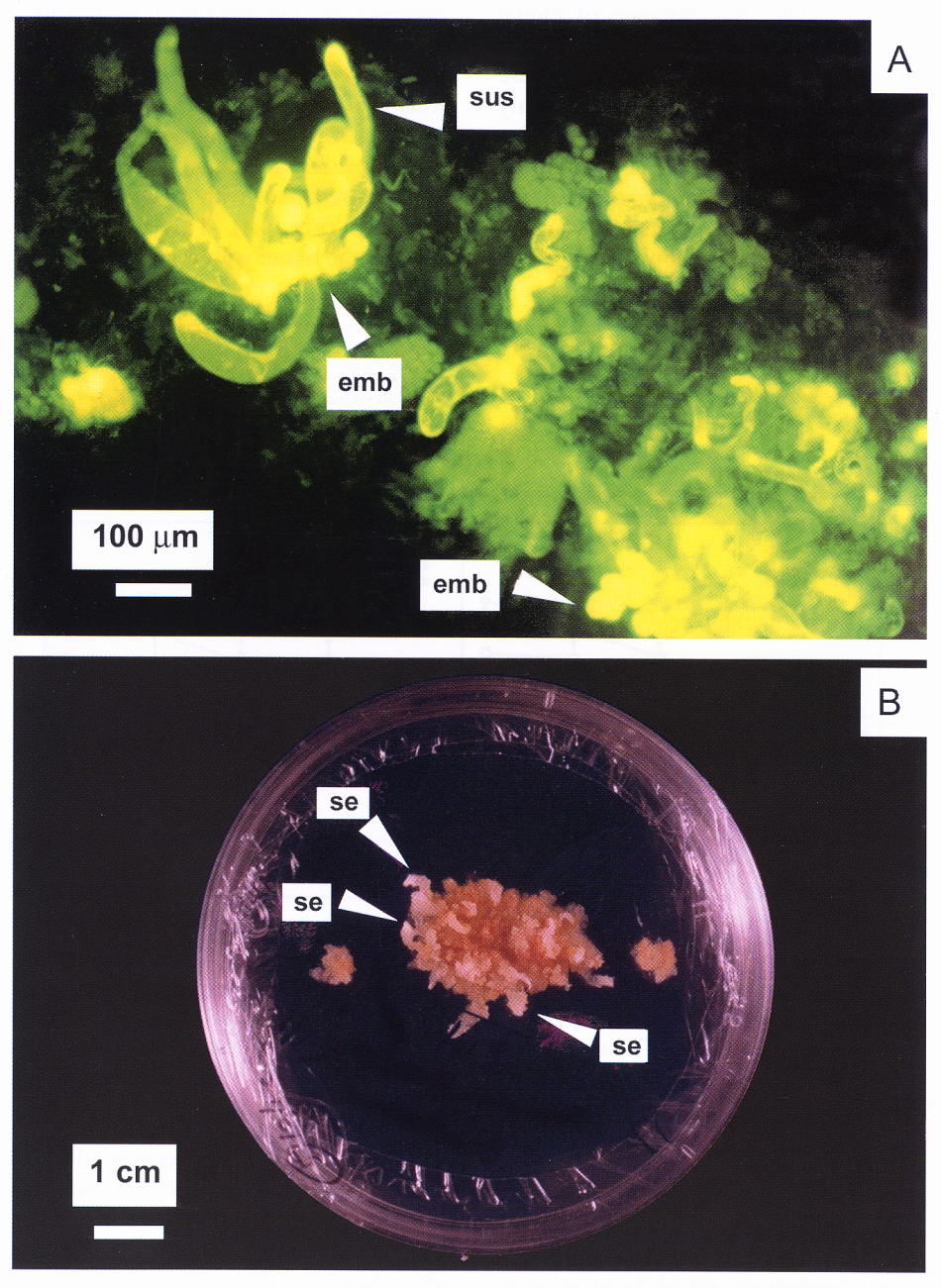


Fig. 2. *A* - Cryopreserved embryogenic culture of *Pinus roxburghii* Sarg. stained with fluorescein diacetate immediately after thawing. Only the embryonal heads (emb) along with surrouding suspensors (sus) take up stain, the dead cells remain unstained.
*B* - Cryopreserved embryogenic culture of *Pinus roxburghii* Sarg. growing on maturation medium and showing normal developing somatic embryos (se).

cells is possible (Kartha *et al.* 1988). The untreated cultures were totally unable to survive and recover aftertheir exposure to freezing temperatures in liquid nitrogen (Fig. 1*A*). A slower re-growth with no healthy recovery of cultures was observed when the cultures were only subjected to slow freezing protocol. This may be attributed to the partial survival of the vacuolated cells that are totally eliminated during the liquid nitrogen storage and only the embryogenic cells with dense cytoplasm survive and are capable of recovering fully.

The slow freezing step (gradually bringing down the temperature to -50 °C) before plunging the culture in liquid nitrogen is also essential. All the cultures that were cryo-stored without undergoing the slow freezing protocol did not re-grow (Fig. 1*B-E*).

One major factor influencing the regrowth of the embryogenic cultures after cryopreservation was the physiological state of the cultures at the time of pre-treatment. Vigourously growing cultures (2 weeks after last subculture) were best suited for cryopreservation. The slow growing, un-subcultured embryogenic cultures pre-treated with 0.3 M and 5 % DMSO and cryopreserved, did not show regrowth after thawing (Fig. 1*B*). Slow proliferating cultures which had attained the stationary phase after being cultured on the same proliferation medium for more than 5 weeks, did not survive the cryopreservation despite pre-treatment with cryoprotectants. This suggests the importance of the physiological state of growth of the culture that is to be utilized for cryopreservation.

Vital staining with FDA and microscopical observations revealed that the cells that retained viability and capacity for regrowth were the embryonal head cells and some of the closely associated suspensor cells. Among the embryogenic mass, several dead cells that have not taken up the fluorescent dye can also be clearly seen (Fig. 2*A*). In the cryopreserved embryogenic culture (using 0.3 M sorbitol and 5 % DMSO) a number of normal somatic embryos can be observed within the culture (Fig. 2*B*).

Once the cryopreserved cultures have been recovered from freezing, the proliferation is comparable to the untreated controls, specially in the cultures that were pre-treated with 0.3 M sorbitol and 5 % DMSO. The recovered and proliferated cryopreserved culture on maturation medium (half strength DCR basal medium + 3 % maltose + 30 μM ABA), produced morphologically similar somatic embryos, as would be produced in un-cryopreserved cell lines (Fig. 2*b*).

Morphological differences and loss of embryogenic potential has been observed in some of the *P. roxburghii* cell lines that we have maintained and subcultured continuously since their induction in 1997 (data not included). This makes it even more essential to cryopreserve the freshly induced embryogenic cell lines. It has also been observed that, the ability of embryogenic cultures to tolerate cryopreservation can be genotype dependent (Nørgaard *et al.* 1993a,b, Häggman *et al.* 1998).

The microscopical examinations revealed that it is only the embryonal heads and the associated suspensors that survive the cryopreservation and are capable of proliferating after thawing (Fig. 2*A*). Kartha *et al.* (1988) observed that the suspensor cells are eliminated during the pre-treatment with sorbitol and DMSO, even before the freezing. Washing the cryoprotectants off from the cryopreserved cultures after thawing was also found to be essential. The unwashed cultures did not recover even after several subcultures (Fig. 1*A*).

An optimization of pre-treatment with cryoprotectants is very much essential for any cryopreservation protocol. It is due to the effect of the cryoprotectants that the embryogenic cultures of *P. roxburghii* Sarg. were able to tolerate slow freezing and cryo-storage. The post-cryopreservation recovery of the cultures, with a minimum lag phase, also depends very much on it. These results will definitely prove to be very useful for the ongoing studies regarding long-term cryopreservation of the embryogenic cultures of various pine species.

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