


Article

Development of Somatic Embryo Maturation and Growing Techniques of Norway Spruce Emblings towards Large-Scale Field Testing

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Abstract: The possibility to utilize non-additive genetic gain in planting stock has increased the interest towards vegetative propagation. In Finland, the increased planting of Norway spruce combined with fluctuant seed yields has resulted in shortages of improved regeneration material. Somatic embryogenesis is an attractive method to rapidly facilitate breeding results, not in the least, because juvenile propagation material can be cryostored for decades. Further development of technology for the somatic embryogenesis of Norway spruce is essential, as the high cost of somatic embryo plants (emblings) limits deployment. We examined the effects of maturation media varying in abscisic acid (20, 30 or 60 μ M) and polyethylene glycol 4000 (PEG) concentrations, as well as the effect of cryopreservation cycles on embryo production, and the effects of two growing techniques on embling survival and growth. Embryo production and nursery performance of 712 genotypes from 12 full-sib families were evaluated. Most embryos per gram of fresh embryogenic mass (296 ± 31) were obtained by using 30 μ M abscisic acid without PEG in the maturation media. Transplanting the emblings into nursery after one-week in vitro germination resulted in 77% survival and the tallest emblings after the first growing season. Genotypes with good production properties were found in all families.

Keywords: Norway spruce; *Picea abies* L. Karst.; somatic embryogenesis; forest biotechnology; forest regeneration material; cryopreservation; maturation; embling production

1. Introduction

In Finland, the increased planting of Norway spruce (*Picea abies* L. Karst.) seedlings and difficulties in seed production has resulted in intermittent shortages of regeneration material of a high breeding value [1]. One solution to this problem is to use vegetative propagation, e.g., somatic embryogenesis (SE), which was observed in Norway spruce for the first time in 1985 [2,3]. Vegetative propagation enables more efficient tree improvement e.g., by capturing non-additive genetic gain [4,5].

Additionally, the cryopreservation of embryogenic tissue (ET) in liquid nitrogen (LN) enables long-term storage of regeneration material in its juvenile state [6–8]. Cryopreservation techniques are available for several conifer species, based on either applying cryoprotectant before freezing, or either on drying embryos or embryogenic tissues in different developmental stages [9–14]. Additionally, for Norway spruce, reliable cryopreservation protocols applicable for large number of samples have been developed [8,15]. As a result, acceptable recovery rates together with high morphological and genetic fidelity have been observed [8,15].

The commercial scale production of conifer emblings, i.e., somatic embryo plants, has been achieved in Denmark, Ireland and France (*Abies*, *Picea* and *Pinus* species) and is being piloted in Sweden

(*Picea abies*). Companies producing conifers (e.g., *Pseudotsuga menziesii*, *Picea glauca engelmannii* complex, *Picea glauca* (Moench) Voss and *Picea sitchensis* (Bong.) Carr., *Pinus radiata* D. Don, *Pinus taeda* L. and *Pinus elliottii* Engelm.) with SE for planting stock exists in North America and New Zealand [6,16,17].

The main reason limiting the commercial application of SE in forestry is the high cost of emblings compared to seedlings [17,18]. Several efforts to reduce costs have been made e.g., producing emblings as donor plants for rooted shoot cuttings, thus fragmenting the high cost of emblings to several hundred rooted shoot cuttings [17,19]. Despite all the efforts, emblings are still rather expensive compared to seedlings, which limits their deployment especially in Nordic conditions [20]. Additionally, the loss of genetic material during SE is a major challenge [21]. However, this could be mitigated by improving production methods in the post cryopreservation phases and in the laboratory-nursery interface [20–23].

Abscisic acid (ABA) is a relatively expensive plant hormone, widely used in conifer SE to promote embryo maturation [24]. It has a positive effect in promoting the maturation of embryogenic tissues, but it can also inhibit the germination and height growth of emblings for several growing seasons after exposure [7,25–27]. The type and amount of ABA concentration significantly affects the maturation results, and the optimal concentration varies between species and genotypes [27–29].

Similar, species and genotype specific, responses in embryo maturation have been reported when various amounts and types of polyethylene glycol has been added to the maturation medium in different conifer species [30–33]. Polyethylene glycol is added to the maturation media to reduce the moisture content of somatic embryos, thus increasing the content of the storage materials in later phases of maturation compared to ABA [31,32,34]. In Norway spruce, polyethylene glycol 4000 (PEG), when added to the maturation media, is known to increase the number of somatic embryos but is also known to have a negative effect on the later growth and development of the embryos [25,35,36]. PEG has also been found to speed up somatic embryo maturation by several weeks [31,36].

The aim of this work was to improve the efficiency of Norway spruce embling production in order to enable large scale testing of numerous SE lines. To achieve this we studied, (I) the effects of different levels of ABA and PEG in maturation media and the effect of an additional cryopreservation cycle on embryo production capacity; and (II) the effects of two different growing techniques on embling yield and early performance. Furthermore, the embryo production capacity and survival rate in the nursery were tested for a wide range of genotypes (712) originating from 12 full-sib families, with the aim to initiate field testing with rooted cuttings. This was done to improve the properties and yield of emblings (I and II) and to evaluate embling production schemes needed for large-scale field testing and variation among full-sib families affecting them (III).

2. Materials and Methods

2.1. Origin of Embryogenic Lines

The embryogenic lines used in this study were initiated in 2014 and 2015 from immature zygotic embryos of full-sibling families of progeny tested plus trees from Southern Finland. The medium and methods developed by Klimaszewska et al. [37], as described by Varis et al. [8] were used for culture establishment. Zygotic embryos without megametophytes, were placed on a modified Litvay's medium (mLM) containing half-strength macroelements [37,38], 10 μ M 2,4-dichlorophenoxyacetic acid (2,4-D), and 5 μ M 6-benzyladenine (BA). The sucrose concentration of the medium was 1% (*w/v*) and the pH was adjusted to 5.8 prior to adding gellan gum (4 g/L, Phytigel™, Sigma-Aldrich, Saint Louis, MO, USA) and autoclaving. The cultures were kept in the dark (at 24 °C) for two to eight weeks without subculturing, until embryogenic tissue (ET) started to grow. Established ETs were subcultured bi-weekly, on a fresh Petri dish of the same medium.

Cryopreservation of ETs was done according to Varis et al. [8]. From each genotype one to four samples were cryopreserved right after initiation. Slowly growing ETs were not cryopreserved. The number of samples per genotype was kept low to increase the number of genotypes in cryostorage.

For maturation in all experiments, from 150 to 200 milligrams of fresh embryogenic mass was weighed and absorbed on filter paper (Whatman # 2), using a Buchner funnel as done by Varis et al. [8].

2.2. Experiment I

The effect of the ABA concentration on embryo production and plant viability was studied using three different trials (1, 2 and 3). In Trial 1, six continuously subcultured Norway spruce embryogenic lines from five families initiated in 2014 were matured in December 2015, and seven lines from four families initiated in 2015 were matured in February 2016. Each line was matured on six filter papers (Whatman # 2) which were placed in petri dishes filled with 28 mL of LM-media containing 60 or 20 μM ABA (Later referred to 60ABA and 20ABA), and three maturation of each treatment (Table 1). Filter papers on the latter media were moved to fresh media two times at one-week intervals at the beginning of maturation. Cotyledonary embryos with visible initial shoot and root meristems and at least four cotyledons were manually counted after eight weeks maturation in the dark at 24 °C room temperature, as was done in previous studies [39,40].

Table 1. Schematic description of experiments and treatments used in different phases of SE production in Experiments I to III.

Exp./Trial	Treatment	Maturation	Germination	1st Growing Period/Growing Season	2nd Growing Season
I/1; 2; 3	60ABA	8 weeks			
I/1	3* 20ABA	8 weeks			
I/2	60ABA + PEG	8 weeks			
I/2	30ABA + PEG	8 weeks			
I/2; 3	30ABA	8 weeks			
II	GT-I	8 weeks 60 μM 30 μM ABA	18:6 Day-night 1 week ⁽¹⁾	Transplanted to Mini-plugs Controlled environment Transplanted to Plantek 81f Winterized and cold stored	Grown outside since June 2017
	GT-II	8 weeks 60 μM 30 μM ABA	18:6 Day-night 1 week ⁽¹⁾	Transplanted to Plantek 81f Nursery greenhouse in March 2017 Grown outside since June 2017	
III	Thawing lots A to D	8 weeks 60 μM ABA	18:6 Day-night 1 week ⁽¹⁾	Transplanted to Mini-plugs Controlled environment Winterized and cold stored	Grown outside since June 2017
	Thawing lot E	8 weeks 30 μM ABA	18:6 Day-night 1 week ⁽¹⁾	Transplanted to Mini-plugs Controlled environment Transplanted to Plantek 81f in March 2017 Grown outside since June 2017	
	Thawing lot F	8 weeks 30 μM ABA	18:6 Day-night 1 week ⁽¹⁾	Transplanted to Plantek 81f Nursery greenhouse in March 2017 Grown outside since June 2017	

In Experiment I, the effect of different concentrations/combinations of abscisic acid (ABA) and polyethylene glycol 4000 (PEG) on the yield of cotyledonary embryos was tested. 3* means a transfer of ET twice to fresh media. In Experiment II, two ex vitro growing techniques for emblings were tested (GT-I and GT-II). In Experiment III, large number of samples from 12 full-sib families was thawed from cryopreservation in lots A to F, to produce emblings (cutting donors) for clone testing. ⁽¹⁾ three days in five $\mu\text{mol}/\text{m}^{-2}/\text{s}^{-1}$, two days in 50 $\mu\text{mol}/\text{m}^{-2}/\text{s}^{-1}$ and two days in 150 $\mu\text{mol}/\text{m}^{-2}/\text{s}^{-1}$.

Trial 2 consisted of seven genotypes (from different full-sib families) which were matured in May 2016. Two of the lines were initiated in 2015 and maintained in a subculture. Five lines initiated in 2014 were thawed from LN. Four different ABA (60 and 30 μM) and PEG (4.75% concentration in media) combinations were used in mLM-media (later referred to 60ABA, 30ABA, 60ABA + PEG and 30ABA + PEG) (Table 1). The filters were kept in the original petri dishes for eight weeks under the same conditions as in Trial 1, after which cotyledonary embryos were counted.

Trial 3 consisted of 120 cryopreserved genotypes from 12 families (10 genotypes per family), which were thawed from LN and matured using LM media with two different ABA concentrations (Table 1). The first lot of 120 genotypes was thawed and matured in 2016. From these genotypes,

samples were cryopreserved again and one sample per genotype was thawed and matured in 2017. From the first lot, samples from 65 genotypes were matured on media containing 60 μM of ABA, and 55 genotypes were matured on media with a 30 μM ABA concentration. In the second lot, all 120 genotypes were matured on media containing 30 μM ABA. The filters were kept in the original Petri dishes for eight weeks in the same conditions as Trial 1, after which cotyledonary embryos were counted.

2.3. Experiment II

Two different growing techniques (later referred to GT-I and GT-II) in a nursery were evaluated by germinating cotyledonary embryos from 18 genotypes (9 families) according to the 1w-filter protocol described by Tikkinen et al. [23]. In short: cotyledonary embryos, cold stored at +3 °C (for at least four weeks in a large refrigerator unit) on the same filter papers in Petri dishes where the maturation was carried out, were germinated one-week in vitro under LED (Light emitting diode) lights (at a temperature from 20 to 23 °C inside the Petri dishes). The emblings were transplanted to a peat-based growth media after germination in vitro, using the ‘pricking out’ method as described by Landis et al. [23,41], in which forceps were used to transfer the emblings and to place them in peat. Peat was gently compressed around the embling to provide sufficient edaphic conditions for developing roots, as demonstrated by Landis et al. [23,41].

In GT-I, 36 emblings from each genotype were transplanted in small containers (Preforma 126/JIF, ViVi Pak, ViVi, Burgh Haamstede, Netherlands) with 126 plugs per container (plug volume 3.4 mL) (Miniplugs), and grown in a controlled environment for 50 days, until the temperature sum reached 1300 degree days (d.d.) (later referred to as the growing period). The controlled environment refers to a growth room, where the light period, temperature and humidity were adjusted to obey suggested levels for the different stages of growth of Norway spruce seedlings [42]. After this artificial growing season, the emblings were transplanted into Plantek 81f containers, winterized and cold stored in a large cooler unit. After cold storage the emblings were transferred outside together with a large lot of seedlings (Table 1).

In GT-II, 81 emblings from each genotype were transplanted straight into Plantek 81f containers (81 separate ventilated compartments of 85 cm³ size) and were grown in a greenhouse as described by Tikkinen et al. [23]. These emblings were grown together with the emblings from thawing lots A to D of Experiment III (Table 1).

2.4. Experiment III

To initiate the field testing of SE-lines, emblings were produced for donor plants for shoot cuttings. This was done with cryopreserved genotypes from 12 full-sib families, initiated in 2014 (Table 2). The aim was to produce emblings from 20 genotypes from each full-sib family to initiate field testing with rooted shoot cuttings.

ETs were thawed from 712 genotypes at six different times, in thawing lots A to F. Thawings were carried out during 2016 (A to E) and 2017 (F) (Table 2). ETs were thawed, subcultured bi-weekly, cryopreserved again and matured (three Petri dishes each) in a five or ten weeks production cycle [8,37,38]. If the ET did not proliferate enough for cryopreservation and maturation in ten weeks it was discarded.

The ETs were cryopreserved again to increase the number of samples from each genotype for future use. Cryopreservation was prioritized, so that maturation was delayed for five weeks, if necessary. In the cases of poorly proliferating ETs, they were matured first and cryopreserved only if enough embryogenic tissue was available after 10 weeks of proliferation. This was done to increase the number of genotypes available for field testing.

Table 2. Description of material included in Experiment III.

Crossing	Explants	Initiated	Cryostored	Thawing	Thawed	Cryo + Maturation	E/gFW	Over 200 E/gFW	Surv. % A to D	Surv. % E to F
E1551 × E2229	99	67	61	III, IV and VI	48	45	94 (±13)	7	28 (±2)	72 (±2)
E162 × E81	200	101	66	II and VI	48	42	89 (±12)	5	14 (±2)	87 (±2)
E18 × E436	200	133	127	III and VI	47	30	110 (±18)	6	62 (±2)	85 (±2)
E207 × E1373	200	145	140	III, IV and VI	50	36	81 (±11)	3	54 (±2)	70 (±5)
E207 × E252	200	60	52	II, IV and VI	51	43	124 (±14)	6	30 (±2)	78 (±2)
E2105c × E2283	200	114	98	II, IV and VI	50	45	40 (±9)	1	14 (±2)	66 (±5)
E212 × E54	200	127	92	I, IV and V	92	61	45 (±7)	2	14 (±2)	80 (±2)
E242 × E222	400	187	106	I, IV, V and VI	106	49	36 (±7)	2	10 (±2)	82 (±2)
E46 × E3222	400	244	209	III and VI	78	53	89 (±12)	5	25 (±2)	88 (±2)
E462 × E64	200	160	137	I and VI	48	41	81 (±13)	5	54 (±2)	81 (±1)
E799 × E1366	45	42	36	II and IV	34	32	144 (±21)	7	24 (±2)	
E9 × E3231	400	132	123	I and VI	60	52	68 (±10)	5	5 (±1)	71 (±2)
Overall	2744	1512	1247	I to VI	712	529	79 (±4)	54	30 (±1)	80 (±1)

Number of explants, genotypes initiated and cryopreserved in 2014. Thawing lot, number of genotypes thawed, cryopreserved again and matured. Yield of cotyledonary embryos (E/gFW) from three maturation dishes (mean ± standard error), as well as the number of genotypes producing over 200 E/gFW and the survival rates (Surv. %) of emblings (mean ± standard error) from different crossings from thawing lots A to D and E to F, in Experiment III.

2.4.1. Thawing, Proliferation Maturation and Germination

After eight weeks of maturation under the same conditions as Experiment I, cotyledonary embryos were manually counted and cold stored on filter papers, as described by Tikkinen et al. [23]. In the case of thawing lot B, the maturation dishes were moved to cold storage before counting. The maturation medium contained 60 μM ABA, in thawing lots A to D, and 30 μM ABA in thawing lots E and F (Table 1). Maturation media was changed between thawing lots D and E, because of the higher yield of cotyledonary embryos in media with a lowered ABA concentration observed in Experiment I.

2.4.2. Embling Production

A one-week in vitro germination protocol was used in all cases following the methods described by Tikkinen et al. [23]. Because of limited resources, e.g., work force and growing space, for thawing lots A to E, only up to 36 cotyledonary embryos from each genotype were selected for cultivation depending on the availability of cotyledonary embryos. The cotyledonary embryos were grown as in GT-1 (Table 1). The emblings from thawing lot E were grown according to GT-I, with the exception that the emblings were transferred straight to the nursery in spring 2017, while they were still growing height (Table 1).

Germinated emblings from thawing lot F were grown as in GT-II (Table 1). From thawing lots E and F, up to 81 cotyledonary embryos were selected for cultivation depending on the availability of cotyledonary embryos.

2.5. Measurements and Data Analysis

Cotyledonary embryos were counted and the embryo productivity was calculated per one gram of fresh cell mass (E/gFM) in all experiments. Mean values are presented with their standard errors (\pm). All measurements and inventory results were analyzed using the IBM SPSS Statistics 22 software package (International Business Machines Corporation, Ammonk, NY, USA). The level of confidence used was 5%.

In Experiment I, nonparametric tests were used because normal distribution could not be assumed. In Trials 1 and 3 of Experiment I, differences between treatments were analyzed with Mann–Whitney U-test. In Trial 2 of Experiment I, the Kruskal–Wallis test (one-way ANOVA on ranks) was used to analyze differences between treatments.

To compare the two growing techniques in Experiment II, the survival of the emblings was inventoried from GT-I after the first growing period in a controlled environment, i.e., before cold storage. The survival and height measurements for the 2017 growing season, were obtained for both growing techniques. Logistic regression was used to examine the differences in survival between growing techniques, after the first growing period and after the growing season of 2017. A non-parametric test (Mann–Whitney U) was used to test the differences in embling height between the two growing techniques, because a normal distribution could not be assumed.

In Experiment III, the differences in embling survival between families were examined with logistic regression after the first growing period and after the growing season of 2017. A crossing covariate was used to investigate a possible parental effect. A thawing covariate was used to distinguish differences between different thawing lots. Among thawing lots, variation occurs in the date of thawing, the ABA concentration in the maturation media, the growing method and the length of the cold storage period. The effect of the thawing lot had no effect on the percentage of cases predicted correctly and was left out from the final models. The effect of location inside the containers was studied by using row and column covariates, which defined the location of a single embling inside a container. This effect was significant, but explained only 0.1% of the correctly predicted cases; hence row and column covariates were excluded from the final models. Embryo production between full-sib families was analyzed with Kruskal–Wallis and Mann–Whitney U nonparametric tests, because normal distributions could not be assumed.

3. Results

3.1. Effect of ABA Concentration and PEG in the Maturation Media

In Trial 1, with the first group of genotypes (Experiment 1), the mean yield of cotyledonary embryos was 80 ± 23 E/gFM when the media contained $60 \mu\text{M}$ ABA, and 185 ± 24 E/gFM when the ETs on filters were twice transferred to fresh media containing $20 \mu\text{M}$ ABA (Figure 1). Reducing the ABA concentration in the maturation media enhanced embryo production by 131% ($p < 0.001$).

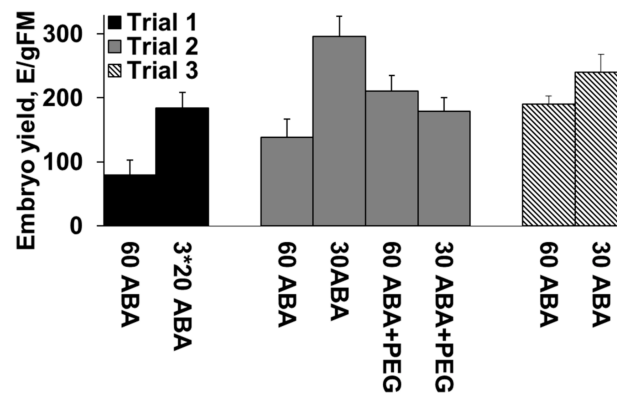


Figure 1. Yield of cotyledonary embryos in different treatments in three Trials in Experiment I. Mean values are presented with standard error bars. In Trial 3, values are presented for genotypes for which maturation with both treatments was available.

In Trial 2, reducing the amount of ABA enhanced the productivity even though the cell mass was not transferred to fresh media. The mean productivity on $30 \mu\text{M}$ ABA was 296 ± 31 E/gFM while on $60 \mu\text{M}$ ABA it was 139 ± 28 E/gFM, with the increase being 113% ($p = 0.001$) (Figure 1). Adding PEG increased the productivity only when combined with $60 \mu\text{M}$ ABA (211 ± 24 E/gFM). With $30 \mu\text{M}$ ABA the number of cotyledonary embryos decreased to 179 ± 22 E/gFM when PEG was included in the media (Figure 1).

In Trial 3, the average yield of cotyledonary embryos among genotypes in the first lot was 180 ± 9 E/gFM. For the 65 genotypes matured on $60 \mu\text{M}$ ABA, the average embryo yield was 191 ± 1 E/gFM. For the 55 genotypes matured on $30 \mu\text{M}$ ABA, the embryo yield was 166 ± 14 E/gFM. In the second lot, following cryopreservation, with $30 \mu\text{M}$ ABA used in the maturation media for all genotypes, the overall embryo yield was 206 ± 19 E/gFM. For the genotypes matured with 60 or $30 \mu\text{M}$ ABA in the first lot, the average embryo yields in the second lot were 240 ± 28 E/gFM and 167 ± 24 E/gFM, respectively. In the second lot, 116 genotypes were successfully regenerated from cryostorage and 114 were matured. No significant change in the average embryo production was found between lots when separately examining the genotypes matured on media containing $30 \mu\text{M}$ ABA in both lots, or between genotypes matured with $60 \mu\text{M}$ ABA in the first lot.

3.2. In Vitro Germination, Survival and Height Growth

In Experiment II, all selected cotyledonary embryos germinated and were transplanted ex vitro in both growing techniques. In GT-I, the average survival rate was $93 \pm 1\%$, after the growing period, before cold storage. After cold storage and the growing season of 2017 in the nursery, the average survival rate and height were $53 \pm 2\%$ and 5.8 ± 0.3 cm, respectively. In GT-II, the average survival rate and height of the emblings after the first growing season (2017) in the nursery were $77 \pm 1\%$ and 10.9 ± 0.2 cm, respectively.

The difference in the embling survival rates between GT-I (after first growing period) and GT-II (after the first growing season of 2017) was significant ($p < 0.001$) (Table 3). The survival and height of the emblings obtained from different growing techniques varied after the growing season of 2017 ($p < 0.001$, in both) (Table 3).

Table 3. Logistic regression models used for analyzing binary response (living or dead) in different growing techniques (GT-I and GT-II), after first growing season in the nursery or artificial growing season in growth room (GP1) and after the growing season of 2017 in the nursery, for Experiments II and III.

Experiment	Measurement	Model	Variable	Sig.	Odds Ratio (95% Confidence Interval)	Correct, %	
Experiment II	After GP1	$\log(p/1-p) =$	$2.839 - 1.372g_1 - 0.076c_2 - 0.437c_4 - 0.748c_6 - 0.820c_7 + 0.470c_8 - 0.748c_9 - 0.163c_{10} - 0.546c_{11}$ Growing technique	<0.001	1	81	
					GT-I		
					GT-II		
					Family		
					1		
					E1551 × E2229		
					0.927 (0.591–1.453)		E18 × E436
					0.623 (0.341–1.138)		E207 × E252
					0.473 (0.293–0.765)		E212 × E54
					0.44 (0.25–0.775)		E242 × E222
0.473 (0.293–0.765)	E462 × E64						
1.6 (0.956–2.676)	E46 × E3222						
0.849 (0.509–1.419)	E799 × E1366						
0.579 (0.324–1.035)	E9 × E3231						
Experiment II	Autumn 2017	$\log(p/1-p) =$	$0.386 + 1.210g_1 + 0.150c_2 - 0.437c_4 - 0.588c_6 - 0.654c_7 + 0.491c_8 - 1.248c_9 - 0.899c_{10} - 1.095c_{11}$ Growing technique	<0.001	1	74.1	
					GT-I		
					GT-II		
					Family		
					1		
					E1551 × E2229		
					1.162 (0.785–1.72)		E18 × E436
					0.646 (0.368–1.134)		E207 × E252
					0.556 (0.361–0.855)		E212 × E54
					0.52 (0.31–0.873)		E242 × E222
0.287 (0.188–0.437)	E462 × E64						
1.634 (1.06–2.52)	E46 × E3222						
0.407 (0.266–0.622)	E799 × E1366						
0.335 (0.203–0.55)	E9 × E3231						
Experiment III	After GP1	$\log(p/1-p) =$	$0.859 + 0.238c_1 + 0.945c_2 + 0.421c_3 + 0.018c_4 + 0.104c_5 + 0.405c_6 + 0.419c_7 + 1.206c_8 + 0.651c_9 + 0.574c_{10} - 0.003c_{11}$ Family	<0.001	1	78	
					E1551 × E2229		
					1.269 (1.019–1.58)		E162 × E81
					2.573 (2.035–3.252)		E18 × E436
					1.523 (1.188–1.954)		E207 × E1373
					1.018 (0.83–1.25)		E207 × E252
					1.11 (0.832–1.48)		E2105c × E2283
					1.499 (1.183–1.899)		E212 × E54
					1.52 (1.197–1.931)		E242 × E222
					3.34 (2.601–4.289)		E462 × E64
1.918 (1.575–2.336)	E46 × E3222						
1.775 (1.371–2.298)	E799 × E1366						
0.997 (0.812–1.225)	E9 × E3231						

Table 3. Cont.

Experiment	Measurement	Model	Variable	Sig.	Odds Ratio (95% Confidence Interval)	Correct, %	
Experiment III	Autumn 2017	$\log(p/1-p) =$	$-2.652 + 0.613b_1 + 1.186b_2 + 0.915b_3 + 3.753b_4 + 3.334b_5 + 0.632c_1 + 1.186c_2 + 0.886c_3 + 0.912c_4 +$ $0.108c_5 + 0.365c_6 + 0.401c_7 + 1.494c_8 + 0.831c_9 + 0.840c_{10} + 0.126c_{11}$	<0.001	1	76.5	
					Thawing	1.846 (1.326–2.571)	A
						6.66 (4.935–8.988)	B
						2.497 (1.714–3.638)	C
						42.655 (29.934–60.782)	D
						28.049 (21.653–36.334)	E
					Family	1	F
						1.881 (1.427–2.479)	E1551 × E2229
						3.275 (2.659–4.034)	E162 × E81
						2.425 (1.931–3.046)	E18 × E436
						2.49 (1.907–3.25)	E207 × E1373
						1.114 (0.776–1.601)	E207 × E252
						1.441 (0.973–2.135)	E2105c × E2283
						1.493 (1.049–2.123)	E212 × E54
						4.456 (3.266–6.08)	E242 × E222
						2.297 (1.905–2.769)	E462 × E64
	2.316 (1.659–3.232)	E46 × E3222					
	1.134 (0.88–1.462)	E799 × E1366					
		E9 × E3231					

In the models g1 is a design variable for growing technique II, c1 to c11 are design variables for full-sib families (Family) and b1 to b5 are design variables for thawing lots (Thawing).

3.3. Donor Plant Production

In Experiment III, 76% (51% to 94% variation among full-sib families) of the thawed genotypes produced enough ET to be matured. Cotyledonary embryos were produced from 67% of the thawed genotypes, varying from 43% to 91% of the thawed genotypes among the full-sib families. The average yield of cotyledonary embryos was 79 ± 4 E/gFW (Table 2).

In thawing lots A to D, the average yield of cotyledonary embryos was 80 ± 4 E/gFW. The embryo yield varied between families ($p < 0.001$). In thawing lot E, in which all the remaining cryopreserved genotypes were thawed from two full-sib families with the lowest number of genotypes producing cotyledonary embryos, on average 46 ± 9 E/gFW were produced. In thawing lot F, the mean yield of cotyledonary embryos was 92 ± 10 E/gFW. In thawing lots E and F, the embryo yield varied among full-sib families ($p = 0.002$).

Overall, 12,910 germinated emblings from thawing lots A to F were transplanted into peat-based growth media. From thawing lots A to D, 8904 germinated emblings from 340 genotypes (varying from 21 to 34 genotypes per family) were transplanted into small growing containers. From thawing lots E and F, 4013 emblings were transplanted straight into Plantek 81f containers. In thawing lots A to F, the embling survival rate after the first growing period or season varied between full-sib families ($p < 0.001$).

From thawing lots A to D, 3837 growing emblings (43%) were recorded after the growing period, varying from 21 to 34 genotypes per family. After cold storage and the 2017 growing season in the nursery, only 1481 (17% overall) of the transplanted emblings from 12 to 28 genotypes per family were alive.

In thawing lots E and F, 4006 emblings from 127 genotypes, from two to 24 genotypes per family, were transplanted into Plantek 81f containers in May 2017. In autumn 2017, the number of vital emblings was 3196 (80% survival), consisting of 121 genotypes, from two to 23 genotypes per family.

In October 2017, a total of 4677 plants were vital from 356 genotypes (50% of all thawed genotypes), from 42 to 18 genotypes among full-sib families. The embling survival rate varied between thawing lots and full-sib families ($p < 0.001$, in both). When the inventories from thawing lots A to D after the first growing period were combined with the inventory from thawing lots E and F after the 2017 growing season, the average survival rate of transplanted emblings was 54%. Survival varied from 50% to 89% among full-sib families. Numbers include emblings from 439 genotypes (62% of the thawed genotypes) varying from 26 to 48 genotypes between full-sib families.

4. Discussion

The embryo yield increased significantly in different groups of genotypes when the ABA concentration in the maturation media was reduced. The increase in embryo yield was over two-fold in Trials 1 and 2 of Experiment I when the reduced amount of ABA was compared to the control 60 μ M ABA treatment. Adding PEG to the semi-solid maturation media increased the embryo yield when compared to 60 μ M ABA content. However, the highest embryo yield was gained without PEG. Using maturation media with 20 μ M ABA and subculturing embryogenic tissues with filter paper resulted in the highest embryo yield. However, the subculturing is labour intensive when used with a large number of samples. For this reason, a maturation protocol, including media containing 30 μ M ABA without subculturing was introduced as standard protocol.

When the ABA content in the maturation media was lowered, the average embryo production increased slightly among a large number of samples in a group of different genotypes (Trial 3 in Experiment I) which had been cryopreserved and thawed at least once before. This happened although more variation between individual genotypes was observed. Additionally, in previous studies, cryopreservation has not systematically reduced embryo production (see e.g., [8,25]). The loss of genetic material by cryopreservation accounted for 5% of the thawed genotypes distributed between five full-sib families. The possible loss of genotypes due to cryopreservation is a potential risk when a small number of samples are initially cryopreserved (see e.g., [8,25]).

The highest survival rate was recorded when germinated emblings were transplanted straight into the nursery (GT-II), although the average survival rate was significantly higher among emblings grown in small containers for the first growing period (GT-I) before cold storage. The decline in embling survival among emblings in GT-I was most likely due to improper cold storage conditions and fungicide treatments, which resulted in a severe infestation of mold (possibly *Botrytis* sp.).

The plants grown in treatment GT-II were significantly taller, although they were grown for one growing period less. This is most likely due to insufficient lighting in the growth room in treatment GT-I combined with the damage caused to initial meristems during cold storage. In treatment GT-I, the growth room light intensity in the photosynthetically active radiation (PAR) region in full light was set to $150 \mu\text{mol}/\text{m}^{-2}/\text{s}^{-1}$. In the greenhouse, emblings were subjected to sunlight filtered through the plastic outer wall of the greenhouse. The suggested minimum light intensity to support height growth and prevent premature terminal bud formation on seedlings is $250 \mu\text{mol}/\text{m}^{-2}/\text{s}^{-1}$ according to Landis et al. [41,42]. Emblings survived well in greenhouse conditions after one-week in vitro germination in treatment GT-II. This implies that a higher light intensity is not harmful for small emblings, as long as the air temperature is favourable. This was also observed previously by Tikkinen et al. [23].

Growing emblings in small containers in artificial conditions is plausible, although special attention needs to be paid to the cold storage conditions, similar to seedlings. Improvements in environmental control are still needed to match the properties of the emblings or seedlings obtained from conventional nurseries. Although, growing space can be saved by using small containers, the method requires additional labour and supplies (i.e., containers, substrate etc.) compared to the method where emblings are transplanted straight into the nursery (see e.g., [23,41]). Additionally, in Nordic conditions supplementary lighting is necessary during a short photoperiod. All the previous matters have an effect on the cost of the emblings. A detailed cost analysis is needed to define the threshold for embling vigor in containers, to determine whether using small containers is profitable.

The embling survival of 77% for cotyledonary embryos selected for in vitro germination is close to the germination and early vigour percentages of unimproved Norway spruce seeds and seedlings reported in earlier studies (see e.g., [43]). The observed mean height of 10.9 cm indicates that the emblings will reach sufficient height standards for planting during the second growing season. By selecting well acclimatizing genotypes for production, survival can be further increased, as suggested by Tikkinen et al. [23].

Genotypes with a high embryo production capacity (over 200 E/gFW, with lowered ABA content) were found, and vigorous emblings were successfully produced from several genotypes from all full-sib families (Figure 1; Table 2). Not all of the cryostored genotypes from the families used were tested, although the number of samples was fairly large varying from 34 to 106 per family. Högberg [44] found that the loss of genotypes during the overall SE process can be vast. According to our results, genotypes with a good embryo production capacity can be found in all families. We thawed a sample of 57% of the 1247 cryopreserved genotypes, representing 12 full-sib families. With the current rates of genotypes successfully initiated (55%) and cryopreserved (45%) of the total number of explants (2744) from selected families, the sample of 712 thawed genotypes accounts for 1292 to 1567 explants (Table 2). In the autumn of 2017, vital emblings were recorded from 356 genotypes, i.e., 23% to 28% of the estimated explants. Without the loss of emblings due to improper storage, emblings from 28% to 34% of the estimated explants could have been obtained. These genotypes could possibly be used for the large-scale propagation of emblings.

Plant loss during the cold storage of emblings from thawing lots A to D was severe, as 61% of emblings which had formed initial buds after the first growing period were lost during cold storage after the first growing period. This was most likely due to the same reasons as those found in the GT-I Experiment II, as the emblings were kept in the same cold storage unit. It is unacceptable to lose emblings at this magnitude after the most critical steps of embling production have been endured.

Hence, controlling the storage conditions and applying chemical treatments against mold must not be overlooked.

The genetic entry and early phases in the SE process, set limits for the later phases of the production (see e.g., [20]). Our results show, that the final output, e.g., the yield and quality of the emblings, together with the genetic diversity of the output of the SE process can be significantly improved by developing the later phases of production.

When a maturation media with a lower ABA concentration was used, the embryo yield increased to such an extent that transplanting germinated emblings in vitro straight into the nursery may be considered the best growing method. This is in line with the findings of [23,45]. Clone testing can be initiated significantly faster by using 12 emblings as cutting donors from each genotype, compared to using seedlings as cutting donors (see e.g., [46]). To succeed, on average 16 cotyledonary embryos need to be germinated for one-week in vitro and transplanted straight into a nursery for each genotype, with a 77% survival rate (Experiment II).

To initiate clone testing directly with emblings, 24 to 32 ramets per genotype are needed in the Finnish spruce breeding programme [47]. To produce this number of emblings, an average of 32 to 42 cotyledonary embryos are needed, with an expected survival rate of 77% from Experiment II. From the poorest family in Experiment III, 37 to 49 cotyledonary embryos are needed with an expected survival rate of 77%.

From the current data, covering 12 families in Experiment III, 394 genotypes reached the embryo production limit of 16 cotyledonary embryos from three maturation dishes (25% to 30% of estimated explants, 27 genotypes in the poorest family) and 336 produced over 32 cotyledonary embryos from three maturation dishes (21% to 26% of the estimated explants, 17 genotypes in poorest family). The current results indicate that large-scale field testing can be initiated from cryostored ETs with the current production protocol. Protocol includes short maturation on semisolid media containing 30 μ M ABA, cold storing cotyledonary embryos on maturation dishes, and transplanting emblings into a nursery after one-week in vitro germination.

5. Conclusions

Reducing the ABA concentration in the maturation media increased the yield of cotyledonary embryos. When combined with state-of-the-art embryo storage and in vitro germination protocols, emblings can be grown and large-scale field testing can be initiated with current nursery protocols with material from a wide genetic background. Intensive planning, considering production in the laboratory and in the nursery, is essential to achieve good results. Despite large variations in embryo/embling production, genotypes with good production properties were found in all families in this study. Furthermore, automation is needed to further increase the production numbers and cost effectiveness, especially in the laboratory-nursery interface, where the embryos and emblings are individually handled.

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