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Research Article

Cryopreservation of *Abies alba* embryogenic tissues by slow-freezing method

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Abstract

Embryogenic tissues of *Abies alba* Mill. were cryopreserved using the slow-freezing approach. Four cell lines were incubated for 24 h on a medium with 0.5 M sorbitol and pre-treated with 5% DMSO. Subsequently, the tissues were frozen at a cooling rate of 1 °C min^{-1} to -40 °C and transferred to liquid nitrogen for 72 hours. After thawing in a water bath at 40 °C, the tissues were cultivated on a proliferation medium. All tested lines recovered, but variations in regrowth frequencies across cell lines were noticed (91.66 to 100%). The recovered tissues showed similar features to the control 2 (non-pre-treated and non-cryopreserved tissues). In the accumulation of fresh and dry mass, no statistically significant differences were observed between cryopreserved cultures and control 2. The cryopreserved tissues produced cotyledonary somatic embryos capable of germination. Microscopic observations revealed considerable structural changes as a consequence of the cryopreservation procedure. The long vacuolated suspensor cells were disrupted, and mostly the meristematic cells of the embryonal region survived. The typical bipolar structure of early somatic embryos has been regained during the post-thaw period. Differences in cryotolerance across cell lines were also observed.

Keywords: conifers; cryo-tolerance; regeneration; silver fir; somatic embryogenesis

Introduction

Somatic embryogenesis (SE) is an asexual process by which an embryo develops without the sexual fusion of gametes from somatic cells of a plant body. The process is based on the totipotency of plant cells, an unique phenomenon characteristic for plant kingdom. The development of somatic embryos requires dedifferentiation of differentiated somatic cells and their subsequent reprogramming leading to SE differentiation (Fehér, 2015). The process has been reported to occur in many plant species under *in vitro* conditions. The first report for somatic embryo formation in conifer trees is dated back to 1985, when the process of somatic embryogenesis was first demonstrated for Norway spruce (Chalupa, 1985; Hakman *et al*., 1985). Since this time, initiation as well as plantlet (somatic seedling) regeneration from early somatic embryos have been reported in a wide range of conifer species belonging to genera *Abies*, *Picea*, *Pinus*, *Pseudotsuga*, *Taxus*,

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Araucaria (reviews Lelu Walter *et al*., 2013; Salaj *et al*., 2015; Klimaszewska *et al*., 2016). In the *in vitro* process of conifer somatic embryogenesis several steps are distinguished as follows: initiation of embryogenic tissues, embryogenic tissue proliferation, somatic embryo maturation, somatic embryo germination leading to plantlet (somatic seedlings) regeneration. The successful maintenance of initiated embryogenic tissue requires regular subculture onto fresh media in one-two-three-week intervals to maintain vigorous growth and regeneration capacity. The long-term maintenance of embryogenic tissues by regular transfers on solid or in liquid media is time-consuming and laborious, and moreover there is a risk of microbial contamination. It has also been observed, that in many species by increasing numbers of subcultures the production of mature somatic embryos decreased or was completely lost (Klimaszewska *et al*., 2009; Li *et al*., 2013). Cryopreservation of embryogenic tissues could be an alternative method for their long-term maintenance. The tissues are cryopreserved in a very early stage of somatic embryo development (stage 1 according to von Arnold and Hakman, 1988), and after removal from liquid nitrogen they are relatively easy to regenerate and used for somatic seedlings production. Moreover, the tissues can be stored in liquid nitrogen until the field tests of planted somatic seedlings indicate the most productive cell lines.

Cryopreservation is a process where plant cells, tissues or more complex structures such as embryos and shoot tips are stored at ultralow temperature (-196 °C) in liquid nitrogen.

Under these conditions, any enzymatic or chemical activity is stopped, preventing the stored biological material from structural and physiological damage. These conditions also allow the storage of plant material for an unlimited time period. At present cryopreservation is a widely accepted biotechnological tool for *ex situ* conservation of a variety of crops, fruit- as well as forest trees (Panis and Lambardi, 2005). Moreover, cryopreservation represents a safe and cost-effective tool, the samples are stored in small volumes, protected from contamination and without the laborious *in vitro* maintenance (Engelmann, 2004).

For conifer embryogenic tissues successful cryopreservation has been reported in *Abies nordmanniana* (Norgaard *et al*., 1993a), *A. cephalonica* (Aronen *et al*., 1999; Misson *et al*., 2006; Krajňáková *et al*., 2011), *A. alba* (Krajnaková *et al*., 2013), *A. fraseri* (Pullman *et al*., 2016), hybrids *Abies alba* x *A. cephalonica* and *Abies alba* x *A. numidica* (Salaj *et al*., 2010; Salaj *et al*. 2016), *Araucaria angustifolia* (Fraga *et al*., 2016), *Picea abies* (Gupta *et al*., 1987; Norgaard *et al*., 1993b; Vondráková *et al*., 2010), *P. glauca engelmanni* (Cyr *et al*., 1994), *P. mariana* (Klimaszewska, 1995), *P. sitchensis* (Find *et al*., 1993; Gale *et al*., 2007), *Pinus caribaea* (Laine *et al*., 1992), *P. nigra* (Salaj *et al*., 2007), *P. patula* (Ford *et al*., 2000), *P. radiata* (Hargreaves *et al*., 2002), *P. sylvestris* (Häggman *et al*., 1998), *P. roxburghii* (Mathur *et al*., 2003), *P. pinaster* (Marum *et al*., 2004; Lelu-Walter *et al*., 2006; Alvarez *et al*., 2012), *Pinus elliottii* x *P. caribaea* (Nunez *et al*., 2017), *Torreya taxifolia* (Ma *et al*., 2012), *Taxus* x *media*, *T. floridana* (Skrlep *et al*., 2008), *Tsuga canadensis, T. caroliniana* (Merkle *et al*., 2014), *Chamaecyparis thyoides* (Ahn *et al*., 2017).

The "classical "slow-freezing method of cryopreservation has been applied for the mentioned species. This technique is convenient for long-term storage of unorganised plant tissues such as calli or suspension cultures as well as embryogenic tissues (Reinhoud *et al*., 2000). The procedure involves the pre-treatment of samples with sucrose or sorbitol, following treatment with cryoprotectant (frequently DMSO in concentration 5 to 15%), subsequently prefrozen at a rate -0.3 to -0.5 °C per minute to -40 °C and finally plunged into liquid nitrogen. The samples should be rapidly thawed in a water bath at a higher temperature (30 to 40 °C) to avoid recrystallization and ensure cell/tissue recovery. Another technique of cryopreservation – vitrification very often used for herbaceous plants (Sant *et al*., 2008) or hardwood trees (Lambardi *et al*., 2005; Guzman Garcia *et al*., 2013; San Jose *et al*., 2015), for conifer embryogenic tissues has been used exceptionally, e.g., in *Picea sitchensis* (Touchell *et al*., 2002) or *Araucaria angustifolia* (Demarchi *et al*., 2014).

The presented study focuses on cryopreservation of *Abies alba* Mill. embryogenic tissues and on their subsequent regeneration, growth parameters of regenerated tissues and control (non-cryopreserved tissues) as well as on somatic embryo maturation. The study is completed by structural characterization during the whole

process of cryopreservation (pre-treatment and post thaw recovery). The relationship between genotype and cryotolerance has also been studied.

Materials and Methods

Plant material

Four cell lines (A01, A30, A31, A32) of embryogenic cultures of silver fir (*Abies alba* Mill.) were initiated from immature zygotic embryos (Salaj *et al*., 2020). The cultures were maintained on DCR (Gupta and Durzan, 1985) proliferation medium by sequential subculturing at 2-3 weeks intervals. The DCR medium was supplemented with 4.4 μM BA. The cultures were incubated in darkness at a temperature of 23 °C. Additional cell lines – altogether 14 – were included to test the relationship between cryotolerance and genotype (A38, A39, A40, A41, A43, A46, A47, A52, A55, A56, A57, A59, A60, A61).

Cryopreservation procedure

The cryopreservation slow-freezing method used in these experiments was described in detail in previous studies (Salaj *et al*., 2010). Briefly, eight days after the last subculture, 3 g of intensively growing embryogenic tissue from cell lines A01, A30, A31, A32 was precultured on a solid DCR proliferation medium supplemented with sorbitol (0.5 M) in Petri plates (2 x 1.5 g) in darkness. 24 hours after incubation, the tissue was resuspended by shaking in plastic tubes containing 9 ml of the same composition liquid medium. Gradually 9 ml of 10% DMSO, dissolved in proliferation medium, was added in three intervals to reach a final concentration of 5% and 18 ml. The tubes with cell suspension were kept on ice. Finally, 1.8 ml of the suspension was pipetted into cryovials (5 x 1.8 ml). The cryovials were placed in Mr Frosty container filled with isopropanol and placed into a deep freezer at -80 °C. The temperature was monitored with a thermometer in one cryovial. When the temperature in the cryovials reached -40 °C, the cryovials were plunged into liquid nitrogen and kept in these conditions for 72 hours. Thawing of samples occurred in a water bath at 40 °C for 3 min. After thawing, the content of cryovials was poured into stacked filter paper discs to absorb the liquid. The filter paper discs with cells on the surface were placed on the solid proliferation DCR medium and cultured as described earlier. The experiment was repeated three times with 5 samples (n=15).

As described earlier, the remaining 9 ml cell suspension was pipetted on stacked filter paper discs and cultivated on DCR proliferation medium. These samples were designed as control 1 (C1), pre-treated but not frozen (cryopreserved) cells. Cell lines proliferating without pre-treatment and without cryopreservation were designed as control 2 (C2).

Growth analysis

The accumulation of fresh and dry mass was evaluated after approximately 3- and 15-months post thaw growth of cell lines A01, A30, A31, A32. Cryopreserved tissues were compared with non-pre-treated and non-cryopreserved tissues control (C2) on proliferation medium. On day eight, after sub-culturing 0.5 g of well growing tissue was transferred to a fresh proliferation medium. The fresh mass and dry mass accumulation were evaluated on seven and fourteen days after the last subculture. Dry mass was measured after embryogenic tissues were dried in an oven at gradually increasing temperatures (from 60 to 105 °C) until a constant dry mass was reached. The experiments were repeated twice with five samples for each cell line and the obtained data were statistically analysed by t-test.

Structural observations

Microscopic observations were done on C2 and cryopreserved-thawed tissues of different cell lines. Small pieces of well growing tissues (1-3 mm in size) were taken, placed on a glass slide, stained with one-two

drops of 2% acetocarmine, squashed and covered by a cover slide. The viability of embryogenic structures was evaluated after staining with 0.5% fluorescein diacetate (FDA) immediately after the sorbitol pre-treatment as well at 8-12 days after thawing, when the first symptoms of regrowing tissues were observed. The preparations were observed under a light microscope Axioplan 2 (Carl Zeiss, Jena, Germany) equipped with a camera system SonyDXC-5500.

Maturation of somatic embryos

Proliferating embryogenic tissues at eight days after subculture were weighed and resuspended in liquid DCR half strength medium without organic additives. Suspension aliquots of 100-120 mg per Petri dish were pipetted on stacked filter paper discs and after absorbing the liquid, the filter paper discs with cells were transferred to a maturation medium. For maturation medium DCR (Gupta and Durzan, 1985) supplemented with 35 μM abscisic acid (ABA), 7.5 % PEG 4000 (polyethylene glycol), 500 $mg.L^{-1}$ enzymatic casein hydrolysate, 500 $mg.L^{-1}$ glutamine, vitamins as thiamine (1 $mg.L^{-1}$), nicotinic acid (0.5 $mg.L^{-1}$), pyridoxine (0.5 $mg.L^{-1}$), myo-inositol (100 $mg.L^{-1}$), and 3% maltose was used. The maturation occurred in darkness at 23 °C. The number of developing embryos was counted by cotyledonary developmental stages (categorization according to: von Arnold and Hakman, 1988). The maturation experiments were repeated two times with 5-6 Petri plates for each cell line. Cell lines A01, A30, A31, A32 were included in the maturation experiments. The embryogenic capacity was estimated as the number of developing somatic embryos per 1 g of fresh weight.

Testing of genotype effect

In this experiment, the relationship between genotype/cell line and cryotolerance was investigated. The cell lines were initiated, proliferated and cryopreserved as described above and stored in liquid nitrogen for 120 min.

Statistical analysis

The obtained data were analysed using a t-test. Accumulation of fresh and dry mass was evaluated and compared in control (non-cryopreserved and non-treated, C2) tissues and in cryopreserved and recovered tissues after 3 and 15 months after thawing.

Results

Tissue regeneration after cryopreservation

Depending on the cell line, control tissues (C1, pre-treated but not cryopreserved) started to grow three to five days after pre-treatment. Their regrowth frequency was 100%. In cryopreserved tissues regrowth started at nine to 10 days after thawing with some differences between cell lines. Tissue regeneration in cell lines A32 and A01 was similar and the regrowing tissues gradually covered the surface of the medium (Figure 1A).

After thawing, many cell groups were necrotised in cell line A30 and later scattered cell clumps regenerated on the surface of the medium (Figure 1B). Despite this, all cryopreserved cell lines regenerated with regrowth (frequency) rates between 92 and 100% (average 96.24%). There was no statistical difference between cryopreserved and C1 tissues (Figure 2).

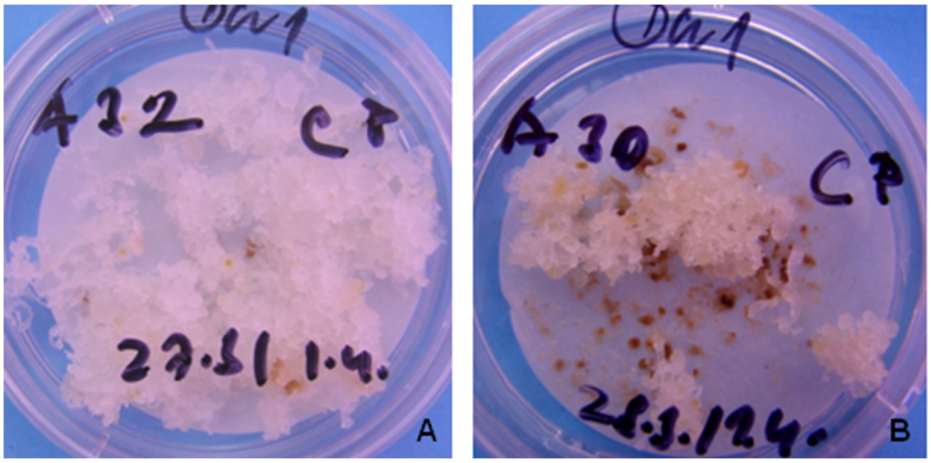

Figure 1. Tissue regeneration in different cell lines approximately 4 weeks after thawing, **A** - cell line A32, **B** - cell line A30

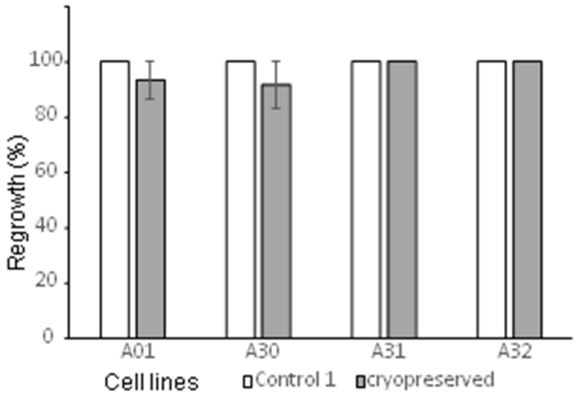

Figure 2. Tissue regrowth (%) in cell lines after cryopreservation (72 hours storage in liquid nitrogen)

Growth parameters after thawing

The cryopreserved tissues intensively proliferated after thawing and looked similar in appearance and colour to C2 tissues. Three months after thawing, the fresh mass of tissues (Table 1) gradually increased and no statistically significant difference in growth was observed between the cryopreserved tissues and C2 (cell lines A01 and A31). Conversely, in cell lines A30 and A32 thawed cryopreserved tissues developed faster than C2 tissues (day 14). Fresh mass accumulation 15 months after thawing showed a similar trend without statistically significant differences between cryopreserved tissues and C2.

Table 1. Fresh mass accumulation (g) in C2 and cryopreserved tissues (CP) at 3 and 15 months after thawing (standard errors of means are in parenthesis) in four cell lines

Cell line	C2		CP tissue at 3 months post thaw		CP tissue at 15 months post thaw	
	day 7	day 14	day 7	day 14	day 7	day 14
A01	0.88 (0.05)	2.46 (0.21)	0.94 (0.03)	2.01 (0.05)	0.66 (0.04)	2.47 (0.06)
A30	1.15 (0.06)	2.09[a,b] (0.09)	1.22 (0.03)	2.37[a] (0.07)	1.57 (0.05)	2.48[b] (0.08)
A31	1.17 (0.09)	2.02 (0.09)	0.73 (0.06)	1.69 (0.16)	0.80 (0.04)	1.87 (0.14)
A32	1.12 (0.07)	2.19 (0.04)	0.94 (0.05)	2.51 (0.12)	0.73 (0.04)	2.58 (0.54)

[a] $P \leq 0.05$; [b] $P \leq 0.01$

To follow dry mass accumulation (Table 2), 500 mg of fresh tissues (inoculum) was dried at day 0. There was no significant difference between inoculum dry mass between different cell lines. Also, dry mass accumulation between cryopreserved tissues and C2 remained the same except for cell line A01 on day 14 when dry mass accumulation after 3 months of thawing was smaller than in C2. These differences disappeared after 15 months (on day 14).

Table 2. Dry mass accumulation (mg) in control 2 and cryopreserved tissues (CP) was evaluated 3 and 15 months after thawing (standard errors of means are in parenthesis) in four cell lines

Cell Line	C2			CP tissue 3 months post thaw			CP tissue 15 months post thaw		
	day			Day			day		
	0	7	14	0	7	14	0	7	14
A01	25.40 (1.62)	32.21 (2.41)	71.18[a] (3.42)	20.77 (2.84)	33.92 (2.09)	59.91[a] (2.54)	21.80 (1.08)	30.24 (1.64)	85.80 (1.83)
A30	25.90 (1.61)	38.19 (1.58)	76.50 (2.27)	25.70 (2.23)	55.31 (1.88)	94.92 (3.16)	24.52 (1.51)	52.01 (2.77)	88.86 (2.99)
A31	26.20 (1.32)	43.38 (2.98)	70.90 (2.88)	28.43 (1.15)	35.92 (1.14)	66.19 (4.02)	21.42 (2.07)	37.10 (1.04)	68.64 (3.15)
A32	22.70 (1.81)	39.65 (2.34)	78.76 (4.63)	25.43 (1.58)	32.59 (1.17)	77.76 (3.18)	21.02 (1.36)	30.85 (2.09)	90.33 (1.83)

[a] $P \leq 0.05$

Structural features

The embryogenic tissues were rapidly growing and composed of stage one bipolar somatic embryos. The cryopreservation procedure (including pre-treatment) was accompanied by structural changes. FDA staining revealed that the long, vacuolated suspensor cells were disrupted and the fluorescent signal was concentrated in meristematic embryonal cells (Figure 3A, B). During the post thaw period, cell division occurred in surviving meristematic cells and the mitotic activity led to the differentiation of meristematic cell clumps (Figure 3C). The subsequent vacuolisation resulted in the formation of early bipolar structures (Figure 3D) and finally, typical somatic embryos (Figure 3E).

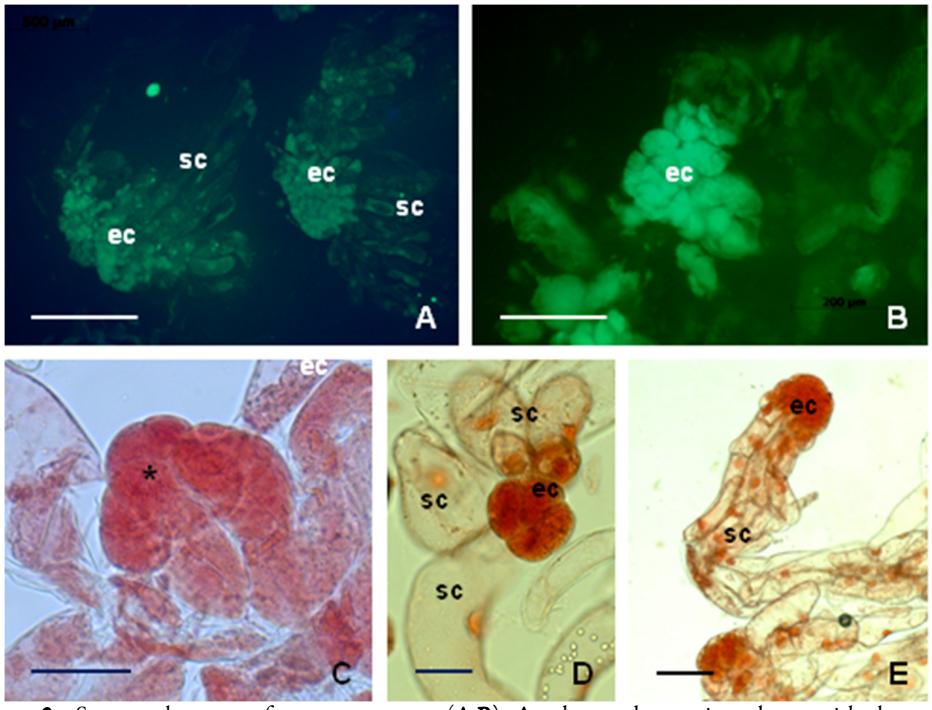

Figure 3. Structural aspects after pre-treatment (**A**,**B**): **A** - damaged somatic embryos with shortened suspensor cells (**sc**) and meristematic embryonal cells (**ec**) concentrating the fluorescence signal, **b** - detailed view of the meristematic embryonal cells with strong fluorescence signal; regeneration of somatic embryos (**C-E**): **C**- cell division in surviving meristematic cells (apparent mitotic figures *), **D** - cellular vacuolisation leading to first symptoms of bipolarity, **E** - fully differentiated somatic embryo with meristematic embryonal cells and long, vacuolated suspensor cells. Scale bars: A, B, E – 200 µm, C, D – 100 µm

Somatic embryo maturation

Maturation of somatic embryo production was tested in cell lines E30, E31, E32. Cell line A01 lost its maturation ability and only abnormal embryos were observed. The remaining three cell lines produced cotyledonary somatic embryos (cse) per 1g as follows: A30 (60 cse, SE 20.05), A31 (64 cse, SE 17.02), A32 (131.5 cse, SE 27.25). The somatic embryo production was cell line dependent and there were considerable differences in maturation capacity even within one cell line (e.g., 0 to 140 or 0 to 200). Moreover, somatic embryo maturation was not synchronised as at the same time different developmental stages (precotyledonary and cotyledonary) were visible (Figure 4A). The well-formed somatic embryos germinated and somatic seedling regeneration occurred (Figure 4B). The germination frequencies ranged from 47 to 55.5%.

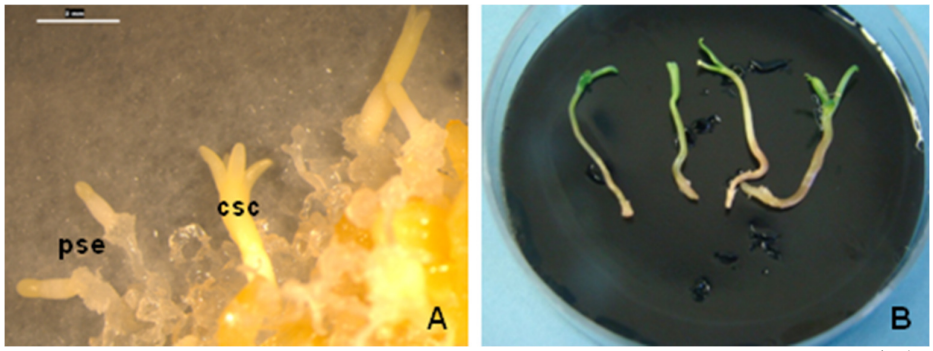

Figure 4. Development of somatic embryos after cryopreservation, **A** - precotyledonary (**pc**) and cotyledonary (**cse**) somatic embryos, **B** - regenerated plantlets (somatic seedlings). Scale bar: 2 mm

Relationship between cryotolerance and genotype

The slow-freezing protocol, previously tested for *Abies* hybrids (Salaj *et al*., 2016), was used to assess the cryotolerance of 14 cell lines. After storage in liquid nitrogen all the tested cell lines regenerated, but variation was observed across genotypes, reaching values of 60 to 100%. The C1 cell lines regenerated in high frequencies and the differences across genotypes were less apparent (Figure 5).

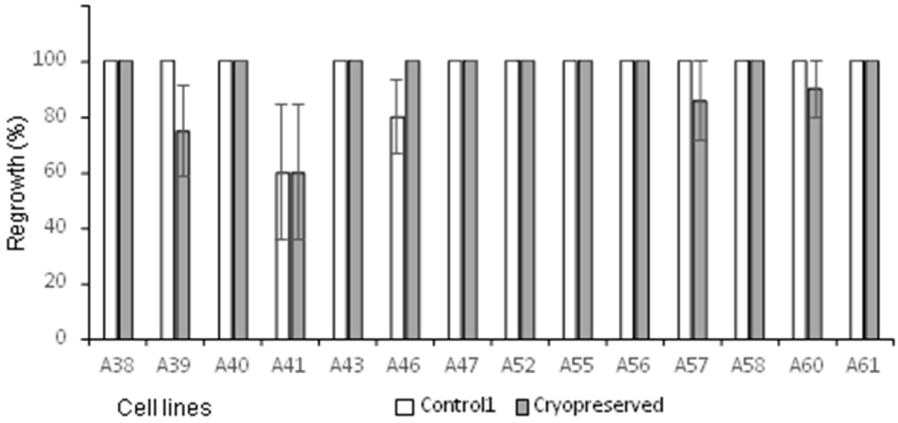

Figure 5. Genotype effect on cryotolerance (tissue regrowth in different cell lines stored for 2 hours in liquid nitrogen)

Discussion

In our previous work, we reported the cryopreservation of seven embryogenic cell lines of *Abies alba* (Salaj *et al*., 2020) using the slow-freezing technique elaborated for hybrid firs (Salaj *et al*., 2010; Salaj *et al*., 2016).

In the present work, embryogenic tissues of *Abies alba* were cryopreserved with the slow-freezing method. Using Mr. Frosty container (Nalgene ™) with a cooling rate of approximately -1 °C per min to -40 °C, recovery of tissues was on average 96.24%, with different frequencies for different cell lines (A01, A30, A31, A32). This is in line with several reports on cryopreservation of conifer embryogenic tissues using this simple and relatively cheap container (Vondrakova *et al*., 2010; Carneros *et al*., 2017; Lineros *et al*., 2018). Varis *et al.* (2017) compared two slow-freezing methods using the Mr. Frosty container and programmable freezer (cooling rates 0.17 °C, 1 °C per minute). Freezing in the programmable freezer resulted in 77% recovery, while freezing in the Mr. Frosty container gave 66% recovery, but in some experiments, 100% recovery could be achieved with both approaches.

Tissues and cells used for cryopreservation should be in their optimal physiological state. Vigorously growing, healthy tissues ensure optimal recovery (Laine *et al*., 1992; Mathur *et al*., 2003). In our experiments, we used tissues at their early exponential growth phase while showing frequently bipolar somatic embryo formation.

The age effect (number of subcultures) on tissue regeneration after cryopreservation has been studied in *Pinus sylvestris* (Latutrie and Aronen, 2013) and *P. pinea* (Carneros *et al*., 2017). In *P. pinea* no relation was observed between age and growth rate due to cryopreservation. Conversely, in *Pinus sylvestris,* the age effect was markant.

Sorbitol pre-treatment (0.5 M) combined with DMSO (5%) was successfully applied to *Abies alba* embryogenic tissues and our results clearly indicate their beneficial effect with respect to tissue regrowth as well

as proliferation. The mild osmotic stress increased desiccation tolerance and subsequent freezing resistance (Touchell *et al*., 2002). Sorbitol as a pre-treatment solution has been applied in a number of conifer embryogenic tissues (Norgaard *et al*., 1993a; Cyr *et al*., 1994; Vondrakova *et al*., 2010) and the success of cryopreservation is based on its stereochemical arrangements (Turner *et al*., 2001). In *Abies alba,* the single cryoprotective treatment (5% DMSO) resulted in high survival as well as satisfactory growth after thawing. In contrast, a mixture of cryoprotectants (PGD1) gave better survival and growth for embryogenic tissues of *Abies nordmanniana* (Aronen *et al*., 1999). In *A. alba,* the high survival rates for C1 tissues (pre-treated but not frozen) indicated that the cryoprotectant had no inhibitory effect on survival.

Cryopreservation studies of conifer embryogenic cultures indicated their relatively high cryotolerance. In interior spruce, 345 cell lines were cryopreserved with 94% survival (Cyr *et al*., 1994), in *Abies cephalonica* 5 weeks after cryostorage, 87% of samples proliferated intensively (Aronen *et al*., 1999). Varis et al. (2017) cryopreserved 136 cell lines in Norway spruce with 100% recovery. Similarly, after cryostorage, high recovery percentages (87.5%) were achieved in *Pinus pinea* (Carneros *et al*., 2017), in *P. pinaster,* the recovery percentages reached 68.3 to 100% (Alvarez *et al*., 2012), and in the hybrid *Pinus elliottii* x *P. caribaea* 25 to 100% recovery rates were observed (Nunez *et al*., 2017) depending on the treatment. These facts suggest cell line and treatment dependence for conifer embryogenic tissues.

Fresh as well as dry mass accumulation evaluated 3 and 15 months after thawing was not adversely affected by cryopreservation of *Abies alba*. In some cell lines, tissue proliferation was different on day 7, but these differences disappeared on day 14 indicating cell line dependence on the cryo-treatment. Similarly, in *Araucaria angustifolia*, treatment and cell line affected the post-thaw-growth. On day 30 after thawing distinct differences were observed depending on the cell line and treatment, but by day 60 the cell lines showed 100% regrowth (Fraga *et al*., 2016).

In the present study, the cryopreservation procedure profoundly affected the somatic embryo structural integrity. Structural observation, based on FDA staining, showed mostly that meristematic embryonal cells survived cryopreservation, the vacuolated suspensor cells were disrupted, resulting in the disintegration of bipolar somatic embryos. The disintegration started already during pre-treatment with sorbitol and showed a very similar pattern immediately after thawing. The same phenomenon was observed in *Pinus sylvestris* (Häggman *et al*., 1998) and *Picea abies* (Vondrakova *et al*., 2010). Detailed structural investigations, using TEM microscopy analysis revealed that different cell types responded differently to cryo-treatment. Small meristematic cells with dense cytoplasm and small vacuoles showed a low level of plasmolysis caused by osmotic dehydration. Contrary, large vacuolated cells exhibit a high level of plasmolysis, and the loss of water from protoplasts may be lethal leading to much damage and death of cells (Volk and Caspersen, 2007). The FDA test to evaluate cell survival after thawing is a frequently used procedure in cryopreserved conifer embryogenic cultures (Häggman *et al*., 1998; Ford *et al*., 2000; Salaj *et al*., 2010; Demarchi *et al*., 2014). However, this test may not be the only reliable indicator of successful tissue recovery and subsequent growth (Perez *et al*., 1997).

In our experiment the purpose of the FDA test was to follow the structural changes occurring after pre-treatment and post-thaw recovery. Our observations indicate that the process of bipolar somatic embryo disintegration (during the cryopreservation procedure) is reversible. Indeed, the surviving meristematic cells from the embryonic part restore mitotic activity leading to the formation of meristematic cell clusters. Vacuolisation of meristematic cells of the cell clusters resulted in the formation of early bipolar structures giving rise to somatic embryos (Salaj *et al*., 2016). During the post-thaw period, the original structure of somatic embryos was regained and the recovered somatic embryos could develop further.

Embryogenic cell lines of *Abies alba* exhibited different responses to cryoprotective as well as freezing treatments. The genotype-dependent response to cryopreservation has been documented in herbaceous plants (Martin *et al*., 2015) as well in hardwood (Bradai and Sanchez-Romero, 2021; O'Brien *et al*., 2021; Pence and Chaiken, 2021) and conifer trees (Norgaard *et al*., 1993a; Ahn *et al*., 2019). The cellular composition of *Abies*

alba embryogenic tissues is very heterogeneous, composed of bipolar structures, long vacuolated cells, meristematic cell clumps (Salaj *et al*., 2020) and the individual cell line responses to cryopreservation may be ascribed to this composition. The different cryotolerance may also be related to different growth characteristics of the tested cultures as it was demonstrated in embryogenic suspension cultures of Norway spruce and Sitka spruce (Find *et al*., 1998).

The cryopreserved tissues produced cotyledonary somatic embryos with variable yields for individual cell lines. The maturation capacity of cryopreserved cell lines of *Abies alba* was comparable to the maturation capacity of non-cryopreserved tissues (Salaj *et al*., 2020), except the cell line A30, where the yield was higher.

The distinct genotype effect on somatic embryo maturation in cryopreserved as well as in control (non-cryopreserved tissues) in conifers is a well-known phenomenon (Alvarez *et al*., 2012; Salaj *et al*., 2012; Latutrie and Aronen, 2013). The effect of the cryopreservation procedure on maturation capacity was demonstrated in several conifer species. In *Pinus radiata,* the maturation capacity was negatively affected by pre-treatment compared to control, but among pre-treated cell lines, no statistically differences in the number of developed mature somatic embryos were observed, albeit the genotype effect was also apparent (Lineros *et al*., 2018). In *Abies alba,* after 6 years of cryopreservation, out of 12 cell lines four cell lines showed regrowth and cotyledonary somatic embryos differentiated in two cell lines (Krajnakova *et al*., 2013). In contrast, our results demonstrate that plantlet (somatic seedlings) regeneration is possible from cryopreserved embryogenic cultures of *Abies alba*. The number of differentiated cotyledonary somatic embryos was similar in cryopreserved and control (non-cryopreserved, no pre-treated) cultures, but to achieve further development of somatic embryos and plantlet survival needs refinements. Results obtained in hybrid firs also demonstrated no negative effect of cryopreservation on the maturation capacity of tested cell lines (Salaj *et al*., 2010). Our results obtained for *Abies alba* or *Abies* hybrids are in agreement with other studies on the cryopreservation of conifer trees (Cyr *et al*., 1994; Hazubska-Przybyl *et al*., 2013; Varis *et al*., 2017; Nunez *et al*. 2017; Ahn *et al*., 2019).

Conclusions

The real evidence of cryopreservation success is the tissue recovery during the post-thaw period and continuous proliferation, as well as the maintenance of the maturation ability of somatic embryos. The presented results demonstrate that the tested *A. alba* cell lines maintain their maturation ability, produce cotyledonary somatic embryos capable of germination, and complete plantlet regeneration.

Authors' Contributions

Conceptualization: TS, KK and JS; methodology: TS, KK, BP and JS; analysis: TS, BP; data interpretation: TS, KK, JS; original draft preparation: TS, KK, BP and JS. All authors have read and agreed to the published version of the manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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