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Improved propagation efficiency in a laboratory–nursery interface for somatic embryogenesis in Norway spruce

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Academic dissertation

To be presented, with the permission of the Faculty of Science and Forestry of the University of Eastern Finland, for public criticism in the auditorium F100 in the Futura Building of the University of Eastern Finland, Yliopistokatu 7, Joensuu, on 20th of December 2018, at 12 noon.

Title of dissertation: Improved propagation efficiency in a laboratory–nursery interface for somatic embryogenesis in Norway spruce

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Dissertationes Forestales 265

https://doi.org/10.14214/df.265

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ISSN 1795-7389 (online)
ISBN 978-951-651-620-5 (pdf)

ISSN 2323-9220 (print)
ISBN 978-951-651-621-2 (paperback)

Publishers:
Finnish Society of Forest Science
Faculty of Agriculture and Forestry at the University of Helsinki
School of Forest Sciences of the University of Eastern Finland

Editorial Office:
Finnish Society of Forest Science
Viikinkaari 6, FI-00790 Helsinki, Finland
http://www.dissertationesforestales.fi

Tikkinen M. (2018). Improved propagation efficiency in a laboratory–nursery interface for somatic embryogenesis in Norway spruce. Dissertationes Forestales 265. 35 p. https://doi.org/10.14214/df.265.

ABSTRACT

The aim of this work was to improve the protocol of somatic embryogenesis (SE) and propagation efficiency in Norway spruce (*Picea abies* (L.) Karst.), which would enable the integration of SE into Finnish breeding programme and the nursery practices applied to seedlings. The studies specifically investigated the following three areas: i) how maturation, cold storage, germination and growing conditions (laboratory–nursery interface) affect the survival and height growth of emblings (Papers I and II); ii) how to improve the efficiency of embling production from genotypes from wide genetic backgrounds (Papers I and II); and iii) how to increase propagation efficiency by rooting cuttings from emblings, and produce field testing material by combining SE and the rooting of cuttings (Papers II and III). To evaluate the possibility of improving the efficiency of SE in the laboratory–nursery interface, a series of experiments were conducted. The cost structure of SE, and the effects of improvements on costs, was estimated.

As a result, the protocol improvements doubled the yield of cotyledonary embryos, nearly doubled embling survival, and increased the height growth of emblings in the nursery by so much that sufficient planting height was reached one year less than before. Emblings were also obtained from 356 genotypes (50% thawed), and embling cuttings rooted well in conditions similar to those used for seedling cuttings. The protocol improvements also reduced embling production costs by 75%. Based on this work, emblings may be grown in nurseries after one week of *in vitro* germination, without any measures that differ from seedlings after transplanting. Propagation efficiency may be further increased by rooting embling cuttings. Furthermore, large-scale clone testing can be initiated with 5–12 emblings acting as cutting donors.

Keywords: embling, maturation, cold storage, germination, rooted shoot cutting, forest biotechnology

ACKNOWLEDGEMENTS

I would like to acknowledge the financial sources that supported this work. This work was part of three projects funded by European Regional Development Fund (A70606, A72814 and A73586).

I would like to express my deepest gratitude to Tuija Aronen, Saila Varis and Heli Peltola for their dedication in guiding me during this educational journey. Tuija and Saila introduced me to somatic embryogenesis and the related laboratory arts. This work would not have been possible without these three people.

I am eternally grateful to Airi Huttunen and Paula Matikainen for their assistance and guidance in the laboratory throughout this work. I would also like to express my gratitude to Tiina Tynkkynen and Kirsi Pulkkinen for their assistance in the nursery work, and to Henna Höglund and the experts in UPM-Kymmene Joroinen nursery for sharing their expertise regarding seedling production. I would like to thank everyone in Luke Punkaharju for the encouraging, supporting and inspirational atmosphere in which to conduct research. I wish to thank prof. Patrick von Aderkas and prof. Scott Merkle for the valuable comments they provided me with during pre-examination of the thesis.

The cheerful bunch of exceptional characters 'Sivusoitimen kukot' [Jacks outside the main mating display], must be acknowledged for providing indispensable peer support and recreational activities. You have truly enrichened my life with all the philosophical, sometimes even poetic, discussions, usually occurring during the afterglow or morning dew.

I express my gratitude to my parents, Heimo and Riitta, and to my sister Suvi. Tyrjä and all the people related to this magnificent Finnish gem: they have kept my feet on the ground. There, it has always been possible to clear my head by 'going back to the roots'. My parents as well as my in-laws, provided indispensable help by occasionally occupying my adorable offspring, for which I am grateful.

Last, but by no means least, my beloved family – daughters Hertta and Lilli and my dear wife Tuuli – who have given me strength and filled my anxious heart with love and joy. These three women have been extremely dedicated to taking my mind out of the lab and the office every day, and enriching those precious moments with their laughter. I'm thankful for you and to you, for bringing the light to the road of life.

In Savonlinna, October 2018

Mikko Tikkinen

LIST OF ORIGINAL ARTICLES

This thesis is based on the following papers, which are referred to in the summary by their Roman numerals. Articles are reproduced with the kind permission of publishers.

I Tikkinen M., Varis S., Peltola H., Aronen T. (2018). Improved germination conditions for Norway spruce somatic cotyledonary embryos increased survival and height growth of emblings. Trees - Structure and Function, *In press*.
https://doi.org/10.1007/s00468-018-1728-6

II Tikkinen M., Varis S., Aronen T. (2018). Development of somatic embryo maturation and growing techniques of Norway spruce emblings towards large-scale field testing. Forests 9(6): 325.
https://doi.org/10.3390/f9060325

III Tikkinen M., Varis S., Peltola H., Aronen T. (2018). Norway spruce emblings as cutting donors for tree breeding and production. Scandinavian Journal of Forest Research 33(3): 207-214.
https://doi.org/10.1080/02827581.2017.1349925

Mikko Tikkinen (Tikkinen M.) was the primary author of all these papers. The primary author was responsible for the planning and implementation of all the experiments, the writing of all the papers, and all the data analysis, with the exception of Experiments I in Papers I and II, which were planned, implemented and the data analyzed together with the co-authors. The co-authors improved the articles by commenting on, and helping to revise, the manuscripts

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SYMBOLS AND ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	Abscisic acid
BA	6-benzyladenine
D.d.	Degree day
E/gFW	Cotyledonary embryos per gram of fresh mass of embryogenic tissue
Growing period	Growing period under controlled conditions (humidity, temperature, irrigation and fertilization), with a temperature sum accumulation of 1300 d.d., in an 18:6 (day:night) photoperiod under LED lights
GT-I	Growing technique I, in which *in vitro* germinated emblings were transplanted into small growing containers, grown in a controlled environment, transplanted into Plantek 81f containers, winterized, cold-stored and moved to the nursery
GT-II	Growing technique II, in which *in vitro* germinated emblings were transplanted into Plantek 81f containers and grown in a nursery greenhouse together with seedlings
LED	Light-emitting diode
KNO_3	Potassium nitrate
M	Moles per liter
mLM	Modified Litvay's medium
Moss	Treated sphagnum moss
NH_4NO_3	Ammonium nitrate
Peat	Medium–coarse, fertilized, light peat substrate (Kekkilä FPM 420 W F6)
PEG	Polyethylene glycol 4000

Peat mix	Medium–coarse, fertilized, light peat substrate (Kekkilä FPM 420 W F6) mixed with vermiculite or perlite in a volumetric mixture of 75% to 25%
Plantek 81f	Growing container consisting of 81 separate ventilated compartments, each 85 cm^3 in size
Rooting box	40×60 cm open-top, grid-based rooting box
SE	Somatic embryogenesis, i.e., a vegetative propagation method by means of tissue culture in which genetically identical embryos are produced from somatic cells of donor individuals. With conifers, somatic embryogenesis is usually initiated from sexually-produced seed embryos, but in some plant species, primordial shoots or other plant tissues can also be used

1 INTRODUCTION

1.1 Background to the study

Forestry will encounter several challenges in the future, e.g., an increasing demand for raw materials and exposure to various biotic and abiotic risks caused by climate change, which are further emphasized by long rotations under Nordic conditions (Pop et al. 2014; Ruotsalainen 2014; Börjesson et al. 2017; Jansson et al. 2017). Tree breeding can contribute to overcoming these negative effects of future challenges (Ruotsalainen 2014).

Breeding is expected to result in genetic gain and to conserve genetic diversity (Lindgren et al. 1997; Ruotsalainen 2002). Besides increasing biomass growth per areal unit and timber quality, breeding can be targeted at other purposes, e.g., to produce tailored raw material for wood products (see, e.g., Harju et al. 2002; Ruotsalainen 2014; Ahtikoski et al. 2018). With the changing climate, breeding can be utilized to increase resilience and resistance against abiotic and biotic factors, e.g. drought, pests and pathogens (Savolainen et al. 2004; Häggman et al. 2013; Rehfeldt et al. 2014; Hamberg et al. 2018).

Tree breeding generally relies on the selection and crossing of individual trees harnessing the best individuals to produce improved forest regeneration material, usually in seed orchards (White et al. 2007). The breeding cycle of conifers is long under Nordic conditions, being approximately 25 to 30 years in Norway spruce (*Picea abies* (L.) Karst.) in Finland (Haapanen and Mikola 2008). Without the use of vegetative propagules, i.e., rooted shoot cuttings, genetic testing would take even longer (Libby 1964; Haapanen 2009; Lelu-Walter et al. 2013). More efficient methods of vegetative propagation, i.e. cloning, could shorten the time required to produce plant material for genetic testing and accelerate the implementation of breeding results in practice (Högberg et al. 1998; Haapanen and Mikola 2008). The utilization of vegetative propagation to produce planting stock would also ease availability problems for improved forest regeneration material (Varis 2018).

Norway spruce is an extensively cultivated conifer species in Europe, and used for a wide range of industrial purposes worldwide (Jansson et al. 2013). For example, during this decade in Finland, Norway spruce has been planted with container seedlings on 46% (circa 120 000 ha) of the annual reforestation area (Official Statistics of Finland 2017). Norway spruce is also currently one of the main tree species in several breeding programmes e.g. in Finland and Sweden (Haapanen et al. 2015; Jansson et al. 2017). On the other hand, in Norway spruce, the old seed orchards in Finland are near the end of their operational lives, while more recently established seed orchards are still juvenile, and not yet in their peak of seed production (Haapanen et al. 2017). Because the uneven age structure of Norway spruce seed orchards and their fluctuating seed crops, the supply of improved seed can change rapidly (Ruotsalainen 2014; Haapanen et al. 2017; Jansson et al. 2017). For these reasons, in Finland the proportion of improved forest regeneration material of Norway spruce has been lower than that of Scots pine (*Pinus sylvestris* L.) (Haapanen et al. 2017).

Shortages of improved forest regeneration material could be eased by producing planting stock through vegetative propagation, which occurs naturally in several woody plants, e.g. *Alnus* spp., *Betula* spp., *Populus* spp., *Prunus padus* L., *Salix* spp. and *Sorbus aucuparia* L. (Heikinheimo 1915; Mikola 1942; Cook 1983; Nestby et al. 2011). Natural conifer clones are rare, but they do occur in high stress environments, e.g., in Arctic areas, and at high altitudes in species such as Black spruce (*Picea mariana* (Mill.) B.S.P.) and Norway spruce (Pereg and Payette 1998; Öberg and Kullman 2011). Vegetative

propagation has been exploited for horticultural crops for millennia, e.g., Cabernet Sauvignon, a cultivar of *Vitis vinifera* L., has been propagated vegetatively since Roman times and existed only in adult form until recent its reappearance in juvenile form due to *in vitro* propagation (Mullins et al. 1979; Bonga 2016). Plant breeding and vegetative propagation have also been applied for dietary purposes, probably for over 10 000 years (Allard 1999).

Vegetative propagation has also been widely used in the genetic testing of forest trees, because clonal testing is considered to be faster and more efficient than progeny testing (e.g. Libby 1964; Haapanen 2009; Lelu-Walter et al. 2013). The capture of non-additive genetic properties is possible with vegetative propagation, thus enabling the exploitation of exceptional individuals, in superior families (Díaz-Sala 2016). High genetic gain makes vegetative propagation an attractive way to produce planting stock (see reviews by Burdon et al. 2008; Ruotsalainen 2014). Several methods of vegetative propagation are also currently available, of which grafting, rooting of shoot cuttings and somatic embryogenesis are mainly applicable to conifer species, with few exceptions (Bonga 2016). Grafting is currently the main method of occupying seed orchards with the desired mother trees (Haapanen and Mikola 2008; Bonga 2016). Cutting propagation is widely used to produce plants for field testing and planting stock for several different species, such as *Eucalyptus* spp. (Zobel 1993), *Picea abies* (Haapanen and Mikola 2008), *Picea sitchensis* (Bong.) Carr. and *Pinus radiate* D. Don (Lelu-Walter et al. 2013).

The ageing of propagation material is one of the key challenges in utilizing vegetative propagation, especially with conifers under the climatic conditions of Nordic countries, where the field testing of genotypes can take a couple of decades (Kleinschmidt and Schmidt 1977; St Clair et al. 1985; Lepistö 1993; Bonga 2016). Efforts to mitigate problems caused by ageing have been made in several vegetative propagation systems such as hedging of donor plants, cryopreservation (see e.g. Sakai 1960; St Clair et al. 1985; Ryynänen 1996; Mason et al. 2002). At the moment cryopreservation of juvenile propagation material appears to be a reliable method for the long-term preservation of genetic material (see e.g. Klimaszewska et al. 1992; Cyr et al. 1994; Find et al. 1998; Krajnakova et al. 2011; Varis et al. 2017).

Somatic embryogenesis is considered to be the most promising method of vegetative propagation to be commercially utilized, and when integrated into breeding, potential gains can be obtained in several phases during field testing and production (Cyr et al. 1994; Thompson 2015; Bonga 2016). It was first discovered among forest trees in Norway spruce (Chalupa 1985; Hakman et al. 1985) and in *Larix decidua* Mill. (Nagmani and Bonga 1985). Somatic embryogenesis provides the option of reliably preserving propagation material in a juvenile state, possibly for decades, when combined with advanced cryopreservation techniques (Park et al. 1993; Cyr et al. 1994; Grossnickle et al. 1996; Park 2002; Lelu-Walter et al. 2013; Varis et al. 2017).

Production of somatic embryos and emblings involves a number of steps initiation, proliferation, maturation, desiccation, germination, and acclimation (see, e.g., Lelu-Walter et al. 2013). In the case of Norway spruce, high initiation rates and recovery of embryogenic tissues from cryopreservation have been observed in previous studies (see, e.g., Nørgaard et al. 1993; Klimaszewska et al. 2001*b*; Varis et al. 2017). The efficiency of somatic embryogenesis has been greatly improved, e.g., by initiating embryogenic tissues from immature zygotic embryos, manipulating the concentration of plant growth regulators and chemical composition of the culture medium (Hakman et al. 1985; Klimaszewska and Smith 1997; Bozhkov and von Arnold 1998; Stasolla and Yeung 2003; Klimaszewska et al.

2007; Hazubska-Przybył et al. 2015; Varis et al. 2017). Various methods concerning the germination, acclimation and selection of emblings have been introduced to improve propagation efficiency (Högberg et al. 2001, 2003; Varis et al. 2014; Llebrés et al. 2018a, b).

Despite impressive developments in the later phases of somatic embryogenesis (e.g. maturation, germination, and acclimation), there remain significant challenges that affect the final outcome such as the loss of plants and genotypes, and the quality of the emblings (see, e.g., Bozhkov and von Arnold 1998; Högberg et al. 1998, 2001; Thompson 2015; von Aderkas et al. 2016; Egertsdotter 2018). Majada et al. (2001) suggested that *in vitro* development and germination are controlled by genes, although they are highly affected by environmental conditions, which can be manipulated (von Aderkas et al. 2008, 2015). Additionally, the temperature during multiplication and embryo maturation has been observed to have a delayed effect (epigenetic memory) on, e.g., survival, height growth and bud set in Norway spruce (Kvaalen and Johnsen 2008; Carneros et al. 2017).

The high cost of emblings limits their deployment as planting stock, together with the potential loss of genetic material during production (Lelu-Walter et al. 2013; Thompson 2015; Bonga 2016). In order to facilitate breeding work, and the availability of highly bred forest regeneration material, the time required for breeding and the implementation of results needs to be shortened (Högberg 2003). This could be done by accelerating the production of clonal material for field testing, and possibly for planting stock (Högberg 2003). This could be achieved by further developing somatic embryogenesis to be effective and efficient enough for tree improvement in practice (Thompson 2015). Developing propagation efficiency, especially in the late phases of somatic embryogenesis (i.e., maturation, cold storage, germination, acclimation, and growing techniques) is essential for making the propagation method desirable for practical use (Thompson 2015). Further measures, such as combining the production of rooted shoot cuttings with somatic embryogenesis, may also be advantageous in increasing the propagation volume and decreasing costs (Park 2002; Lelu-Walter et al. 2013; Bonga 2015; Thompson 2015).

The time required for the breeding of Norway spruce, and the implementation of results, could be shortened by improving the production of field test material, and planting stock with modern vegetative propagation methods, such as somatic embryogenesis (SE); however, large bottlenecks in SE still exist, such as in the cold storage, germination, and acclimation phases, which greatly affect the yield, properties, and cost of emblings. Rooting cuttings from emblings could also improve propagation efficiency.

1.2 Aim of the study

The aim of this work was to improve the protocol for somatic embryogenesis and propagation efficiency in Norway spruce (*Picea abies*), in order to enable the integration of somatic embryogenesis into current Finnish breeding programme and nursery practices applied to seedlings. More specifically, the study addressed: i) how the maturation, cold storage, germination and growing conditions (protocols) affect the survival and height growth of emblings (Papers I and II); ii) how to improve the efficiency of embling production for genotypes from wide genetic backgrounds (Papers I and II); and iii) how to increase propagation efficiency by rooting cuttings from embling donors, and producing field testing material by combining somatic embryogenesis and the rooting of cuttings (Papers II and III). To evaluate the possibility of improving the efficiency of SE at the

laboratory–nursery interface, a series of experiments were conducted. Also, the SE cost structure, and the effects of protocol improvements on production costs, were estimated.

2 MATERIAL AND METHODS

2.1 Origin of propagation material

Embryogenic lines for all of the sub-studies were initiated from immature seeds originating from full-sib families of progeny-tested Norway spruce plus-trees from southern Finland. Embryogenic lines were initiated in 2011 and 2014 according to a method developed by Klimaszewska et al. (2001a), as described by Varis et al. (2017). In short, zygotic embryos, without megagametophytes, were placed on modified Litvay's medium (mLM) containing half-strength macroelements (Litvay et al. 1985; Klimaszewska et al. 2001a), 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D), and 5 µM 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 (Phytagel™, Sigma, 4 g/l). The cultures were kept in the dark (24 °C) for 2 to 8 weeks without subculturing, until embryogenic tissue started to grow. Established embryogenic tissues were subcultured bi-weekly, on fresh Petri dishes of the same medium.

Cryopreservation of the established embryogenic cultures was performed according to the approach of Varis et al. (2017: (i) pretreatment of fresh embryogenic tissues on a semi-solid medium with increasing sucrose concentration – 0.1 M for 24 h, 0.2 M for another 24 h; (ii) use of cryoprotectant – a mixture of polyethylene glycol 6000, glucose, and dimethylsulfoxide, 10% w/v each; and (iii) slow cooling of the samples before subjecting them to liquid nitrogen). SE initiation, propagation, cryopreservation, and storage were carried out in the laboratory facilities of the Natural Resources Institute Finland (Luke) in Punkaharju, Southern Finland.

2.2 Means used to improve embryo yield, embling survival and height growth

Maturation of the somatic embryos was carried out, according the protocol developed by Lelu-Walter (2008), as modified by Varis et al. (2017). In short, approximately 200 mg of fresh embryogenic tissue was weighed, suspended in 3 ml of liquid maturation medium, and poured onto filter paper placed in a Büchner funnel. The liquid was drained by suction, and the filter was placed on a semi-solid maturation medium in Petri dishes (9 cm in diameter), which were sealed with Parafilm. After eight weeks in the maturation medium at +24 °C in darkness, the embryos were counted and cold-stored Papers **I** and **II**). Embryos that had formed a visible initial shoot and root meristems and at least four cotyledons each, were counted as a cotyledonary embryo under a stereomicroscope, as in studies by Klimaszewska et al. (2001*b*) and Belmonte and Yeung (2004). In Paper **I**, the abscisic acid (ABA) concentration in the maturation medium was 60 µM. In Paper **II,** 20 µM and 30 µM ABA concentrations were also used, as well as a medium with 4.75% polyethylene glycol 4000 (PEG) containing 60 µM and 30 µM ABA.

Cold storage protocols were applied, as follows: 1) cotyledonary embryos were moved from the filter paper and placed in direct contact with the semi-solid maturation medium during counting, and were then cold-stored in maturation vessels until the start of

germination 2) the cotyledonary embryos were kept on the filter paper during counting and cold storage in the maturation vessels (Paper **I**). In addition, the refrigerator unit where the cold storage was carried out was changed to a significantly larger one for Experiment II of Paper **I**. This larger cooler unit was used in all of the experiments in Paper **II**.

In vitro germination protocols were tested in Paper **I**. In Experiment I of Paper **I**, two germination media were tested: 1) 28 ml of semi-solid mLM without plant growth regulators, supplemented with 20 g/l of sucrose, as used in studies by Litvay et al. (1985) and Klimaszewska et al. (2001); and 2) the same medium, but without ammonium nitrate (NH_4NO_3). In addition, both media contained 950 mg/l of potassium nitrate (KNO_3) and 500 mg/l of L-glutamine, as other sources of nitrogen. In all of the other experiments in Papers **I** and **II**, a germination medium without NH_4NO_3 was used. In the experiments of Paper **II**, slightly less (20 ml) semi-solid mLM was used.

Germination *in vitro* was carried out in a growth room, where the temperature and photoperiod were adjustable (Papers **I** and **II**). In Paper **I**, the growth room temperature was set to 24°C for Experiments I and II. For Experiments III and IV in Paper **I**, and for all of the experiments in Paper **II**, the growth room temperature was lowered so that the temperature inside the germination vessels conformed to the suggested optimal temperature for the germination of Norway spruce seedlings (20°C to 23°C; see, e.g., Bergsten 1989; Leinonen et al. 1993; Landis 2010a; Rikala 2012). Fluorescent lights (Philips Master TL-D 36W/840) were used in Experiments I, II and IV of Paper **I**. In Experiment III in Paper **I**, and in all of the experiments in Paper **II**, LED (light-emitting diode) tubes (Valoya AP67 L14) were used.

The light intensity was modified with cloths placed between the light source and the germination vessels. In Paper I, four durations of in vitro germination were used – 5, 3, 2, and 1 week/s. In the five-week in vitro germination treatment, the following concentrations were applied: 5 $\mu mol/m^2/s^1$ for the first two weeks, 20 $\mu mol/m^2/s^1$ for the third week, 50 $\mu mol/m^2/s^1$ for the fourth week, and 150 $\mu mol/m^2/s^1$ for the fifth week, according to Varis et al. (2014, 2017). In the shorter treatments (3, 2 and 1 week/s), light intensities of 5, 50, and 150 $\mu mol/m^2/s^1$ were used in time proportions of 43%, 28.5%, and 28.5%, respectively.

Growing techniques for emblings were tested in Papers **I** and **II**. *In vitro* germinated emblings were either grown in small containers in a controlled environment before moving them to the nursery (GT-I), or transplanted straight into large containers and grown in the nursery under the protocols used for Norway spruce seedlings (GT-II).

In GT-I, the emblings were transplanted into small containers filled with peat-based growth media and grown in a controlled environment (18:6 photoperiod under LED lights, controlled temperature and humidity) until the temperature sum reached 1300 d.d. (growth period), after which the emblings were winterized and cold-stored in a cooler unit (Papers **I** and **II**).

In GT-II, the method was closer to that used in conventional seedling production, whereby emblings were transplanted into Plantek 81f containers (81 separate, ventilated compartments, 85 cm^3 in size) filled with peat and grown in a nursery greenhouse with seedlings. An acclimation period of three weeks was used in Experiment II of Paper **II**. During this acclimation period, the emblings were kept in a greenhouse where relative humidity was controlled with fogging devices, and temperature was controlled. In all the other experiments in which GT-II was used, the emblings were transplanted straight to a nursery greenhouse and grown under the same conditions as seedlings. Emblings produced by GT-II were cold-stored in open storage, under a consistent layer of snow (Landis et al. 2010b).

2.3 Donor plant production and rooting of shoot cuttings from emblings

Donor plant production was carried out by thawing embryogenic tissues from 712 genotypes from 12 full-sib families, with the aim of producing emblings that could be used as donor plants for shoot cuttings. After rooting, these cuttings could be used to initiate clone testing. Thawing was carried out in six thawing lots – A to E (Paper **II**). After thawing, the embryogenic tissues were subcultured bi-weekly, cryopreserved again, and maturated (three Petri dishes each) in a five- or 10-week production cycle. Embryogenic tissues that had not proliferated enough for cryopreservation and maturation in 10 weeks were discarded.

The embryogenic tissues were cryopreserved again in order to increase the number of samples from each genotype for future use. Cryopreservation was chosen over maturation to avoid physiological ageing the samples during continuous subculture. The embryos were matured on media containing 60 µM (thawing lots A to D) or 30 µM (thawing lots E and F) ABA. Maturation was carried out by the same method, and under the same conditions, as defined earlier.

Up to 36 cotyledonary embryos were selected for *in vitro* germination from thawing lots A to D. From thawing lots E and F, up to 81 cotyledonary embryos were selected for *in vitro* germination. All embryos selected for *in vitro* germination were transplanted into containers. Embling production was carried out by using GT-I and GT-II. In addition, emblings from thawing E were produced using GT-I, with the exception that emblings were transplanted to Plantek 81f containers and grown in a nursery during the growing season without a dormancy period.

The *rooting of shoot cuttings from emblings* was investigated by using shoot cuttings from 36 genotypes from 12 full-sib families (including two specially formed genotypes: pendulous branching habit and red needles on the newest shoots at the beginning of the growing season) in the successive years 2015 and 2016 (Paper **III**). The cuttings were collected during dormancy from an experimental plantation situated in Punkaharju Finland.

Rooting was carried out in a greenhouse equipped with fogging devices and heated sand beds. The relative humidity was kept at over 80%. The temperatures of the air and rooting media were +17 °C and +22 °C, respectively (Lepistö 1976). Fungicide treatments were carried out in two-week cycles to avoid grey mould (*Botrytis cinerea*).The cuttings were not fertilized during the rooting period. The level of water availability was kept in the recommended range to support growth of seedlings by determining weight limits for each rooting vessel and media combination (Heiskanen 1993). No additive plant hormones were used to induce rooting.

In the rooting experiment carried out in 2015, two different types of containers were tested: 1) a 40×60 cm open-top, grid-based rooting box (hereafter referred to as ‘rooting box’); and 2) Plantek 81f containers. Two rooting media were tested: 1) a mixture of medium–coarse, fertilized, light peat substrate (Kekkilä FPM 420 W F6, hereafter referred to as ‘peat’) and vermiculite in a volumetric mixture of 75% to 25% (hereafter referred to as ‘peat mix’); and 2) sand, from which the larger particles were filtered out using a 4-mm-mesh steel sieve.

In the rooting experiment carried out in 2016, all of the cuttings were rooted in Plantek 81f containers. Two different rooting media were used: 1) a peat and perlite in a volumetric mixture of 75 % to 25 %, respectively (hereafter referred to as ‘peat mix’); and 2) treated sphagnum moss (hereafter referred to as ‘moss’).

2.4 Evaluation of cost structure for different phases of somatic embryogenesis

The cost structure of embling production was estimated in different phases – planning, thawing, proliferation, maturation, and germination. The work time consumed by each phase of the process was estimated during the operational work to produce donor plants from a large number of genotypes (Paper **II**). The estimation covers the processing of 120 samples of embryogenic tissues, 10 genotypes from each of the 12 full-sib families. The genotypes produced a high number of embryos, which had a high survival rate in the nursery (Experiment III in Paper **II**). All cotyledonary embryos were considered to have germinated in the estimation.

Planning included the selection of samples from cryostorage, and surveillance of their production from the beginning of laboratory production all the way to the point where the emblings were transferred to the nursery. In addition, planning included the scheduling of all the other phases of the production, and scheduling of the transfer to the nursery at the required time.

Time consumption for the thawing was estimated from preparation of the media and equipment, recovery of samples from cryogenic storage, and processing of the samples all the way through to the proliferation media. Thawing included the use of liquid nitrogen during recovery of the samples from cryogenic storage, the semi-solid media needed before transferring the embryogenic tissues to the proliferation media, sterilized water, and disposable items (e.g., pipette tips, filter paper, etc.).

Proliferation included subculturing the embryogenic tissue to fresh proliferation media bi-weekly, three times, until maturation. The consumption of work time includes preparing the media and equipment needed during the process. In the estimation, the amount of embryogenic tissue was expected to increase by one Petri dish per genotype, bi-weekly, on average. Enough embryogenic tissue to cover maturation on 10 Petri dishes was estimated to be obtained in seven weeks after proliferation, as cryopreservation is not necessary in production, but was performed in Experiment I of Paper **III**. The work time and material costs were estimated for the maturation phase in the same way as for the previous phases. After eight weeks of maturation, the cotyledonary embryos were cold-stored on Petri dishes until germination.

In the germination phase, the individual cotyledonary embryos were manually picked from the Petri dishes and placed on the germination dishes. After placing the embryos in the germination medium, the embryos were germinated *in vitro* for one week (Paper **II**). After germination *in vitro*, the germinated emblings were expected to be assigned to an operator (e.g., in the nursery) who would take care of the production from that point forward.

Costs attributable to the initiation of the cultures, initial cryopreservation, laboratory testing for embryo yield, and producing emblings for field testing, were taken into account. This was done by estimating the overall cost of the production phases mentioned, and distributing them evenly among the maximum amount of vegetative propagules in the '*Parents of families*' type of basic material in '*qualified*' category in Finland, which is limited to 4 000 000 propagules per family (1055/2002 § 5). Also, the overhead costs (including e.g.: initial costs of specialized equipment needed for large-scale in vitro propagation, development and maintenance of infrastructure, management, administration, information and communication technology (ICT) services etc.) of Luke in 2017 were taken into account in the estimation. List prices from 2017 were used in the estimation for the chemicals and disposable products needed for embling production.

2.5 Laboratory measurements

Embryo yield – cotyledonary embryos per gram of fresh mass (E/gFM) – was defined by dividing the amount of counted cotyledonary embryos by the weight of embryogenic tissue (in grams) placed on maturation media (Papers **I** and **II**). Embryos were counted after maturation and before cold storage, except in the case of thawing lot B in Experiment III of Paper **II**, in which the embryos were moved to cold storage before counting.

Cotyledonary embryos were photographed after cold storage, right before *in vitro* germination, in Paper **I** (Experiments II and III). The photographs were taken with a microscopic camera (Zeiss AxioCam ERc 5s, ZEN 2011 blue edition) connected to a Zeiss Discovery.V8 stereomicroscope. For photographing, the embryos were systematically placed onto germination media, to enable individual identification, from laboratory to nursery and regeneration site. The lengths and widths of the shoots and root apices, and the widths of the crowns of the cotyledons on the embryos, were measured using ImageJ software (version 1.48v, Java 1.8.0_51 [32-bit]; see, e.g., Svobodová et al. 1999).

The shoot and root lengths of the emblings were measured after *in vitro* germination in Paper **I** (Experiments I and II). In Experiment I (Paper **I**), the measurements were carried out by manually placing the emblings against a ruler. In Experiment II (Paper **I**), the emblings were photographed (Canon Powershot G5 PC1049) after *in vitro* germination, with a scale enabling comparison of root and shoot length among treatments. When reliable measurements could not be determined due to improper orientation of the embryo/embling, these cases were excluded from the measurement data.

2.6 Data analysis

Differences among embryo yields, dimensions and dimension ratios of the cotyledonary embryos and emblings between treatments were tested statistically (Papers **I** and **II**). For analysis of variance, one-way ANOVA was used, when an assumption of normality could be made; otherwise, non-parametric tests – Kruskal–Wallis (one-way ANOVA on ranks) or Mann–Whitney *U* – were used. All statistical analyses were performed using IBM SPSS Statistics, v. 22 or newer. The level of confidence used was 5% in all tests.

Embling survival was determined after each growing season or period, depending on the growing method (Papers **I** and **II**). Embling height was measured after each growing season in the nursery, and before planting. For Experiment II in Paper **I**, measurements were available only after the second growing season. In Experiment IV in Paper **I**, height was not measured before planting. Logistic regression was used to estimate the effects of test treatments on the survival of the emblings. The spatial effect inside the test setting was investigated using block, row, and column as covariates. The effect of embryo length on embling survival was investigated using Spearman's correlation because normality could not be assumed (Paper **I**).

The rooting capacity of the shoot cuttings was assessed after eight weeks of rooting by both tests in Paper **III**. Logistic regression was also used to estimate the effects of the rooting media, container type, full-sib family, and clone in the rooting capability of shoot cuttings (Paper **III**). Also, the spatial effect inside the test setting was taken into account by using the covariates block, row, and column. In 2015, different rooting media/container interactions were tested using the $\chi 2$-test.

3 RESULTS

3.1 Yield, survival, and height growth after improvements (Papers I and II)

Maturation results were greatly improved when lower ABA concentrations in the maturation media were applied (Paper **II**). The highest yields of cotyledonary embryos were recorded when the ABA concentration was reduced to 20 µM (two subcultures to fresh media during maturation) or 30 µM (no subcultures during eight week maturation). The increases in the yield of cotyledonary embryos were 131% (20 µM ABA) and 113% (30 µM ABA), when compared to control treatment (60 µM ABA). Adding PEG to the maturation media had a positive effect on the yield of cotyledonary embryos only when the ABA concentration was 60 µM. Significant differences in embryo yield were found between cryopreserved and continuously subcultured embryogenic tissues (Paper **I**).

Keeping the embryos on filter paper during *cold storage*, before three-week *in vitro* germination, resulted in a higher survival in the nursery (GT-II), compared to embryos that were cold-stored in direct contact with semi-solid media, at 41% and 31%, respectively (Experiment III in Paper **I**).

In vitro germination for only one week in a semi-solid medium, without inorganic nitrogen, increased the survival by 88% and height growth by 28% of emblings in the nursery compared to the poorest reference treatment in the same test year (**Fig. 1**; Paper **I**). Lowering the overall nitrogen level in the germination media increased the root-to-shoot length ratio by 73% (Paper **I**). In GT-II, among the embryos cold-stored on filter paper, shorter *in vitro* (one week) germination improved the survival (62%) and height growth (17.9 cm) of emblings in the nursery, when compared to *in vitro* germination of three (41% survival, 15.6 cm length) and five (32% survival, 14.9 cm length) weeks (**Fig. 1**; Paper **I**). In Experiment III in Paper **I**, cotyledonary embryos selected for one week of *in vitro* germination treatment were significantly shorter after cold storage than the cotyledonary embryos selected for other treatments. When the cotyledonary embryos were cold-stored in direct contact with semi-solid media, the length of the *in vitro* germination period affected the survival and growth of the emblings less (Paper **I**). A survival of 77% was obtained when cold storage on filter paper and *in vitro* germination of one week was applied to different genotypes before transplanting to the nursery (Paper **II**).

Emblings were grown with GT-I in Experiment IV in Paper **I** and in Experiment II in Paper **II**. With GT-I, embling survival after the growing period varied from 40% to 65% among different growing substrates, and remained stable until planting at the forest regeneration site (Paper **I**). The average height of the emblings in Experiment IV was 12.4 cm after one growing season in the nursery (Paper **I**). In paper **II**, where different sets of genotypes were germinated *in vitro* for one week, instead of two weeks, an average survival of 93% was obtained after the growing period. Survival of the emblings decreased to 53% after cold storage and one growing season in the nursery, and average height was 5.8 cm (Paper **II**). With the same set of genotypes, an embling survival of 77% was obtained with GT-II after one growing season in the nursery (Paper **II**).

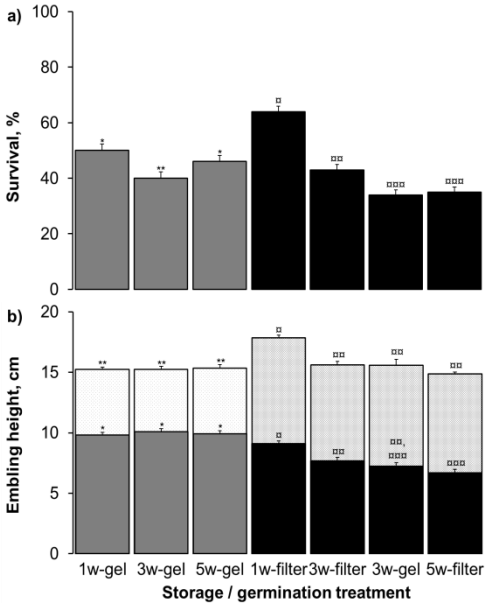

Figure 1 a) Survival of emblings after the first growing season (fall 2015 and 2016 for Experiments II and III of Paper **I**). b) Mean height of emblings following different germination treatments in fall 2016 (lower bars), and growth during 2017 before planting (upper bars). Statistical differences ($p < 0.05$, Kruskal–Wallis nonparametric test (one-way ANOVA on ranks)) among treatments are marked with *, **, and *** in Experiment II, and with ¤, ¤¤, and ¤¤¤ in Experiment III. Treatments are named according to the duration of *in vitro* germination, in weeks (1w, 3w, and 5w); '-gel' and '-filter' refer to cold storage in direct contact with semi-solid media or on filter paper, respectively. Average values, with standard error bars, are presented for all genotypic treatments (Paper **I**)

3.2 Impact of full-sib families on embling production

In Experiment III in Paper **II**, 2744 zygotic embryos, from 12 full-sib families, were used as explants, and of the 1 512 initiated genotypes, 1247 grew enough to be cryopreserved. From the 712 thawed genotypes, 76% (51% to 94% variation among full-sib families) produced enough embryogenic tissue to be matured (Paper **II**). Cotyledonary embryos were produced from 67% of the thawed genotypes, varying from 43% to 91% of the thawed genotypes among the full-sib families (Paper **II**). The average yield of cotyledonary embryos was 79 E/gFW. From 712 thawed genotypes, *in vitro*-germinated emblings were obtained from 50%, from 18 to 42 genotypes among full-sib families, after the first growing season in the nursery (Paper **II**). Embling survival varied among thawing lots and full-sib families (Paper **II**).

From thawing lots A to D, 8904 emblings from 340 genotypes (varying from 21 to 34 genotypes per family) were grown with GT-I (Paper **II**). Overall, 3837 (318 genotypes varying from 21 to 34 genotypes per family) emblings survived the growing period in a controlled environment, and were transplanted to larger growing containers (Paper **II**). Only 1481 (varying from 12 to 28 genotypes per family) emblings survived cold storage and the first growing season in the nursery (Paper **II**).

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 (Paper **II**). After the first growing season in the nursery, the number of vital emblings was 3196 (80% survival), consisting of 121 genotypes, from two to 23 genotypes per family (Paper **II**).

Figure 2 Rooted shoot cutting of Norway spruce, after a rooting period of eight weeks (Paper **III**).

Rooted cuttings were obtained from all genotypes and treatments (Paper **III**). Typically, following the eight-week rooting period, the rooted shoots had 1–14 roots, approximately 1–10 cm in length (**Fig. 2**; Paper **III**). The use of rooting containers consistently resulted in a higher rooting percentage (79%) than the boxes (47%), regardless of the rooting medium (Paper **III**). Rooting percentages in peat mix were superior in both tests (71% in 2015 and 73% in 2016), when compared to either sand (56%) or moss (48%). Rooting varied between rooting media in both test years (Paper **III**).

The highest rooting rates were obtained when the rooting containers were filled with the peat–vermiculite mixture; on average, 91% of the scions rooted, varying from 55% to 100% among genotypes (Paper **III**). The two specially-formed clones showed an average rooting success of 91%. The rooting percentages for the specially-formed genotypes, 11Paf-6017 and 11Paf-6020, were 96 (from 89% to 100%) and 87 (from 78% to 100%), respectively.

A decreasing trend in rooting was found among cuttings that were collected from the same donor plant in both tests (from 84% in 2015 to 63% in 2016). The average rooting among genotypes used only in 2016 was 70% (Paper **III**).

3.3 Production costs of different phases in somatic embryogenesis

Labor costs accounted for the majority of the costs in all of the production phases (**Table 1**). The effect of the production phase on overall costs increased in the latter phases of SE, where the number of units to be handled per genotype increased (**Table 1**). In the maturation phase, the ABA concentration in the media largely affected the costs.

The average yield of cotyledonary embryos was 206 ± 19 E/gFM among the 106 genotypes that produced embryos (Paper **III**). On average, 158 mg of embryogenic tissue was dispersed per maturation dish, enabling harvesting of approximately 32 cotyledonary embryos. With an estimated amount of 1567 explants entering the initiation, the cost of testing genotypes in the laboratory and nursery, and of producing emblings for field testing, accounted for approximately 0.3% of the cost of an embling after *in vitro* germination.

Table 1. Cost structure in embling production for different phases of production. Estimation of labor and material costs in different phases together with the proportion of the overall costs in each phase

Production phase	Labor cost, %	Material cost, %	Overall cost, %
Planning	100		6
Thawing	92	8	4
Proliferation	88	12	10
Maturation	83	17	20
Germination	98	2	60

4 DISCUSSION

4.1 Evaluation of approaches and methodology applied

In Paper **I**, the effect of removing inorganic nitrogen from the germination medium, thus decreasing the overall nitrogen content in the medium, was studied with 10 genotypes from six full-sib families. The root growth of the emblings during *in vitro* germination increased significantly on the medium without inorganic nitrogen. Unfortunately, the measurement method proved to be destructive for the emblings, and the effect of enhanced root growth could not be verified in the nursery. This led to the development of a measurement method based on photographs, which enabled the handling of a larger amount of emblings, and allowed the measurements to be taken after the emblings were moved to the nursery.

In Experiments II and III in Paper **I**, three durations (one, three, and five weeks) of *in vitro* germination were studied. In addition, in Experiment III, two cold storage methods (embryos on filter paper or in direct contact with semi-solid maturation media) were studied. The newly introduced cold storage method in the latter experiment made a comparison of the results between experiments difficult. A comparison of the two cold storage methods was made in Experiment III, in the three-week *in vitro* germination treatment. Evaluation of the individual effects of the protocol's improvements, i.e., storage time and embryo position, is difficult, but the overall effects are clear.

In Experiment IV of Paper **I**, the growing of emblings in small containers after two-weeks of *in vitro* germination was tested with three growing substrates. In this experiment, there was no previous routine treatment. Embling survival varied between substrates, and the surviving emblings were transplanted into larger containers. The results indicate the potential to grow emblings in a controlled environment during the winter months, and for this reason, a comparison of growing techniques GT-I and GT-II was made in Paper **II**.

In paper **II**, maturation media with different ABA concentrations and the presence of PEG were tested in three trials in Experiment I. The first two trials comprised an evaluation of several different media. In the third trial, the maturation medium containing 30 µM ABA was compared with that containing 60 µM ABA for 120 genotypes. The third trial was carried out on two thawing lots. After the first lot, the embryogenic tissues were cryopreserved and thawed again as the second lot. Hence, the results include a possible

effect from ageing of the samples before cryopreservation, and a possible effect of the additional cryopreservation cycle. In the first lot, 65 genotypes were matured on media containing 60 µM ABA; these same genotypes were matured on media containing 30 µM ABA in the second lot. The remaining 55 genotypes were matured on media containing 30 µM ABA in both lots. The additional cryopreservation cycle weakened the comparison between treatments in the third trial, but the presence of 55 genotypes that maturated with the same media in both lots enabled an evaluation of the effect of the cryopreservation cycle and ABA concentration in the maturation media.

In Experiment II of Paper **II**, the growing methods GT-I and GT-II were compared among 18 genotypes. High mortality, due to improper cold storage in GT-I, complicated the evaluation of the true potential of the growing technique, compared to GT-II.

In Paper **III**, the rooting potential of cuttings from embling donors was estimated, in different rooting media, in two experiments in consecutive years. The change from vermiculite to perlite in the rooting medium complicated the comparison of the results between test years. The rooting experiments were carried out by using mainly the same genotypes in both test years. From the 36 overall genotypes rooted, 20 were used in both test years. Seedlings from the same full-sib families were not available to be used as donor plants to enable comparison between rooted cuttings from embling and seedling donors. Analysis between the numbers of roots per cutting in different treatments was not performed because the data was not available from the treatments with highest rooting rates in each test year. This data was not available because only the dead cuttings were removed from the Plantek 81f containers filled with Peat-mix.

4.2 Yield, survival and height growth after improvements (Papers I and II)

Based on this work, improvements made in the maturation, cold storage, and germination phases increased efficiency in Norway spruce somatic embryogenesis (SE). From a practical standpoint, it was possible – with the same amount of work – to produce more than twice the amount of embryos. The number of surviving emblings per germinated cotyledonary embryo nearly doubled, and the emblings' growth rates increased, compared to the control treatments. The results were valid for emblings from a wide genetic background. When the laboratory–nursery interface was planned and coordinated well, the emblings did not require any special treatment in the nursery compared to seedlings after transplanting.

The yield of cotyledonary embryos could be greatly increased by lowering the ABA concentration from 60 µM in the maturation medium (Paper **II**). The 30 µM ABA concentration was introduced as the standard protocol, and no additional subculturing during maturation was needed. In addition, adding PEG to the semi-solid maturation media increased the embryo yield only when compared to the 60 µM ABA content, thus the amount of expensive chemicals could be reduced (Paper **II**).

The survival and height growth of emblings can be significantly increased by improving the cold storage and germination conditions (Paper **I**). The cold storage of embryos on filter paper in maturation dishes, and shortening of the *in vitro* germination period to one week, increased embling survival by 88% and height growth by 28% in the nursery after the first growing season, compared to the poorest treatment in the test year (Paper **I**). The cold storage of embryos on filter paper, instead of in direct contact with a semi-solid medium, increased survival by 23%, but had no significant effect on height growth after one growing

season in the nursery, when applied with *in vitro* germination of three weeks (Paper **I**). Besides introducing the cold storage of cotyledonary embryos on filter papers, the change of refrigerator unit to a significantly larger one, which had more stability during cold storage, may have also increased embling survival, by reducing premature germination (Morel et al. 2014).

Visual changes in the cotyledonary embryos (i.e., increased size, premature germination during cold storage, and curving) were observed after cold storage of 20 weeks in direct contact with a semi-solid medium in Experiment IV in Paper **I**. This may be an indication of a change in the ABA concentration, and the accumulation and premature use of storage compounds in the embryos, due to direct contact with the gel medium leading to high water availability (see, e.g., von Arnold and Hakman 1988; Lelu et al. 1994; Klimaszewska and Smith 1997; Bozhkov and von Arnold 1998; Högberg et al. 2001; Morel et al. 2014).

Removing inorganic nitrogen from the germination media, which led to a decrease in the total amount of nitrogen in the germination media, also had a large impact on root growth during five weeks of *in vitro* germination (Paper **I**). The results herein support previous findings, in which the growth of emblings and seedlings is enhanced when less nitrogen is used (see, e.g., Brouwer 1962; Ingestad and Kähr 1985; Kaakinen et al. 2004; Gruffman et al. 2012; Dahrendorf et al. 2018; Llébres et al. 2018a, b). Due to the improved root growth, a germination medium without inorganic nitrogen was introduced in the standard protocol.

Shortening the *in vitro* germination from five weeks to one week resulted in higher survival rates and significantly taller emblings when the embryos were cold-stored on filter paper, compared to longer *in vitro* germination times. The difference in height and height growth between the one- and five-week germination treatments remained significant until planting at the regeneration site. As the physiological ageing of embryogenic tissues may affect Norway spruce similarly as found in maritime pine (*Pinus pinaster* Ait.), even higher embling survival could be achievable by using one-week *in vitro* germination on cotyledonary embryos, cold-stored on filter papers, than was achieved in Experiment III in Paper **I** (Lelu-Walter et al. 2016). This may partially explain the higher survival rate observed in Experiment II, and in thawing lot F in Experiment III in Paper **II**, in addition to the differences among genotypes.

The effects of initial sampling of cold-stored cotyledonary embryos after one-week *in vitro* germination cannot be completely ruled out in Experiment III in Paper **I**; however, the observed higher embling survival rates in Experiment II, and in thawing lot F in Experiment III in Paper **II** support the positive effect of a short *in vitro* germination, despite the differences in genotypes. In addition, the higher survival of shorter cotyledonary embryos in Paper **I** may also be an indication of too long a maturation period, which has been reported to decrease embling survival in later phases of SE (Morel et al. 2014). Shorter maturation treatments have already been applied in different SE protocols in Norway spruce (see, e.g., Bozhkov and von Arnold 1998; Högberg et al. 2001; Hazubska-Przybył et al. 2015).

Survival in the nursery after the improvements is close to the early vigor of unimproved Norway spruce seeds (Papers **I** and **II**). Height growth in the nursery varies between genotypes (Paper **I**). Survival and height growth in the nursery can be further improved by selecting genotypes with good production properties. More height growth and higher survival can be obtained by further improving laboratory and nursery protocols, but not to the same magnitude as achieved in the current work (Papers **I** and **II**).

4.3 Implications of full-sib families in embling production (Paper II)

Genotypes with high embryo production capacity were found in all of the 12 full-sib families (Paper **II**). Vigorous emblings were obtained from a large number of genotypes (Paper **II**). Emblings were obtained from 356 genotypes, i.e., from 23% to 28% of the estimated original explants entering the initiation (Paper **II**). Without major embling mortality in thawing lots A to D, due to improper storage conditions and fungicide treatments, the emblings yield could possibly be close to the survival rate of transplanted emblings (75%) obtained in Experiment IV in Paper **I**. As embling survival was significantly higher in thawing lots E and F (80%), similar survival rates might have been expected by applying GT-II. Hence, the obtained survival rates may underestimate the true potential of the SE genotypes and full-sib families. During the first growing period, or first growing season, in the nursery, 23% of the genotypes were lost. Without the massive loss of emblings due to fungal infestation (possibly *Botrytis* sp.), the loss of genotypes during those production phases may have been as low as 5%. Current results support the findings of Park et al. (1998) and Park (2002), suggesting that genetic variance components decrease greatly in the late phases of SE, compared to initiation and proliferation.

It is possible to estimate the mean and variation of the genotypes producing emblings per explants in the entire SE process among the tested families by using the mean estimated values from all tested families and the best (E799 × 1366) and poorest (E242 × E222), which were both nearly fully tested. From the full-sib family E799 × 1366, 34 genotypes of the 36 (94%) cryopreserved ones were tested; they ranked highest in initiation (93%), cryopreservation (80% of the original explant genotypes), and had a 94% regeneration rate from cryopreservation. The mean yield of cotyledonary embryos was also the highest (144 E/gFW) in genotypes of the family E799 × 1366. In addition, all the cryopreserved genotypes from the full-sib family (E242 × E222) were tested during Experiment III in Paper **II**. This full-sib family produced the least embryos in thawing lots A to D, ranked poorly in initiation and regeneration after thawing (third poorest initiation, at 47%, and second poorest cryopreservation, at 27% of overall initiations), and the mean yield of cotyledonary embryos was the lowest (36 E/gFW).The number of genotypes with emblings was in the range of 28% to 34% (55% initiation rate and 45% of overall initiations grew enough embryogenic tissue to be cryopreserved; Paper **II**), varying from 7.5% to 60% among full-sib families.

These results indicate that from three to four immature zygotic embryos are needed to get a cryopreserved genotype that produces emblings. To succeed in this, two explants were needed with the absolute best material for the protocol. By comparison, it took 14 explants with the poorest material for the protocol.

4.4 Implications of rooting of cuttings on test plant production (Papers II and III)

Shoot cuttings taken from emblings rooted well under similar conditions used for seedling cuttings (Paper **III**). In both test years highest rooting was achieved in Plantek 81f containers filled with peat mixed with vermiculite or perlite (75/25% v/v). Rooting rates varied between rooting vessels, rooting media and genotypes.

With an average survival of 64% to 77% in GT-I, the requirement for 12 emblings to perform as cutting donors could potentially be achieved with approximately 16 to 19

germinated emblings (Papers **I**, **II** and **III**). To establish clonal field testing according to the testing protocol used by the Finnish tree breeding programme, 59 cuttings are needed for rooting, which can be produced from 12 emblings in two years in the nursery (Paper **III**).

By using rooted cuttings derived from emblings, the demand for cuttings to establish field testing can be achieved two to three years faster than using rooted cuttings derived from seedlings (Paper **III**). Even more time (one to two years) can be saved in the production of test material if field tests are established directly using emblings (Paper **III**). Högberg (1998) estimated that three years could be saved by using emblings to establish clone testing, which is supported by the current results.

Based on this work, the requirement of 20 genotypes per family for clone testing (see Haapanen and Mikola 2008) could be achieved by initiating SE from 40 to 280 explants at the current success rates. From 60 to 80 explants are enough to propagate emblings from 20 cryopreserved genotypes.

In this work, emblings were obtained from larger proportion of the original explants than previously estimated, but in production, it needs to be taken into account that propagation rates of under 10% can introduce a risk of reduced genetic gain (Högberg 2003). It has been estimated that initiation rates below 30% may result in reduced genetic gain (see review by Bonga 2016).. Haines and Woolaston (1991) concluded that a substantial reduction in genetic gain will occur in a propagation system when one of following coincides with a high retained proportion and/or a low propagation rate: (i) a low number of genotypes can be propagated; (ii) a low number of genotypes can be propagated from a high number of retained genotypes; and (iii) economic and reproductive traits have a strongly adverse correlation. Although the propagation rate was only 7.5% in E242 × E222, the result that 30 genotypes could be propagated (40 without the fungal infestation, possibly *Botrytis*) also indicates that, in this full-sib family, significant genetic gain may be obtained. In Norway spruce, there is no evidence of adverse correlations between SE success and economically important traits (Högberg 2003).

4.5 Implications of protocol improvements on the cost of embling production

The success rate of late phases of SE could be improved without increasing labor costs while simultaneously decreasing material costs, e.g. the amount of ABA per maturation dish was reduced to half and the *in vitro* germination period shortened from five weeks to one week.. It can be estimated that the cost of a cotyledonary embryo was decreased by 53% due to an average increase in embryo yield by 113% (Trial 2 in Experiment I in Paper **II**), even when the material cost of ABA reduction is ignored. The obtained 88% increase in survival of emblings, due to improved cold storage and germination conditions, resulted in a 47% saving in the cost of a surviving embling when compared to the poorest treatment. Thus was the case even when the saving in cost due to a shorter *in vitro* germination was ignored. When combined, the cost of a single surviving embling decreased by 75% as an impact of these rather simple improvements in the protocol.

The estimated cost structure emphasizes the labor intensity of SE production, especially for the maturation (large numbers of samples per genotype are handled) and germination (individual embryos are handled) phases, which are in line with the findings of Thompson (2015). The costs resulting from the selection of genotypes with good production properties, and the production of emblings for field testing, were marginal, when the costs were allocated to the maximum amount of emblings allowed to be produced in the ‘*Parents*

of families‘ type of basic material in the ‘*qualified*‘category in Finland (1055/2002 § 3). It is unknown whether the maximum amount of emblings can be produced in practice. Therefore the actual effect of genotype testing on production costs may be underestimated in this method.

From the perspective of the embling producer, the cost effect of genotype testing could be distributed among the samples in cryostorage. In this way, the cost of genotype testing would account for approximately 28% of the cost of an embling germinated *in vitro*, when assuming 10 samples per genotype in cryostorage, with the current protocol and embryo yield. If the testing of genotypes is fragmented in cryopreserved samples, the initial cryostorage ought to be larger in order to decrease the cost of genotype testing. Another way to decrease the cost effect of genotype testing would be to increase the number of times a particular sample of embryogenic tissue was matured following initial removal from cryopreservation. We based our estimation based on only ten successive maturations. However, by increasing the number of maturations, the physiological characteristics of the embryogenic tissue eventually become relevant as the embryogenic tissue ages. From an operational perspective, the embling producer must define a threshold in the maturation efficiency at which point to select suitable genotypes for production, and must also apply the threshold when the selected genotypes lose their capability of producing embryos (see, e.g., Lelu-Walter et al. 2016; Egertsdotter 2018).

In addition, the efficiency and final cost of an embling are greatly affected by the number of emblings produced per unit. This can also be improved by selecting highly productive genotypes for production. The selection of genotypes for production should also consider genetic diversity, genetic gain, and production cost, as the breeding value of a single genotype does not correlate with its production properties (see, e.g., Park 2002). By selecting highly productive genotypes, with good breeding value, for production, decreased production costs and increased genetic diversity may be simultaneously achieved, compared to a situation where the selection of genotypes is based entirely on high breeding value. Despite the significant reduction in production costs for the manual production of emblings, automation is still urgently needed to further reduce production costs, and to reach practically significant production numbers. Hopefully, the great efforts undertaken to automate embling production, such as in Sweden with SE Fluidics System, will soon be available on a larger scale, so as to benefit tree improvement globally (see, e.g., Aidun and Egertsdotter 2018).

5 CONCLUSIONS AND FUTURE PROSPECTS

In this work, significant improvements in the yield of cotyledonary embryos were achieved by reducing the ABA concentration in maturation media. In addition, significant improvements in the yield and properties of Norway spruce emblings were achieved by improving the cold storage and germination conditions of somatic embryos. Shortening the *in vitro* germination period to one week, together with the use of improved cold storage and germination conditions, increased both the number of surviving plants and amount of shoot growth. Despite large variations in embryo and embling production, genotypes with good production properties were found in all families. Protocol improvements resulted in a 75% reduction in the production costs of an embling. Shoot cuttings from embling donors rooted

well in similar rooting conditions used for cuttings originating from seedlings; however, there are substantial differences in rooting capability between different rooting media and containers, as well as between clones.

Based on this study, large-scale clone testing could be initiated by producing five to 12 emblings to perform as cutting donors for rooted shoot cuttings. This method also enables the comparison of ramets originating from seedlings and emblings from an even set point. In addition, rooted cuttings for field testing can be produced significantly faster from emblings than from a single seedling donor from each genotype.

Basic material from 12 full-sib families (Paper **II**) was also registered as basic material during 2017, under type '*Parents of family*' in category '*qualified*'. The field testing of genotypes will be initiated in 2019. Two lots of *in vitro*-germinated emblings were sold to nurseries in 2018; however, production capacity will need to be significantly increased in the future to enable embling production on a large scale. New initiations from full-sib families will also be made in the near future.

Despite the significant improvements achieved in this study, further research is still needed to improve the efficiency of somatic embryogenesis. This need is urgent for decreasing the cost of emblings to make them competitive with seedlings. Automation, especially in the final steps of embling production, i.e. germination and transplanting, is also essential for increasing the propagation volume to a commercial scale. Initiating SE from adult trees would enable even faster implementation of breeding results, so that future challenges can be met in the fastest way possible.

REFERENCES

Aidun C.K., Egertsdotter U. (2018) SE Fluidics System. In: Jain S. M., Gupta P. (eds), Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants, Volume 1, second edition, Forestry Sciences 84. Springer International Publishing AG. Cham, Switzerland. p. 211-227. https://doi.org/10.1007/978-3-319-89483-6_19

Ahtikoski A., Haapanen M., Hynynen J., Karhu J., Kärkkäinen K. (2018) Genetically improved reforestation stock provides simultaneous benefits for growers and a sawmill, a case study in Finland, Scandinavian Journal of Forest Research 33:1 484-492. https://doi.org/10.1080/02827581.2018.1433229.

Allard R.W. (1999) History of plant population genetics. Annual Review of Genetics 33:1-27. https://doi.org/10.1146/annurev.genet.33.1.1

Belmonte M.F., Yeung E.C. (2004) The effects of reduced and oxidized glutathione on white spruce somatic embryogenesis. In Vitro Cellular Developmental Biology - Plant 40:1 61-66.

Bergsten U. (1989) Temperature tolerance of invigorated seeds of *Pinus sylvestris* L. and *Picea abies* (L.) Karst. using TTGP-test. Forestry Supplement 62: 107-115.

Bonga J.M. (2015) A comparative evaluation of the application of somatic embryogenesis, rooting of cuttings, and organogenesis of conifers. Canadian Journal of Forest Research. 45:379–383. doi:10.1139/cjfr-2014-0360.

Bonga J.M. (2016) Conifer clonal propagation in tree improvement programs. In: Park Y.-S., Bonga J.M., Moon H-K. (eds) Vegetative propagation of forest trees. Seoul: Korea Forest Research Institute. p. 3-31.

Börjesson P., Hansson J., Berndes G. (2017) Future demand for forest-based biomass for energy purposes in Sweden. Forest Ecology and Management 383:1 17-26. https://doi.org/10.1016/j.foreco.2016.09.018.

Bozhkov P.V., von Arnold S (1998) Polyethylene glycol promotes maturation but inhibits further development of *Picea abies* somatic embryos. Physiologia Plantarum 104:2 211-224.

Brouwer R. (1962) Nutritive influences on the distribution of dry matter in the plant. Journal of Agricultural Science 10: 399-408.

Burdon R.D., Carson M.J., Shelbourne C.J.A. (2008). Achievements in forest tree genetic improvement in Australia and New Zealand 10: *Pinus radiata* in New Zealand. Australian Forestry. 71:4 263-279. https://doi.org/10.1080/00049158.2008.10675045.

Carneros E., Yakovlev I., Viejo M., Olsen J.E. Fossdal C.G. (2017) The epigenetic memory of temperature during embryogenesis modifies the expression of bud burst-related genes in Norway spruce epitypes. Planta 246: 553-566.

Chalupa V. (1985) Somatic embryogenesis and plantlet regeneration from cultured immature and mature embryos of *Picea abies* (L.) Karst. Commun. Inst. Forest. Czech Republic. 14:57-63.

Cook R.E. (1983) Clonal Plant Populations: A knowledge of clonal structure can affect the interpretation of data in a broad range of ecological and evolutionary studies. American Scientist 71:3 244-253.

Cyr D.R., Lazaroff W.R., Grimes S.M.A., Quan G., Bethune T.D., Dunstan D.I. Roberts D.R. (1994) Cryopreservation of interior spruce (*Picea glauca engelmanni* complex) embryogenic cultures. Plant Cell Reports 13:574-577.

Dahrendorf J., Clapham D., Egertsdotter U. (2018) Analysis of Nitrogen Utilization Capability during the Proliferation and Maturation Phases of Norway Spruce (*Picea abies* (L.) H.Karst.) Somatic Embryogenesis. Forests 9:6 288. https://doi.org/10.3390/f9060288.

Díaz-Sala C. (2016) Physiological, cellular, molecular and genomic analysis of the effect of maturation on propagation capacity. In: Park Y.-S., Bonga J.M., Moon H-K. (eds) Vegetative propagation of forest trees. Seoul: Korea Forest Research Institute. p. 75-96.

Egertsdotter U. (2018) Plant physiological and genetical aspects of the somatic embryogenesis process in conifers. Scandinavian Journal of Forest Research 1-10. https://doi.org/10.1080/02827581.2018.1441433.

Find J.I., Kristensen M.M.H., Norgaard J.V., Krogstrup P. (1998) Effect of culture period and cell density on regrowth following cryopreservation of embryogenic suspension cultures of Norway spruce and Sitka spruce. Plant Cell, Tissue and Organ Culture 53:1 27-33. https://doi.org/10.1023/A:1006070729489.

Finlex (2018) 1055/2002 Maa- ja metsätalousministeriön asetus metsänviljelyaineiston kaupasta. http://www.finlex.fi/fi/laki/alkup/2002/20021055#Pidp452488960. [Cited 31 May 2018].

Grossnickle S.C., Cyr D., Polonenko D.R. (1996) Somatic embryogenesis tissue culture for the propagation of conifer seedlings: a technology comes of age. Tree Planters' Notes 47(2): 48-57.

Gruffman L., Ishida T., Nordin A., Näsholm T. (2012) Cultivation of Norway spruce and Scots pine on organic nitrogen improves seedling morphology and field performance. Forest Ecology and Management 276: 118-124. https://doi.org/10.1016/j.foreco.2012.03.030.

Haapanen M., Mikola J. (2008) Metsänjalostus 2050 ─ pitkän aikavälin metsänjalostusohjelma. Metlan työraportteja / Working Papers of the Finnish Forest Research Institute 71. 50 p.

Haapanen M. (2009) Clones in Finnish tree breeding. Proceedings of the Nordic meeting held in September 10th-11th 2008 at Punkaharju, Finland. Working papers of the Finnish Forest Research Institute 114: 16-19.

Haapanen M., Jansson G., Nielsen U.B., Steffenrem A., Stener L-G. (2015) The status of tree breeding and its potential for improving biomass production – A review of breeding activities and genetic gains in Scandinavia and Finland. In: Stener L-G, (ed.). Uppsala, Sweden: Skogsforsk. 56 p.

Haapanen M., Leinonen H., Leinonen K. (2017) Männyn ja kuusen siemenviljelyssiemenen taimitarhakäytön kehitys 2006–2016: Alueellinen tarkastelu. Metsätieteen aikakauskirja [1241] 4p. http://dx.doi.org/10.14214/ma.7716.

Häggman H., Raybould A., Borem A., Fox T., Handley L., Hertzberg M., Lu M.Z., Macdonald P., Oguchi T., Pasquali G., Pearson L, Peter G., Quemada H., Séguin A., Tattersall K., Ulian E., Walter C., McLean M. (2013) Genetically engineered trees for plantation forests: key considerations for environmental risk assessment. Plant Biotechnology Journal 11:7 785-798. https://doi.org/10.1111/pbi.12100.

Haines R. J., Woolaston R. R. (1991) The influence of reproductive traits on the capture of genetic gain. Canadian Journal of Forest Research 21: 272-275. https://doi.org/10.1139/x91-034.

Hakman I., Fowke L.C., von Arnold S., Eriksson T. (1985) The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce). Plant Science. 38:53-59.

Hamberg L., Velmala S.M., Sievänen R., Kalliokoski T., Pennanen T. (2018) Early root growth and architecture of fast- and slow-growing Norway spruce (*Picea abies*) families differ—potential for functional adaptation, Tree Physiology. 38:6 853–864. https://doi.org/10.1093/treephys/tpx159.

Harju A., Kainulainen, P., Venäläinen, M., Tiitta M., Viitanen H. (2005) Differences in Resin Acid Concentration between Brown-Rot Resistant and Susceptible Scots Pine Heartwood. Holzforschung 56:5 479-486. https://doi.org/10.1515/HF.2002.074.

Hazubska-Przybył T., Wawrzyniak M., Obarska A., Bojarczuk K. (2015) Effect of partial drying and desiccation on somatic seedling quality in Norway and Serbian spruce. Acta Physiologia Plantarum 37:1735 1-9. https://doi.org/10.1007/s1173.

Heikinheimo O. (1995) Kaskiviljelyn vaikutus Suomen metsiin. Keisarillisen senaatin kirjapaino. Helsinki. 263p.

Heiskanen J. (1993) Variation in water retention characteristics of peat growth media used in tree nurseries. Silva Fennica 27: 77-97.

Högberg K-A. (2003) Possibilities and limitations of vegetative propagation of Norway spruce. Acta Universitatis Agriculturae Sueciae, Silvestria 294. Uppsala: Swedish University of Agricultural Sciences.

Högberg K.-A., Ekberg, I., Norell L., von Arnold S. (1998) Integration of somatic embryogenesis in a tree breeding programme: a case study with *Picea abies*. Canadian Journal of Forest Research 28:10 1536–1545. https://doi.org/10.1139/x98-137.

Högberg K-A., Bozhkov P.V., Grönroos R., von Arnold S. (2001) Critical Factors Affecting *Ex Vitro* Performance of Somatic Embryo Plants of *Picea abies.* Scandinavian Journal of Forest Research 16: 295-304.

Högberg K-A., Bozhkov P.V., von Arnold S. (2003) Early selection improves clonal performance and reduces intraclonal variation of Norway spruce plants propagated by somatic embryogenesis. Tree Physiology 23: 211-216.

Ingestad T., Kähr M. (1985) Nutrition and growth of coniferous seedlings at varied relative nitrogen addition rate. Physiologia Plantarum 65: 109-116.

Jansson G., Danusevičius D., Grotehusman H., Kowalczyck J., Krajmerova D., Skrøppa T., Wolf F. (2013) Norway Spruce (*Picea abies* (L.) H.Karst.). In: Paques L.E. (ed.), Forest Tree Breeding in Europe: Current State-of-the-Art and Perspectives. Managing Forest Ecosystems, Volume 25. Springer. Dordrecht Heidelberg New York London. p. 123-176 https://doi.org/10.1007/978-94-007-6146-9_3.

Jansson G., Hansen J.K., Haapanen M., Kvaalen H., Steffenrem A. (2017) The genetic and economic gains from forest tree breeding programmes in Scandinavia and Finland. Scandinavian Journal of Forest Research 32:4 273-286. https://doi.org/10.1080/02827581.2016.1242770.

Kaakinen S., Jolkkonen A., Iivonen S., Vapaavuori E. (2004) Growth, allocation and tissue chemistry of *Picea abies* seedlings affected by nutrient supply during the second growing season. Tree Physiology 24:6 707-719. http://dx.doi.org/10.1093/treephys/24.6.707.

Kleinschmidt J., Schmidt J. (1977) Experiences with Picea abies cutting propagation in Germany and problems connected with large scale application. Silvae Genetica 26: 197-203.

Klimaszewska K., Ward C., Cheliak W. (1992) Cryopreservation and Plant Regeneration from Embryogenic Cultures of Larch (*Larix* x *eurolepis*) and Black spruce (*Picea mariana*). Journal of Experimental Botany 43:1 73-79. https://doi.org/10.1093/jxb/43.1.73

Klimaszewska K., Smith D.R. (1997) Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. Physiologia Plantarum. 100:4 949-957. https://doi.org/10.1111/j.1399-3054.1997.tb00022.x.

Klimaszewska K., Lachance D., Pelletier G., Lelu M.-A., Seguin A. (2001a) Regeneration of transgenic *Picea glauca*, *P. mariana*, and *P. abies* after cocultivation of embryogenic tissue with *Agrobacterium tumefaciens*. In Vitro Cellular Developmental Biology - Plant. 37: 748-755.

Klimaszewska K., Park Y.-S., Overton C., Maceacheron I., Bonga J.M. (2001b). Optimized somatic embryogenesis in *Pinus strobus* L. *In Vitro* Cellular Developmental Biology - Plant. 37:3 392-399. https://doi.org/10.1007/s11627-001-0069-z.

Klimaszewska K., Tront in J-F., Becwar MR., DeVillard C., Park Y.-S., Lelu-Walter M.-A. (2007) Recent progress in somatic embryogenesis of four Pinus spp. Tree and Forestry Science and Biotechnology 1: 11-25.

Krajňáková J., Sutela S., Aronen T., Gömöry D., Vianello A., Häggman H. (2011) Long-term cryopreservation of Greek fir embryogenic cell lines: Recovery, maturation and genetic fidelity. Cryobiology 63:1 17-25. https://doi.org/10.1016/j.cryobiol.2011.04.004.

Kvaalen H., Johnsen O. (2008) Timing of bud set in *Picea abies* is regulated by a memory of temperature during zygotic and somatic embryogenesis. New Phytologist 177: 49-59. https://doi.org/10.1111/j.1469-8137.2007.02222.x.

Landis T.D., Dumroese R.K., Haase D. (2010*a*) The Container Tree nursery manual. Vol. 6. Seedling Propagation. Washington, DC: U.S. Department of Agriculture, Forest Service. Agricultural Handbook 674.

Landis T.D., Dumroese R.K., Haase D. (2010*b*) The Container Tree nursery manual. Vol. 7. Seedling Processing, Storage, and Outplanting. Washington, DC: U.S. Department of Agriculture, Forest Service. Agricultural Handbook 674.

Libby W.J. (1964) Clonal selection and an alternative seed orchard scheme. Silvae Genetica 13: 32-40.

Lindgren D., Mullin T.J. (1997) Balancing Gain and Relatedness in Selection. Silvae Genetica 46:2-3 124-129.

Litvay J.D., Verma D.C., Johnson M.A. (1985) Influence of loblolly pine (*Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of wild carrot (*Daucus carota* L.) Plant Cell Reports 4:6 325-328. https://doi.org/10.1007/BF00269890.

Leinonen K., Nygren M., Rita H. (1993) Temperature control of germination in the seeds of Norway spruce (*Picea abies* (L.) Karst.). Scandinavian Journal of Forest Research 8:1-4 107-117. https://doi.org/10.1080/02827589309382759.

Lelu M.-A., Bastien C., Klimaszewska K., Charest P.J. (1994) An improved method for somatic plantlet production in hybrid larch (*Larix½leptoeuropaea*): Part 2. Control of germination and plantlet development. Plant Cell, Tissue and Organ Culture 36: 117-127.

Lelu-Walter M.-A., Bernier-Cardou M., Klimaszewska K. (2008) Clonal plant production from self- and cross-pollinated seed families of *Pinus sylvestris* (L.) through somatic embryogenesis. Plant Cell, Tissue and Organ Culture 92: 31-45. https://doi.org/10.1007/s11240-007-9300-x.

Lelu-Walter M.-A., Thompson D., Harvengt L., Sanchez L., Toribio M., Pâques L.E. (2013) Somatic embryogenesis in forestry with a focus on Europe: state-of-the-art, benefits, challenges and future direction. Tree Genetics & Genomes 9:4 883-899. https://doi.org/10.1007/s11295-013-0620-1.

Lelu-Walter M.-A, Klimaszewska K, Miguel C, Aronen T, Hargreaves C, Teyssier C, Trontin J.-F. (2016) Somatic embryogenesis for more effective breeding and deployment of improved varieties in Pinus spp.: bottlenecks and recent advances. In: Loyola-Vargas V.M., Ochoa-Alejo N. (eds), Somatic Embryogenesis - Fundamental Aspects and Applications. Springer Verlag, Chapter 19, pp. 319-365. ISBN 978-3-319-33704-3. http://dx.doi.org/10.1007/978-3-319-33705-0_19.

Lepistö M. (1976) Vegetative Propagation by Cuttings of *Picea abies* in Finland. The Foundation for Forest Tree Breeding in Finland 1976.

Lepistö M. (1993) Genetic variation, heritability and expected gain of height in Picea abies in 7 to 9-year-old clonal tests. Scandinavian Journal of Forest Research 8:480-488. doi: 10.1080/02827589309382794.

Llebrés M.T., Avila C., Cánovas F.M., Klimaszewska K. (2018a) Root growth of somatic plants of hybrid *Pinus strobus* (L.) and *P. wallichiana* (A. B. Jacks.) is affected by the

nitrogen composition of the somatic embryo germination medium. Trees 32:2 471-484. https://doi.org/10.1007/s00468-017-1635-2.

Llebrés M.T., Pascual M.B., Debille S., Trontin J.-F., Harvengt L., Avila C., Cánovas F.M. (2018b) The role of arginine metabolic pathway during embryogenesis and germination in maritime pine (*Pinus pinaster* Ait.), Tree Physiology 38:3 371-381. https://doi.org/10.1093/treephys/tpx133.

Majada J.P., Sierra M.I., Sanchez-Tames R. (2001) Air exchange rate affects the *in vitro* developed leaf cuticle of carnation. Scientia Horticulturae 87: 121-130.

Mason W.L., Menzies M.J., Biggin P. (2002) A comparison of hedging and repeated cutting cycles for propagation of Sitka spruce. Forestry 73:2 149-162.

Mikola P. (1942) Koivun vesomisesta ja sen metsänhoidollisesta merkityksestä. Referat: Ûber die Ausschlagbildung bei der Birke un ihre forstliche Bedeutung. Acta Forestalia Fennica 50. 92-101.

Morel A., Trontin J.-F., Corbineau F., Lomenech A-M., Beaufour M., Reymond I., Le Metté C., Ader K., Harvengt L., Cadène M., Label P., Teyssier C., Lelu-Walter M.-A. (2014) Cotyledonary somatic embryos of *Pinus pinaster* Ait. most closely resemble fresh, maturing cotyledonary zygotic embryos: biological, carbohydrate and proteomic analyses. Planta 240:5 1075-1095. https://doi.org/10.1007/s00425-014-2125-z.

Mullins M.G., Nair Y., Sampet P. (1979) Rejuvenation in vitro: Induction of Juvenile Characters in an Adult Clone of Vitis vinifera L. Annals of Botany 44:5 623-627.

Nagmani R., Bonga J.M. (1985) Embryogenesis in subcultured callus of *Larix decidua*. Canadian Journal of Forest Research 15:6 1088-1091. https://doi.org/10.1139/x85-177.

Nestby R., Percival D., Martinussen I., Opstad N., Rohloff J. (2011) The European Blueberry (*Vaccinium myrtillus* L.) and the Potential for Cultivation. A Review. The European Journal of Plant Science and Biotechnology 5: 17-34.

Nørgaard J.V., Duran V., Johnsen Ø., Krogstrup P., Baldursson S., von Arnold S. (1993) Variations in cryotolerance of embryogenic *Picea abies* cell lines and the association to genetic, morphological, and physiological factors. Canadian Journal of Forest Research 23:12 2560-2567. https://doi.org/10.1139/x93-317.

Öberg L. Kullman L. (2011) Ancient Subalpine Clonal Spruces (*Picea abies*): Sources of Postglacial Vegetation History in the Swedish Scandes. Arctic 64:2 183-196. https://doi.org/10.14430/arctic4098.

Official Statistics of Finland (OSF) (2017) Silvicultural and forest improvement work [internet publication] Helsinki: Natural Resources Institute Finland [cited: 1.2.2018]. http://stat.luke.fi/en/silvicultural-and-forest-improvement-work-2016_en.

Park Y.-S., Pond S.E., Bonga, J.M. (1993) Initiation of somatic embryogenesis in white spruce (*Picea glauca*): genetic control, culture treatment effects, and implications for tree breeding. Theoretical and Applied Genetics 86:4 427-436. https://doi.org/10.1007/BF00838557.

Park Y.-S., Barrett J.D., Bonga J.M. (1998) Application of somatic embryogenesis in high-value clonal forestry: deployment, genetic control, and stability of cryopreserved clones. In Vitro Cellular and Developmental Biology - Plant 34:3 231–239. https://doi.org/10.1007/BF02822713.

Park Y.-S. (2002) Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. Annals of Forest Science 59:5-6 651-656. https://doi.org/10.1051/forest:2002051.

Pereg D., Payette S. (1998) Development of black spruce growth forms at treeline. Plant Ecology 138: 137-147. https://doi.org/10.1023/A:1009756707596.

Pop J., Lakner Z., Harangi-Rákosa M., Fáric M. (2014) The effect of bioenergy expansion: Food, energy, and environment. Renewable and Sustainable Energy Reviews 32 559-579. https://doi.org/10.1016/j.rser.2014.01.056.

Rehfeldt G.E., Jaquish B.C., Sáenz-Romero C., Joyce D.G., Leites L.P., St Clair J.B., López-Upton J. (2014) Comparative genetic responses to climate in the varieties of *Pinus ponderosa* and *Pseudotsuga menziesii*: reforestation. Forest Ecology and Management 324: 147-157. https://doi.org/10.1016/j.foreco.2014.02.040.

Rikala R. (2012) Metsäpuiden paakkutaimien kasvatusopas. Suonenjoki: The Finnish Forest Research Institute. 247 p.

Ruotsalainen, S. (2002) Managing breeding stock in the initiation of a long-term tree breeding program. Metsäntutkimuslaitoksen tiedonantoja 875: 95 p.

Ruotsalainen S. (2014) Increased forest production through forest tree breeding, Scandinavian Journal of Forest Research, 29:4 333–344. https://doi.org/10.1080/02827581.2014.926100.

Ryynänen L. (1996) Survival and regeneration of dormant silver birch buds at super-low temperatures. Canadian Journal of Forest Research 26:4 617-623. https://doi.org/10.1139/x26-071.

Sakai A. (1960) Survival of the twig of woody plants at -196°C. Nature 185: 392-394

Savolainen O., Bokma F., García-Gil R., Komulainen P., Repo T. (2004) Genetic variation in cessation of growth and frost hardiness and consequences for adaptation of *Pinus sylvestris* to climatic change. Forest Ecology and Management 197:1–3 79-89. https://doi.org/10.1016/j.foreco.2004.05.006.

Stasolla C., Yeung E.C. (2003) Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell, Tissue and Organ Culture 74: 15-35.

St Clair J.B., Kleinschmit J., Svolba J. (1985) Juvenility and serial vegetative propagation of Norway spruce clones (*Picea abies* Karst.). Silvae Genetica 34:1 42-48.

Svobodová H., Albrechtová J., Kumstýřová L., Lipavská H., Vágner M., Vondráková Z. (1999) Somatic embryogenesis in Norway spruce: Anatomical study of embryo development and influence of polyethylene glycol on maturation process. Plant Physiology and Biochemistry 37:3 209-221. https://doi.org/10.1016/S0981-9428(99)80036-9.

Thompson D. (2015) Challenges for the large-scale propagation of forest trees by somatic embryogenesis – a review. In: Park Y.-S., Bonga J.M. (eds) Proceedings of the 3rd International Conference of the IUFRO unit 2.09.02 on 'Woody plant production integrating genetic and vegetative propagation technologies.' September 8–12, 2014. Vitoria-Gasteiz, Spain, pp 81-91.

Varis S., Heiska S., Aronen T. (2014) Kuusen solukkolisäys [Somatic embryogenesis of Norway spruce]. Working Papers of the Finnish Forest Research Institute 310. 50 p.

Varis S., Ahola S., Jaakola L., Aronen T. (2017) Reliable and practical methods for cryopreservation of embryogenic cultures and cold storage of somatic embryos of Norway spruce. Cryobiology 76:8-17. https://doi.org/10.1016/j.cryobiol.2017.05.004.

Varis S. (2018) Norway Spruce *Picea abies* (L.) Karst. In: Jain S. M., Gupta P. (eds), Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants, Volume 1, second edition, Forestry Sciences 84. Springer International Publishing AG. Cham, Switzerland. p. 255-267. https://doi.org/10.1007/978-3-319-89483-6_19.

von Aderkas P., Kong L., Hawkins B., Rohr R. (2007) Effects of non-freezing low temperatures on quality and cold tolerance of mature somatic embryos of interior spruce (*Picea glauca* (Moench) Voss x *P. engelmanii* Parry ex Engelm. Propagation of Ornamental Plants 7:3 112–121.

von Aderkas P., Teyssier C., Charpentier J-P., Gutmann M., Pâques L., Le Metté C., Ader K., Label P., Kong L., Lelu-Walter M-A. (2015) Effect of type of light conditions on anatomical and biochemical aspects of somatic and zygotic embryos of hybrid larch (*Larix* x *marschlinsii*). Annals of Botany 115:4 605-615. https://doi.org/10.1093/aob/mcu254.

von Aderkas P., Kong L., Prior N.A. (2016) In vitro techniques for conifer embryogenesis In: Park Y.-S., Bonga J.M., Moon H-K. (eds.) Vegetative propagation of forest trees. Seoul: Korea Forest Research Institute. pp 335–350.

von Arnold S., Hakman I. (1988) Regulation of somatic embryo development in *Picea abies* by abscisic acid (ABA). Journal of Plant Physiology 132:2 164-169. https://doi.org/10.1016/S0176-1617(88)80155-X.

White T.L., Adams W.T., Neale D.B. (2007) Forest genetics. Wallingford: CABI Publishing.

Zobel B.J. (1993) Clonal forestry in the eucalypts. In: Ahuja M.R., Libby W.J. (eds) Clonal Forestry II: Conservation and Application. Springer Verlag, Berlin, pp139-148.

99 references