

regulator in experiments with *Q. robur* reported earlier (MANZANERA, 1992), as compared with combinations of BA and α -naphthaleneacetic acid (NAA). Other authors (LELU and BORNMAN, 1990; SUNG et al., 1988) have observed the role of 2,4-D in cell division and embryogenic potential. However, 2,4-D is also an inhibitor of further embryo development (HALPERIN, 1970). In contrast with our results, CHALUPA (1990) obtained embryogenesis in *Q. robur* embryos using BA, alone or combined with gibberellic acid (GA₃), whereas 2,4-D was inefficient.

The effect of desiccation by the addition of sorbitol or by air-drying on the germination of cork oak somatic embryos was negligible in our case. On the contrary, a high concentration of osmoticum produced an effect similar to that of abscisic acid (ABA) in rapeseed (FINKELSTEIN and CROUCH, 1986). In interior spruce, a pulse of mannitol in combination with ABA doubled the production of mature somatic embryos compared with the standard ABA treatment (ROBERTS, 1991). GINGAS and LINEBERGER (1989) obtained germination of red oak somatic embryos by desiccation with either sorbitol or by air-drying. In pendunculate oak, somatic embryo germination was also stimulated after desiccation with 6% sorbitol (CHALUPA, 1990), but was hampered in olive (RUGINI, 1988). In interior spruce, partial drying at high humidity promoted germination of the somatic embryos up to 90% (ROBERTS et al., 1990).

Cold storage of somatic embryos matured "in vitro" at 5°C for 10 weeks and at 2°C for 2 weeks were the best treatments for breaking dormancy. These conditions are similar to those necessary for zygotic embryos of other *Quercus* species to break dormancy.

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Genotypic Differences in the Ability of Embryogenic *Abies nordmanniana* Cultures to Survive Cryopreservation¹⁾

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Abstract

Embryogenic cultures of *Abies nordmanniana* were cryopreserved after preculture in 0.4 M sorbitol and pretreatment with 5% DMSO (dimethyl sulfoximide). After cryopreservation, only a few meristematic cells in the embryo heads survived. Following an initial lag-phase, growth resumed and complete embryos were formed by the surviving cells. Regrowth capacity depended strongly on genotype as determined by frequency of survival, length of the lag-phase, and growth rate after cryo-

preservation. Only one of five genotypes resumed growth at same rate as before cryopreservation. Possible causes of differential cryotolerance among genotypes are discussed.

Key words: *Abies nordmanniana*, cryopreservation, somatic embryogenesis, tissue culture.

Introduction

In conifers the use of somatic embryogenesis for plant propagation purposes is limited since it can only be induced from juvenile material with unknown genetic properties. Maintenance of stock cultures in the laboratory during field evaluation of the clones is laborious, and prolonged subculture also increases the risk of losing clones due to microbial contamination and mislabelling.

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Cryopreservation is the best method for long term storage of tissue cultures due to the reduction and subsequent arrest of the metabolic functions by imposition of ultralow temperatures while viability is maintained (KARTHA, 1985).

Because of their fast growth and large population of meristematic cells embryogenic cell suspensions are well suited for cryopreservation. Several coniferous embryogenic cell cultures have been successfully cryopreserved: *Picea glauca* (KARTHA et al., 1988), *Picea abies* (Galerne and DEREUDDRE, 1988; GUPTA et al., 1987), *Pinus taeda* (GUPTA et al., 1987), *Picea sitchensis* (FIND et al., 1993).

Abies nordmanniana, a Christmas tree species, cannot be propagated on large scale by cuttings. However, somatic embryogenesis has been demonstrated (NØRGAARD and KROGSTRUP, 1991) and cryopreservation is of interest, since field evaluation can be carried out in 5 to 10 years. In this paper, we describe the successful cryopreservation of embryogenic cultures of *Abies nordmanniana* with focus on genotype effects and quantitative analysis of regrowth. The method is based on the method of KARTHA et al. (1988) and modified for use with solid medium cultures.

Materials and Methods

Embryogenic cultures were initiated and maintained as described by NØRGAARD and KROGSTRUP (1991), except that BAP concentration in the proliferation medium was increased to 10 μ M. Five different genotypes (8, 10, 12, 14, 15) were selected for cryopreservation experiments. Genotypes 8 and 14 were characterized by a high growth rate and good regeneration ability. Genotype 10 was characterized by a high growth rate and low regeneration ability, and genotypes 12 and 15 were intermediate in both characters (NØRGAARD, 1992). All experiments were initiated two weeks after subculture and repeated 3 to 4 times with 2 Petri dishes per genotype per treatment.

Two independent experiments were conducted. Experiment A involved only genotype 8. In this experiment the effect of preculture, pretreatment, freezing to -35° C, and cryopreservation on regrowth was investigated. In experiment B the four remaining genotypes were frozen in order to investigate susceptibility of different genotypes to cryopreservation. In this experiment growth of cryopreserved samples was compared to growth of untreated and pretreated samples.

For preculture treatment, 1 g of actively growing embryogenic cultures was dispersed with a scalpel and transferred to 20 ml liquid proliferation medium in a 50 ml Erlenmeyer flask. The cell culture was further dispersed by pumping through a 2 mm wide sterile disposable transfer pipette. Two flasks were established for genotypes 10, 12, 14, and 15, and 3 for genotype 8. One flask per genotype was not treated further (untreated control). The other flasks were precultured with sorbitol: 10 aliquots of 0.105 ml 4 M sterile sorbitol solution was added to the flasks every 3 min for $\frac{1}{2}$ hour to reach a final concentration of 0.2 M sorbitol. The flasks were shaken after each addition. The cultures were closed with an aluminium cap, sealed with 2 rounds of polyethylene film and left on a shaker for 24 hours (120 rpm). The next day 10 aliquots of 0.117 ml 4 M sorbitol was added in the same way resulting in a final concentration of 0.4 M sorbitol. The flasks were then incubated on a shaker for another 24 hours. One flask (genotype 8) was not treated further (precultured control).

For pretreatment, the flasks were placed in an ice bath and 10 aliquots of 0.116 ml DMSO were added to each flask to reach a concentration of 5% DMSO. The cultures were then poured into 25 ml measurement cylinders and allowed to settle in the ice bath for 30 min. The supernatant was withdrawn to give a concentration of 75% settled cell volume (SCV). The cultures were resuspended, and 1 ml of each was transferred to 1 ml plastic ampoules (Nunc) in an ice bath. The ampoules were sealed with Cryoflex (Nunc), placed on canes and left in the ice bath until transfer to the freezing chamber. Samples for pretreated controls were left in the ice bath until plating.

Freezing was performed in a programmable freezer, 1610 Scientific Biological Freezer, Sy-Lab, Austria. The ampoules were inserted at -0.5° C and frozen at a rate of 0.3° C/min to -35° C. A plateau (14 min at -15.9° C) was inserted to avoid rapid cooling after crystallization. One thermocouple was inserted into an ampoule and another was left in the air. At the end of the freezing program, the canes were transferred to a liquid nitrogen container (-196° C). Two ampoules of genotype 8 were thawed and plated directly after the freezing program (frozen -35° C control).

After 2 hours in liquid nitrogen the ampoules were thawed quickly by swirling them in a water bath at 37° C, until melting had begun (2 to 3 min). The ampoules were surface sterilized with 70% (v/v) ethanol, and the contents were poured onto a filter paper on the medium. The filter paper + cells were transferred to fresh medium after 1 hour and 18 hours. After 18 hours, the weight of filter paper + cells was noted. All controls were also adjusted to 75% SCV before plating and plated controls were transferred to fresh medium after 1 hour and 18 hours.

To determine viability, regrowth was followed by weighing the filter paper + cells every week (wet weight, WW). Every 2 weeks the cells were transferred to fresh medium. The regrowth was determined for 8 weeks after plating. Cells from genotype 8 were stained with fluorescein diacetate (FDA) (WIDHOLM, 1972) 4 hours after plating (thawing), and at day 11.

Regrowth data were analysed using Non-linear Regression, SAS PC. The model was created to allow estimation of a linear growth rate after an initial lag-phase. The mathematical formula for the model was: $\text{weight} = a + b \cdot (\text{time} - c) \cdot (\text{time} > c + 0.5) + b \cdot 0.5 \cdot (\text{time} > c - 0.5) \cdot (\text{time} < c + 0.5) \cdot (\text{time} - c + 0.5)^2$, where a = the initial weight, b = linear growth rate, c = length of the lag-phase. The model is composed of three parts. From $\text{time} = 0$ to $\text{time} = c - 0.5$ the function is a constant, a . From $\text{time} = c - 0.5$ to $\text{time} = c + 0.5$ the function increases quadratically. From $\text{time} = c + 0.5$ the function is linear with a growth rate of b . The quadratic part of the function was inserted to make the curve smooth and give the function a continuous first derivative. If this condition is not fulfilled, the non-linear regression procedure will not estimate standard errors.

Results from the 3 or 4 repetitions were pooled before performing statistical analysis. Results from contaminated Petri dishes were omitted before statistical treatment. For experiment B, data were analyzed both with and without data from non-surviving cultures.

Results

Crystallization of the samples took place at temperatures varying between -11.5° C and -16° C. There was no

obvious correlation between the crystallization temperature in the sample and subsequent regrowth of the frozen samples.

1. Experiment A

The untreated and the precultured cultures resumed growth 1 week after plating. The two other controls initiated growth after 2 weeks, and samples frozen in liquid nitrogen had commenced growth 3 weeks after plating. The lag-phase for the frozen samples varied from 2 to 4 weeks in the 3 repetitions. The growth curves (Figure 1) indicated a linear growth for all treatments after the initial lag-phase. The length of the lag-phases and linear growth rates were estimated by non-linear regression and it was confirmed that the 5 growth curves were parallel (Table 1). As noted by the growth curves (Figure 1) there are only small differences in lag-phase among the first 3 treatments. Freezing the samples to -35°C increased the lag-phase by about one week, and freezing in liquid nitrogen increased it by another week (Table 1).

FDA staining of the cells 4 hours after plating showed that embryos and suspensor cells from the untreated and sorbitol precultured controls were alive. The suspensor cells from the DMSO pretreated control showed almost no fluorescence. In the cryopreserved and thawed samples only a few isolated cells in the embryo heads and scattered in the cell mass showed fluorescence. One week after thawing, regrowth was visible using a dissecting microscope, and at day 11 complete stage 1 embryos with live suspensor cells were found in the cryopreserved and thawed samples.

2. Experiment B

All genotypes exhibited linear growth with different lag-phases preceding the onset of growth (Figure 2). The lag-phase for the untreated control was \leq one week and for the pretreated control it varied depending on genotype (Table 2).

Cryotolerance was determined by 3 different parameters: the fraction of cryopreserved samples that resumed growth, the length of the lag-phase, and the growth rate after the lag-phase. Analysis of regrowth of the cryopreserved samples showed that there were large genotype differences. Additionally there was a significant variation among the different samples and repetitions. In genotype 12, only one sample of 6 (3 repetitions) survived cryopreservation. One dish was lost due to contamination. In

Mg wet weight

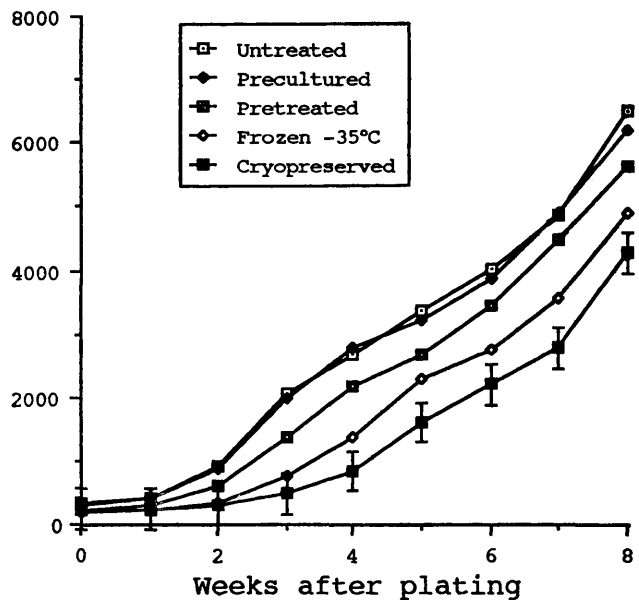


Figure 1. — Regrowth data from cryopreserved and control samples of embryogenic *Abies nordmanniana* cultures (Genotype 8). Each point is an average of 3 replications with 2 Petri dishes per replication. For clarity \pm standard errors have only been shown on the lower curve. Standard errors for the other curves are of the same order of magnitude.

genotype 15, the samples from two replications did not survive. In genotypes 10 and 14, all uncontaminated samples survived and resumed growth. Growth was resumed in genotype 10 after $2\frac{1}{2}$ weeks, in genotype 14 after $3\frac{1}{2}$ weeks, in genotype 15 after $4\frac{1}{2}$ weeks and in genotype 12 the one surviving sample resumed growth after 6 weeks (Table 2).

In all cases the estimated growth rates for the cryopreserved samples were lower than for the two controls (Table 2). The estimate for genotype 12 is of limited value due to the long lag-phase and low fraction of surviving samples. In genotype 15 the estimates of the growth rates included data on non-surviving samples. Restricting the analysis to the surviving samples, the estimate increased from 268 mg/week to 530 mg/week or 65% of the growth rate of the untreated culture. In genotype 14 the growth rate was 76% of the rate before cryopreservation. Despite

Table 1. — Statistical treatment of regrowth of *Abies nordmanniana* (genotype 8). Results were analysed by nonlinear regression. Shown are the estimates of the 3 parameters of the model and their 95% confidence limits.

Treatment	Lag-phase weeks	Growth rate mg WW/week
Untreated	1.32 ± 0.95	844 ± 134
Precultured	1.23 ± 0.81	814 ± 103
Pretreated	1.77 ± 1.19	809 ± 178
Frozen -35°C	2.55 ± 0.66	794 ± 123
Cryopreserved	3.48 ± 0.85	803 ± 206

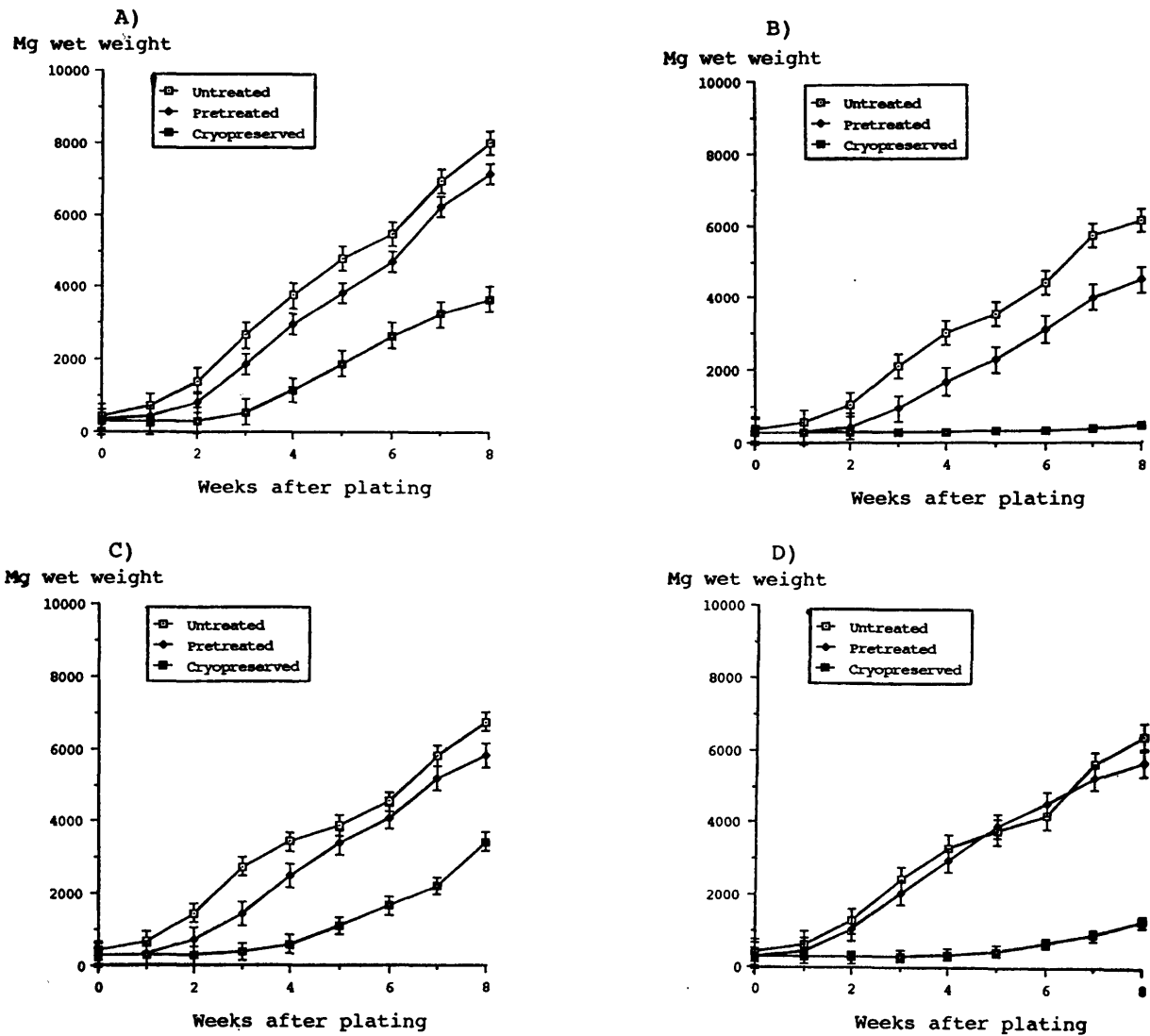


Figure 2. — Growth curves for pretreated and cryopreserved samples of embryogenic *Abies nordmanniana* cultures (Genotypes 10, 12, 14, 15). The mean wet weight of planted cells and filter paper (\pm standard error) is plotted against time after plating. The curves are means of 3 to 4 replications and 2 Petri dishes per replication.

- a) Cryopreservation of Genotype 10. The genotype was cryopreserved 4 times. One cryopreserved dish was lost due to contamination.
- b) Cryopreservation of Genotype 12. The genotype was cryopreserved 3 times. Only one dish from replication 2 showed regrowth within the time frame of this study. The other dish from replication 2 was lost due to contamination.
- c) Cryopreservation of Genotype 14. The genotype was cryopreserved 3 times. All samples showed regrowth and none were lost because of contamination.
- d) Cryopreservation of Genotype 15. The genotype was cryopreserved 4 times. The samples from the 3rd and 4th replication showed no regrowth within 8 weeks after thawing.

Table 2. — Statistical analysis of regrowth data from cryopreserved *Abies nordmanniana* cultures (Experiment B). Upper figure is the estimate for lag-phase in weeks and lower figure is growth rate in mg wet weight per week \pm confidence limits (95%). * Estimates do not include non-surviving samples.

Genotype	Untreated	Pretreated	Cryopreserved
10	1.03 \pm 0.86	1.60 \pm 0.59	2.55 \pm 1.10
	1066 \pm 133	1048 \pm 113	646 \pm 172
12	1.14 \pm 0.98	2.14 \pm 1.16	5.97 \pm 2.34
	845 \pm 122	722 \pm 169	88 \pm 132
14	0.60 \pm 0.84	1.55 \pm 0.80	3.75 \pm 0.96
	831 \pm 87	867 \pm 127	632 \pm 187
15	0.74 \pm 1.15	0.76 \pm 1.18	4.55 \pm 1.38
	811 \pm 119	789 \pm 149	268 \pm 149
15*			4.75 \pm 0.67
			530 \pm 143

the short lag-phase, genotype 10 grew at a rate of only 61% of the rate before cryopreservation.

Discussion

Embryogenic *Abies nordmanniana* cultures could be cryopreserved using the method of KARTHA et al (1988). However, cryotolerance as measured by survival, length of lag-phase, and regrowth was genotype dependent.

GUPTA et al. (1987) noted that no growth was observed in cryopreserved cells of *Picea abies* and *Pinus taeda* for the first 5 weeks following thawing, but GALERNE and DEREUDDRE (1988) and FIND et al (1993) reported regrowth from cryopreserved suspension cultures of *Picea abies* and *Picea sitchensis* after only 3 to 4 days. Such short lag-phases were not observed in our study, but suspension cultures may be more cryotolerant, because they are more uniform and grow in an environment of higher osmotic potential.

In the present work, growth rate was identical before and after cryopreservation in only 1 of the 5 genotypes. In genotypes 12 and 15, very long lag-phases resulted in poor estimates for the linear part of the growth curve. In genotype 14, the growth curve (Figure 2c) indicated that growth increased with time suggesting that this genotype recovered slowly from cryopreservation. Possibly, a true estimate of growth rate for genotypes with low cryotolerance requires a longer observation period.

The observed change in growth rate could result from selection at the cellular level. Most of the cells are killed during cryopreservation and only cells from the embryo head survive (KARTHA et al., 1988; GUPTA et al., 1987; GALERNE and DEREUDDRE, 1988). Although suspensor cells are regenerated by the surviving cells, cultures may need longer time to reestablish their original morphology. The time required for this reestablishment could be genotype dependent as indicated by the present results. To determine whether this is the case, comparisons of morphology and growth rate of cryopreserved and unfrozen cultures should be done several months after thawing. JALONEN and VON ARNOLD (1991) have shown that the morphology of embryogenic *Picea abies* cultures is genotype-dependent and related to regeneration ability. Such information is not yet available in *Abies nordmanniana*, but in the present work cell lines with good regeneration ability (8 and 14) also had the highest cryotolerance.

A change in growth rate following cryopreservation has not been reported in other members of *Pinaceae*. Statistical analysis of regrowth has only been done by FIND et al. (1993) who showed that the growth rate of cultures after cryopreservation was identical to that of unfrozen cultures in one genotype of *Picea sitchensis*. GALERNE and DEREUDDRE (1988) and DURAN (1991) plotted growth curves of cryopreserved and control cultures but made no statistical comparison.

No other publications have reported genotype difference in cryotolerance of embryogenic cultures of *Pinaceans*. Using the same experimental protocol as in the present

study, we have found a large genotype variation in cryotolerance for 70 different embryogenic *Picea abies* cultures. Some genotypes showed fast regrowth and others did not survive cryopreservation (NØRGAARD et al., submitted). DURAN (1991) also tested 3 different genotypes of *Picea abies* in 6 independent replications and found one of the genotypes to have a consistently low cryotolerance. Genotype differences in cryotolerance have also been found in angiosperms (McLELLAN et al., 1990).

Efficient cryopreservation of most genotypes is essential in forest tree breeding in order not to impose unintended selection pressure on the breeding populations. Future research on cryopreservation of embryogenic cultures of *Abies nordmanniana* and other members of *Pinaceae* should focus on developing methods for a broad range of genotypes. It is possible that the methods have to be optimized for groups of genotypes with differing cryotolerance to ensure survival.

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