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SHORT COMMUNICATION

Long-term cryopreservation of embryogenic *Pinus sylvestris* cultures

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Abstract
Long-term cryopreservation is important for producing clones from somatic embryogenesis (SE) since it maintains the regeneration ability of the SE lines during the field tests required for clone selection. In the present work, we report recovery, proliferation and embryo production capacity of embryogenic Scots pine (*Pinus sylvestris* L.) cultures following 2–12 years of cryopreservation. Altogether 108 SE lines from three donor trees were used as material. The SE cultures were originally cryopreserved using two different mixtures of polyethyleneglycol 6000, glucose and dimethylsulphoxide (PGD) as cryoprotectant. Good recovery rates (80–93%) were observed for the lines stored for up to 10 years whereas after 12 years recovery rate was lower (59%). The length of cryostorage did not affect the proliferation rate of the SE cultures. Percentage of the SE lines producing mature embryos varied from 56 to 100, without connection to duration of cryostorage. The average embryo production of the maturing lines varied, but not significantly, from 326 per gFW of tissue following 2 years of cryostorage, to 107–111 per gFW after 9–10 years, and to 55 per gFW after 12 years of storage. Genotypic variation and the age of the cultures at the moment of cryopreservation probably contribute to the observed differences in embryo production. Thus, long-term cryopreservation of Scots pine SE cultures seems possible using PGD as cryoprotectant, and good recovery combined with satisfactory embryo yield can be expected at least up to 10 years of storage.

Keywords: *Maturation, PGD mixtures,* Pinus sylvestris, *proliferation, recovery.*

Introduction

Somatic embryogenesis (SE) is considered the most potential vegetative propagation method for Scots pine (*Pinus sylvestris* L.). The first embryogenic cultures of Scots pine were established already during 1990s from precotyledonary embryos isolated from immature seeds (Keinonen-Mettälä et al., 1996; Sarjala et al., 1997). Since that time, protocols have been improved, and currently, all the stages from SE initiation till the plant regeneration are well known (Aronen et al., 2009; Lelu-Walter et al., 2008).

The existing SE protocols for Scots pine are based on the use of immature zygotic embryos as explants for culture initiation. When using juvenile explants, the final performance of the SE clones can be evaluated only by field tests of regenerated plants taking 5–10 years at the minimum. The Scots pine SE cultures have, however, been found to gradually lose their embryo production capacity with 1–2 years of continuous *in vitro* culture. Cryopreservation of Scots pine SE cultures is thus a pre-requisite for successful clone delivery, being able to maintain regeneration ability during field testing.

Cryopreservation consists of the storage of material at very low temperature, usually in liquid nitrogen (LN) at -196°C. Cryopreservation of plant material started in the 1960s with the works of Sakai (1960, 1965). This technique of storage is based on the reduction and subsequent standstill of the metabolic activities of cells at temperatures of LN. Based on the slowed cell activity, it was postulated that cryopreserved material could be stored without alteration or modification for a theoretically unlimited period with very limited maintenance (Engelman, 2011). Following the theory, there is no clear evidence that morphological, cytological or genetic alterations could be found in plant cells as a consequence of cryopreservation, as reviewed by Harding (2004). However, results obtained by Walters et al. (2004) question the idea of all biological activity being ceased during cryopreservation. In their study with

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the dried seeds of 42 species stored in LN, measurable decrease of germination percentages could be observed after 10 years of storage.

Cryopreservation has been performed with many embryogenic cultures of woody plants (Lambardi et al., 2008) but only a few articles report long-term cryostorage in conifers. After 4 years of cryopreservation of *P. radiata* D. Don SE cultures, Hargreaves et al. (2002) showed a good recovery (100%) on nurse tissue and all the lines proliferated well. More recently, Krajnáková et al. (2011) showed that in *Abies cephalonica* Loudon SE cultures cryostored for 6 years, there was no significant difference in the proliferation of the cultures or the maturation of somatic embryos before and after cryopreservation.

Efficient cryopreservation techniques have been developed for Scots pine SE cultures (Häggman et al., 1998; Lelu-Walter et al., 2008), but there are no reports on their long-term cryostorage. Successful cryostorage is, however, needed to maintain regeneration ability of SE cultures during clonal field testing lasting up to 10 years. The aim of the present work was to study the viability, proliferation ability and embryo production capacity of Scots pine SE lines following long-term (for 2–12 years) cryopreservation.

Materials and methods

Plant material

Altogether 108 different embryogenic lines of Scots pine were used in this study. All the SE lines originate from three open pollinated elite Scots pine clones (K818: 34 lines, K884: 37 lines and K908: 37 lines) growing in the Punkaharju clone collection (61°48′N, 29°17′E, 90 m above sea level). The embryogenic cultures cryostored in the years 1997–2000 were derived from zygotic embryos taken from one-year-old immature cones of Scots pine, initiated and sub-cultured on DCR-based media, as described by Häggman et al. (1998). The SE lines cryostored in 2007 were initated and proliferated as described by Aronen et al. (2009), using also DCR-based media.

Cryopreservation

All the SE lines were cryopreserved using the pretreatments (24 h on proliferation medium supplemented with 0.2 M sucrose, followed by another 24 h on the same medium with 0.4 M sucrose) and slow cooling procedure according to Häggman et al. (1998), with minor modifications, as follows:

In 1997, eight months after their initiation, 8 SE lines from K884 and 15 lines from K908 were cryostored using PGDI (1:1) cryoprotectant mixture: 0.4 ml of polyethyleneglycol 6000, glucose and dimethylsulphoxide (PGD) cryoprotectant mixture (10% polyethyleneglycol 6000, 10% glucose and 10% dimethylsulfoxide, Me2SO in water) was added drop by drop onto tissue incubated in 0.4 ml of growth regulator-free liquid DCR medium containing 0.4 M sucrose in 2 ml cryotube on ice over a period of 30 min. The cryotubes were then let to stand for 30 min on ice before freezing to −38°C at a rate of 10°C/h in a programmable freezer, and finally, immersion into LN.

In 1999, 10–15 months after their initiation, eight SE line from K818, 14 lines from K884, and 11 lines from K908 were cryostored using PGDI cryoprotectant mixture, as described above.

In 2000, 10 months after their initiation, 22 SE lines from K818 and 7 lines from K884 were cryostored using PGDII (1:2.5) cryoprotectant mixture: The samples were incubated in 0.4 ml of growth regulator-free liquid DCR medium with 0.4 M sucrose in 2 ml cryotubes on ice, and 1 ml of the PGD cryoprotectant mixture was added dropwise over a period of 30 min. Then the cryotubes were let to stand on ice for 30 min before freezing to −38°C at a rate of 10°C/h in a programmable freezer, and finally, immersion into LN.

In 2007, 6–7 months after their initiation, 3 SE lines from K818, 8 lines from K884 and 11 lines from K908 were cryostored using PGDI cryoprotectant mixture, as described above.

Thawing and recovery of the cryostored samples

All the cryopreserved samples were regenerated in 2009. Cryotubes, one per line, taken from LN were let to stay for 1–3 minutes in a water bath (37°C), and immediately after that the cryoprotectant was removed quickly, and tissues washed with liquid proliferation medium (LM-K Solution). The tissues were placed on a filter paper and transferred three times, every 24 hours, on proliferation media with decreasing concentration of sucrose (0.4, 0.2 and 0.09 M of sucrose) to rehydrate the tissues. The proliferation medium used was a LM-based medium (Litvay et al., 1985) according to Lelu-Walter et al. (2008) containing 2.2 μM 2, 4-D and 2.3 μM Benzyladenine (BA) (mLV-L), and the thawed tissues were transferred onto fresh medium every second week. After 9–12 weeks on proliferation medium, the number of proliferating lines was determined based on visual observation to give the final recovery percentage.

Evaluation of viability and embryogenic nature of the thawed samples

Immediately after thawing, all the tissues were examined under research microscope. For the viability evaluation, tissues were stained with 0.5% of Fluorescein diacetate in acetone (FDA) diluted 25-fold with water for 25 min, after which observations were made under white light (BF) and under UV light. The viable tissues will be fluorescent under UV and the dead tissues will stay dark. The embryogenic nature of the tissues was observed using acetocarmine staining: staining solution was prepared by dissolving 1 g of carmine into 50 ml of acetic acid (45%) followed by filtering. The samples were stained for approximately 30 min, and observed under white light. The tissue was considered embryogenic, if proembryos consisting of brightly stained, small and densely packed embryogenic head cells with larger and vacuolated suspensor cells could be distinguished.

Proliferation experiments

The growth of the embryogenic lines recovered from cryostorage was tested by using filter paper proliferation according to Aronen et al. (2009): About 200 ($\pm$10) mg of embryogenic tissue was mixed in LM-based liquid proliferation medium, the suspension poured on a sterile filter paper (Whatman #2; 5,5 cm of diameter) in a Buchner funnel, the liquid drained off by suction, and the filter paper placed onto LM proliferation medium. For each line, there were three replicates. The tissues were transferred onto fresh medium after 3 weeks. The proliferation experiment lasted for 6 weeks, after which the tissues were reweighted and stained with acetocarmine. The proliferation of each line is presented as an average growth ratio: Final weight/Initial weight (W_1/W_0).

Maturation experiments

Filter paper maturation according to Lelu-Walter et al. (2008) was used for evaluating embryo production capacity of the lines recovered from cryostorage. About 200 ($\pm$10) mg of embryogenic tissue taken 1–2 weeks after the last subculture was mixed in 5 ml of liquid LM maturation solution (with 10% activated charcoal) before being poured on a filter paper, and placed onto LM maturation medium with 80 μM abscissic acid (ABA) and 0.2 M sucrose (Lelu-Walter et al., 2008). After 9 weeks, the total number of cotyledonary embryos per dish was counted for three Petri dishes per line. Then a maximum of 30 embryos per line was transferred for germination and acclimation in greenhouse following the procedures of Aronen et al. (2009). The quality of the produced embryos was evaluated visually, based on two categories of "good/slim" and "bad/stub" types of embryos according to Aronen et al. (2009).

Proliferation and embryo production after short term or no cryopreservation

Unfortunately, all the SE lines included in the present study were not tested for their proliferation or embryo production capacity prior to cryopreservation or after short-term cryostorage, so these data are available only for part of the material, as follows: proliferation data obtained using DCR medium was published for 1997 materials by Häggman et al. (1998), for 2000 materials by Aronen et al. (2001) and for 2007 materials by Aronen et al. (2009), as shown in Table I. For 1999 materials, embryo production data without cryopreservation is available for 5 SE lines from K818, for 10 lines from K884 and for 7 lines from K908. Maturation experiment was performed as described by Niemi and Häggman (2002) with minor modifications: embryogenic cell masses were first cultivated on growth regulator-free DCR medium for 1 or 2 weeks, then 500 mg ($\pm$20 mg) of tissue was suspended and transferred onto filter paper in a Buchner funnel, as described above, and the filter placed on DCR medium supplemented with 90 uM ABA and 7% (w/v) Polyethyleneglycol (PEG 4000 and 176 mM sucrose for 2 weeks, followed by the cultivation on the same medium without PEG for another 2 weeks. After this, the maturing cultures were cultivated on the DCR medium containing 88 mM sucrose, and transferred onto fresh medium with a 3-week interval. For 2000 materials, embryo production data is available for 26 SE lines from K818 and 17 lines from K884 without cryopreservation, and for 9 lines from K818 and 1 line from K884 following short term, four-week cryostorage using PGDII cryoprotectant mixture. Maturation experiments were done similarly as for 1999 materials, except that the cultures were kept on hormone-free DCR medium for 1 week and that 5% PEG was used. For 2007 material, embryo production data without cryopreservation has been published by Aronen et al. (2009). Maturation of two lines from K818, three lines from K884 and four lines from K908 was performed as tissue clumps on MB4 medium (Aronen et al., 2009).

Statistical analysis

The effect of cryopreservation year and donor tree on the proliferation of the thawed samples of Scots

Table I. Growth ratio (W_1/W_0) and embryogenecity of the Scots pine cultures cryostored in 1997–2007 and thawed in 2009 after a 6-week proliferation experiment on LM medium.

	Long-term cryopreservation			Short-term or no cryopreservation	
	Number of SE lines	Mean ± SE	Percentage of embryogenic lines	Number of SE lines	Mean ± SE
Year of cryopreservation					
1997	11	20.6 ± 1.8	91	No cryo: 6[a]	4.9 ± 0.7[a]
				24h cryo: 6[a]	7.7 ± 2.2[a]
1999	27	20.5 ± 1.3	93	Not available	
2000	26	24.0 ± 1.5	100	No cryo: 2[b]	5.2 ± 3.7[b]
				4w cryo: 2[b]	10.0 ± 0.9[b]
2007	13[d]	17.4 ± 2.1		No cryo: 13*[c]	20.8 ± 2.0[c]
	19[e]	19.1 ± 1,7	84		
Donor tree[f]					
K884	29	21.1 ± 1.2			
K908	21	19.0 ± 1.5			
K818	27	23.0 ± 1.6			

Note: As a comparison, proliferation data prior to cryopreservation or following short-term cryostorage on DCR medium is also given, when available for the yearly sets of the SE lines.
[a]Häggman et al. (1998).
[b]Aronen et al. (2001).
[c]Aronen et al. (2009).
[d]Same genotypes compared prior to and after cryopreservation.
[e]All genotypes cryostored.
[f]Only the 13 genotypes from 2007 included in the analysis.

pine embryogenic lines were studied using analysis of variance, and the means was compared using the Student–Newman–Keuls multiple range test, the data showing normal distribution. Embryo production data did not follow normal distribution, and was therefore analysed by non-parametric Kruskall–Wallis test. The cryopreservation year and donor tree were treated separately in the analysis.

Results

Viability and recovery of the thawed samples

Directly after thawing, more than 90% of all the lines seemed to be viable according to FDA staining. Then, proliferation of tissue was observed after 9–12 weeks following thawing to evaluate the real recovery: 83% of the SE lines cryopreserved in 2007, 80% of the lines from 2000 and 93% of the lines from 1999 were proliferating. A lower recovery rate, 59%, was observed for the lines preserved in 1997. Part of the 1997 material, 6 out of 23 lines, was lost due to media contamination, and not included in the analysed data.

Proliferation experiments

After 6 weeks of proliferation, the average growth ratios of Scots pine embryogenic lines varied 17–24 times the initial weight of the tissue (Table I). The cryostorage time and the donor tree did not impact significantly ($F = 2.04$ and $p = 0.12$ and $F = 1.76$ and $p = 0.18$, respectively) the growth ratio of the SE lines. More than 84% of the lines contained only embryogenic tissue, or embryogenic tissue together with some non-embryogenic cells (Table I). For the material cryostored in 2007, it was possible to compare the 13 same genotypes after 2 years of cryopreservation and without cryopreservation, and no significant difference ($F = 1.47$ and $p = 0.237$) in their growth ratio was observed. The proliferation results after long-term cryopreservation (i.e. for materials stored in 1997, 1999 and 2000) show higher mean values than for the corresponding materials without or after short cryopreservation (Table I). This can be explained by the evolution of the techniques of proliferation, especially the composition of the medium used and potentially also by genotypic differences due to limited availability of the old data.

Maturation experiments

The average number of cotyledonary somatic embryos produced per gFW of tissue varied remarkably in the Scots pine embryogenic lines cryostored for 2–12 years (Table II). Twenty-four of 81 tested lines did not produce mature embryos at all, but on the other hand, in the best line 977 embryos per gFW were obtained. The average embryo production was high after 2 years of cryostorage, on a good level after 9–10 years, and lower after 12 years (Table II). No significant differences were, however, found among the cryopreservation years (Chi-Square 6.97,

Table II. Production of cotyledonary somatic embryos per gFW in the SE lines cryopreserved in 1997–2007, thawed in 2009, and filter-maturated on LM medium according to the cryopreservation year and the donor tree.

	Long-term cryopreservation			Short-term or no cryopreservation		
	Number of tested lines	Number of maturing lines	Mean ± SE in maturing lines	Number of tested lines	Number of maturing lines	Mean ± SE in maturing lines
Year of cryopreservation						
1997	9	9 (100%)	55.2 ± 21.2	Not available		
1999	27	15 (56%)	106.9 ± 34.7	No cryo: 22	14 (64%)	9.2 ± 1.6
2000	26	16 (62%)	110.8 ± 45.8	No cryo: 43	31 (72%)	19.5 ± 3.3
				4w cryo: 10	9 (90%)	5.3 ± 1.3
2007	19	17 (89%)	326.4 ± 75.6	No cryo: 9[a]	9 (100%)	14.7 ± 4.0[a]
Donor tree						
K884	31	24 (77%)	171.3 ± 52.0			
K908	23	19 (83%)	217.3 ± 57.8			
K818	27	14 (52%)	84.4 ± 29.5			

Note: As a comparison, maturation data prior to cryopreservation or following short-term cryostorage on DCR (1999-2000) or MB4 (2007) medium is also given, when available.
[a]Maturation as tissue clumps, Aronen et al. (2009).

$p = 0.073$) or donor trees (Chi-Square 4.38, $p = 0.112$). In general, all the cryostored materials have higher mean values for embryo production than the lines from same donors without cryopreservation. As suggested also for the proliferation results, this is probably a consequence of the quick evolution of the maturation protocols and different media composition used, but also genotypic differences might be involved.

The picked up embryos were germinated and transferred to greenhouse. Majority of the produced embryos were of good quality. The best conversion rate into plants was observed in the lines cryopreserved only for 2 years, and the lowest conversion rate in the lines kept in cryostorage for the longest period, 12 years. The conversion of the slim-type embryos in all the materials was remarkably better than that of stub-type embryos (Table III). The performance of the regenerated emblings in greenhouse during the first growing season was normal, and they grew, on an average, 85.8 (±2.8 SE) mm, and showed remarkable differences among the lines with the means varying from 26.0 (±3.5) to 185.2 (±7.8) mm.

Discussion

In the present study, we report the viability, proliferation and embryo maturation capacity of Scots pine SE lines following long-term (for 2–12 years) cryopreservation. For most of the conifer species, the maximum recovery after short-term cryopreservation is between 60% reported for *Pinus patula* Schiede ex Schltdl. & Cham. and 100% observed e.g. for *Pinus caribaea* Morelet 'Hondurensis', *Picea glauca* (Moench) Voss, *P. engelmannii* Parry ex Engelm. and *Pinus radiata* (Lambardi et al., 2008). In Scots pine, Häggman et al. (1998) reported 78% of the embryogenic lines remaining viable after 24 hours of cryopreservation and being recovered within 7 weeks, while Lelu-Walter et al. (2008) reached 100% recovery from cryopreservation lasting up to 3 years. In the present study, over 90% of the lines were viable when observed directly after thawing, and recovery rates observed 9–12 weeks later, were from 80 to 93% after 2–10 years of cryostorage. A lower recovery rate, 58%, was however recorded for the samples stored for 12 years.

The proliferation experiment performed with the present materials showed that a period of 2 years of cryopreservation did not have an effect on the proliferation ability of the 13 lines studied both prior to and following cryostorage. Proliferation ratios (21–24 x) comparable with the published ones (Aronen et al., 2009) were observed also after 9, 10 or 12 years of cryopreservation suggesting that a cryostorage time does not affect the growth of the recovered lines. Then, as suggested by Aronen et al. (2009), our observations do not show any significant differences in the proliferation of lines originating from different donor trees.

However, we observed differences in the production of mature embryos in the present materials depending on the cryopreservation year: the quantity of mature embryos produced was higher, although not statistically significantly, after 2 years of cryostorage than after longer periods of 9–12 years. In a deciduous tree, *Betula pendula*, differentiation ability measured as regrowth rate of the thawed buds was found to be significantly lower after 5 years of cryostorage than following shorter cryopreservation periods (Ryynänen, 1999). On the other hand, Lelu-Walter et al. (2008) showed that the embryo production capacity of the Scots pine SE cultures

Table III. Quality and conversion rate of the somatic embryos produced in the embryogenic lines of Scots pine following cryostorage of 2–12 years (from 1997–2007 to 2009).

Cryopreservation year	1997	1999	2000	2007
Number of embryos studied	69	57	95	318
Embryo type				
Good/slim (%)	65	73	81	73
Bad/stub (%)	35	27	19	27
Conversion rate into plants				
Of slim embryos (%)	60.0	73.0	68.8	84.9
Of stub embryos (%)	29.2	16.7	16.7	24.4
Overall (%)	49.3	58.0	58.9	68.8

decreases significantly when the cultures age from 8 to 24 months. In the present study, the SE lines cryostored for 2 years were cryopreserved at the age of 6–7 months, while the lines cryostored for 9–12 years were older (8–15 months) at the time of cryopreservation.

Overall the quantities of cotyledonary embryos produced (from 55 to 326 per gFW of tissue) in the thawed SE cultures in the present study were comparable with the numbers reported for Scots pine without cryostorage. According to Aronen et al. (2009), the embryo yield in the filter maturation comparable with the present study was on an average $321 \pm$SE 130 per gFW, and in the study by Lelu-Walter et al. (2008), with the same maturation method as in the present study, the mean number of embryos produced varied from 196 to 406 per gFW depending on the age of the SE culture. Thus, the embryo maturation capacity of the present SE lines can be considered good or satisfactory for a period of conservation up to 10 years.

In their study, Lelu-Walter et al. (2008) suggested that there is a positive interaction between the capacity for embryo production and the conversion rate of the embryos. This interaction can be seen also when comparing the yearly lots of the present material: the better the average embryo production of the lines, the higher the conversion rate too. The present study also confirms the observation made by Aronen et al. (2009), who showed that the quality of the somatic embryo is an essential factor for the rooting and the conversion into plant: slim-type embryos show better conversion rates.

When evaluating the effects of the length of cryopreservation in the present study, it should be remembered that different genotypes were cryostored yearly, and thus also genotypic variation has to be counted as a potential factor. We have, however, used the same families when comparing the cryostorage years, i.e. the SE lines from the same donor trees, and as big number of the lines as possible to balance the genotypic variation. In addition to preservation time, culture age and genotypic effect, also original quality of the SE lines at the time of cryostorage, might have influenced the results, the SE technique for Scots pine being much enhanced (Aronen et al., 2009; Lelu-Walter et al., 2008) since the establishment of the oldest cryopreserved cultures. The present results also suggest that the LM medium widely used for pine SE cultures (Klimaszewska et al., 2001; Lelu-Walter et al., 2006; Percy et al., 2000 Park et al., 2006; Lelu-Walter et al., 2008) seem to be more favourable for the Scots pine embryogenic cultures than the DCR-based media used in former studies.

As a conclusion, the long-term cryopreservation of Scots pine SE cultures seems possible when using PGD mixture as cryoprotectant. On the basis of the present observations, the recovery and proliferation of the embryogenic lines after thawing is good up to 10 years of cryostorage, and also embryo maturation capacity remains on satisfactory level. In order to ensure good embryo production, it would be recommendable to control the original quality and embryogenecity of the cultures prior to cryopreservation, as well as to perform cryopreservation before the cultures begin to age.

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