Dissertationes Forestales 360

Tissue culture and cryopreservation in the utilization and conservation of genetic resources of Norway spruce (*Picea abies*) and elms (*Ulmus glabra, U. laevis*)

Sakari Välimäki

Faculty of Biological and Environmental Sciences

University of Helsinki

Academic dissertation

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Author: Sakari Välimäki

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Thesis Supervisors:
Docent Tuija Aronen, D.Sc. (For.) (main supervisor)
Natural Resources Institute Finland (Luke), Savonlinna, Finland

Professor Ari Pekka Mähönen
Faculty of Biological and Environmental Sciences, University of Helsinki, Finland

Pre-examiners:
Professor Jorge Canhoto
Department of Life Sciences, University of Coimbra, Portugal

Professor Scott Merkle
Warnell School of Forestry and Natural Resources, University of Georgia, Athens, Georgia, USA

Opponent:
Professor Elina Oksanen
Department of Environmental and Biological Sciences, University of Eastern Finland, Finland

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ABSTRACT

In this work, tissue culture techniques were developed for two purposes: the production of Norway spruce (*Picea abies* [L.] Karst.) forest regeneration material with somatic embryogenesis (**I**–**III**) and the conservation of genetic resources of European white elm (*Ulmus laevis* Pall.) and wych elm (*Ulmus glabra* Huds.) with cryopreservation and organogenesis (**IV**, **V**).

In *P. abies* somatic embryogenesis, zygotic embryos are used as explants to produce embryogenic tissue, which can be matured into copies of the initial embryo for clonal propagation. In Paper **I** Plantform temporary immersion system bioreactors were tested for *P. abies* somatic embryogenesis. The production of cotyledonary embryos in bioreactors required adding steps to the protocol and the bioreactors were laborious to use. In Paper **II** growing embryogenic tissue in suspension culture was evaluated. Suspension culture is more scalable than propagation on a semi-solid media. However, it required rinsing the tissue before maturation and it could benefit from optimizing the media composition. Higher hydrogen peroxide content was observed in embryogenic tissue grown in suspension than on semi-solid media, but no conclusive evidence of higher oxidative stress in suspension culture was found. In Paper **III**, filter disc cultures grew faster than clumps on semi-solid media and produced slightly more embryos. The storage compound profile of somatic embryos was most similar to the zygotic embryos at 4–8 weeks of cold storage.

In organogenesis, plant organs and new plants are clonally propagated from meristematic tissues or through a callus phase. In Paper **IV**, a micropropagation protocol was developed to regenerate *U. laevis* and *U. glabra* dormant buds from cryostorage. Both elm species are endangered in Finland and globally threatened by Dutch elm disease. The main challenges were surface sterilization and poor regeneration of *U. glabra* from cryostorage. In Paper **V**, dehydration was successfully implemented to improve the regeneration rate.

Keywords: somatic embryogenesis, organogenesis, embryogenic tissue, dormant buds, vegetative propagation, forest biotechnology

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STATEMENT OF AUTHOR'S CONTRIBUTION

Paper I: LP had the main responsibility for designing and carrying out the experiments, in participation with MT, FS, SaiV and TA. SakV had the main responsibility for the data analysis and writing the manuscript. MT participated in statistical analysis. LP, MT, FS, SaiV and TA participated in writing the manuscript.

Paper II: As stated in the contribution statement of the article: "SakV had the main responsibility for designing and conducting the experiments, analyzing the data, and writing the manuscript. TH-P and ER planned and conducted the indicators of stress condition experiments and participated in writing the manuscript. MT, SaiV, and TA participated in planning the experiments, data analysis, and writing the manuscript. All the authors have read and agreed to the published version of the manuscript."

Paper III: As stated in the contribution statement of the article: "SakV had the main responsibility for designing and conducting the filter disc experiments and analyzing their results. MT, SaiV and TA participated in designing the experiments and data analysis. CT, MT, and TA planned, and CT, AD, and NB carried out, the storage compound analysis. SakV and CT did the interpretation of the storage compound data. SakV and CT wrote the first draft of the manuscript, and MT, SaiV, M-AL-W, and TA participated in writing the manuscript. All the authors have read and agreed to the published version of the manuscript."

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ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	Abscisic acid
ANOVA	Analysis of variance
BA	6-benzylaminopurine
DED	Dutch elm disease
DKW	Juglans plant media
EDTA	Ethylenediaminetetraacetic acid
ET	Embryogenic tissue
FW	Fresh weight
GA	Gibberellin A
H_2O_2	Hydrogen peroxide
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
LED	Light-emitting diode
mLM	Modified Litvay's medium
Mt	Meta-topolin
MS	Murashige and Skoog plant medium
NAA	1-naphthaleneacetic acid
NaDCC	Sodium dichloroisocyanurate
PEG	Polyethylene glycol
PEM	Proembryogenic mass
PGR	Plant growth regulator
POX	Guaiacol peroxidase
PVP	Polyvinylpyrrolidone
RFO	Raffinose family oligosaccharides
SE	Somatic embryogenesis
TCA	Trichloroacetic acid
TDZ	Thidiazuron
TIS	Temporary immersion system (bioreactor)
WPM	Woody plant medium

INTRODUCTION

The Finnish forests

Forestry areas encompass 86% of Finland's total land area (Korhonen et al. 2021). After the Second World War, the raw materials harvested from forests played a crucial role in both kickstarting the country's economy and aiding its reconstruction. Today, over 90% of Finland's forested lands are under active management, with protected areas being predominantly concentrated in northern Finland (Korhonen et al. 2021). The majority of commercially utilized forests are artificially regenerated, with 65% of forest regeneration carried out through planting (Sikström et al. 2020). In Finland, ensuring the forest regeneration after the felling of a stand is mandatory. Given the growing need for bio-based raw materials, enhancing the yield of commercially utilized forests in their most optimal growing zones could free forest areas for other uses, such as the conservation of biodiversity. The proposed EU regulation on nature restoration (COM/2022/304) that aims to mitigate biodiversity loss, could decrease the amount of land area available for forestry use. Other competing forms of land use include constructing energy infrastructure, housing, and recreational use. In the face of reduced area available for forestry, it is important to utilize it as efficiently as possible. This can be achieved by using high quality bred forest regeneration material.

Tree breeding of the commercially important species is carried out as a statutory task by Natural Resources Institute Finland (Luke) as a governmental institution. The breeding of forest trees in the northern hemisphere is an extremely slow process, with generations spanning decades and the desired traits showing years after planting. However, the current 1.5-generation Norway spruce (*Picea abies* (L.) Karst.) seed orchards produce seed with a projected increase of 36.9% in volume growth compared to unimproved trees (Haapanen 2020).

Finnish forests are dominated by two conifers, Scots pine (*Pinus sylvestris* L.) and *P. abies*. *P. sylvestris* is the dominant species in 64% and *P. abies* in 26% of Finnish forests (Korhonen et al. 2021). The remainder are dominated by deciduous trees, mainly birches (*Betula pendula* Roth and *Betula pubescens* Ehrh.). Altogether, Finland has around 30 native tree species. In addition to the most common and economically important species, rare tree species play an important role in Finnish forests as they provide unique ecological niches and habitats for many different species. The endangered tree species in Finland include the elms native to Finland, European white elm (*Ulmus laevis* Pall.) and wych elm (*Ulmus glabra* Huds.).

Picea abies (L.) Karst.

Norway spruce is a large gymnosperm species that is native to northern and central Europe, including Finland. It is a coniferous evergreen tree of the family Pinaceae (Caudullo et al. 2016). *P. abies* reaches reproductive maturity at 20–30 years. *P. abies* is a monoecious tree with separate megastrobili and microstrobili and the pollen is spread long distances by the wind. The seed production is irregular and good seed years are often followed by large amounts of seed pests (Nikkanen and Ruotsalainen 2000). *P. abies* is a highly commercially important species in Finland for construction, pulp, and paper production.

In 2022, over 113 million *P. abies* seedlings were domestically produced for forest regeneration, of which over 91% were bred seed (categories “tested” or “qualified”) (Finnish Food Authority 2023). Out of the planted *P. abies* seedlings, 6.8% were of foreign origin (Finnish Food Authority 2023). The cultivation of spruce has risks, including susceptibility to fungi of the species complex *Heterobasidion annosum s.l.* that cause root rot. The warming climate is also disadvantageous for planting spruce, due to increased droughts, storms, and pest, like bark beetle, breakouts. However, they are less affected by browsing of moose and other deer species (Ruotsalainen et al. 2022).

Ulmus laevis (Pall.) and Ulmus glabra (Huds.)

The elm species native to Finland are European white elm (*Ulmus laevis*) and wych elm (*Ulmus glabra*) Elms are mid-sized monoecious hermaphrodite deciduous tree species, with wind-pollinated flowers (Caudullo and De Rigo 2016). The distribution of *U. laevis* is limited to rare riparian forests in southern Finland, whereas *U. glabra* grows also in more northern latitudes. Both species are endangered and protected in Finland, where they are found at the northernmost edge of their distribution. For *U. laevis*, there is reduced genetic variation and high differentiation in the marginal populations in Finland (Vakkari et al. 2009). The genetic diversity of the native elms in Finland is conserved in ex situ field collections, which have grafted trees from different populations, allowing the production of more diverse seed.

Globally, both species have been severely affected by Dutch elm disease (DED), which is caused by fungi (*Ophiostoma ulmi*) and (*Ophiostoma novo-ulmi*) and is spread by bark beetles of the genus *Scolytus* (Brasier 1991). Both *U. glabra* and *U. laevis* are affected by DED, but *U. laevis* is generally considered less attractive for the bark beetles (Collin et al. 2020). Finland currently remains free of DED, but that can well change in the future due to climate change causing the environment to become more suitable for the bark beetle (Hannunen and Marinova-Todorova 2016).

Plant tissue culture

Plants can adapt to environmental changes through developmental plasticity. Plant cells can acquire totipotency, allowing the generation of new genetically identical individuals from somatic cells without sexual reproduction, i.e., vegetatively. This inherent totipotency is the basis for tissue culture techniques. Plant tissue culture is a form of vegetative propagation and defined as the in vitro culture of cells, tissues, and organs under aseptic conditions (Thorpe 2007). The expression of totipotency generally requires external stimuli in the form of plant growth regulators (PGRs) or stressors. The material introduced to in vitro needs to be sterilized to avoid fungi and bacteria taking over the culture. Furthermore, the cultures need to be subcultured regularly, managed under aseptic conditions, and grown with sterilized media. The tissue-cultured plants are generally not fully autotrophic and require in addition to micro- and macronutrients (salts) and a carbon source in the media. If semisolid media is used, it needs to be solidified with a gelling agent like agar or gellan gum. The external culture conditions such as temperature, light intensity, day and night cycle, and subculture frequency need to be optimized for the propagated plant material.

Somatic embryogenesis in conifers

The existence of somatic embryogenesis (SE) is a powerful expression of plant cell totipotency. It represents a complete rejuvenation of the individual plant, where somatic cells transform into embryos with no vascular connection to the original plant (von Arnold et al. 2002; Correia et al. 2016; Su et al. 2021). In SE, a genetic program is launched in somatic cells, which leads them down an alternative developmental path toward polar somatic embryos (Smertenko and Bozhkov 2014; Vondráková et al. 2016). SE can occur through either direct or indirect pathways. In indirect embryogenesis, the process involves an unorganized callus phase, whereas, in direct embryogenesis, somatic embryos develop directly from the explant tissue (von Arnold et al., 2002; Smertenko and Bozhkov, 2014). However, in conifer SE the embryos do not form directly from the explant, the emerging tissue is already either embryogenic or not (von Arnold et al., 2002).

SE is the most successful tissue culture method for conifers, and the first conifer SE protocols were published in the 1980s for *P. abies* (Chalupa 1985; Hakman et al. 1985). Since then, protocols have been developed for many other conifer species, most of them in the family Pinaceae but also for a few species in Cupressaceae, Taxaceae, Cephalotaxaceae, and Araucariaceae (Klimaszewska et al. 2016). Plant regeneration through SE has five steps: initiation, proliferation, prematuration, maturation, and germination (von Arnold et al. 2002). The efficacy of each subsequent step is influenced by the preceding ones (Klimaszewska and Cyr 2002).

In addition, SE closely resembles zygotic embryogenesis and can be used as a model system to study it (Larsson et al. 2008; Hakman et al. 2009). Most information on plant embryo development is derived from angiosperm species, like *Arabidopsis*, and there are differences between angiosperm and gymnosperm embryo development. There are also differences between zygotic and somatic embryogenesis, although, after the initial steps of the maturation, SE follows a pattern very similar to zygotic embryogenesis. In contrast to zygotic embryos, somatic embryos can develop from a group of cells instead of a single fertilized egg (Smertenko and Bozhkov 2014). The nutrients to the developing embryo are provided by the media instead of a megagametophyte, and exogenous PGRs are used to direct the process. Unlike zygotic embryos, somatic embryos do not necessarily require desiccation (Smertenko and Bozhkov 2014), although it improves their further viability (Eliášová et al. 2022).

Culture initiation and proliferation

For conifers, SE is predominantly induced with either immature or mature seed embryos as explants (Hakman et al. 1985; Klimaszewska and Cyr 2002; Stasolla and Yeung 2003). Immature embryos generally have higher success rates than mature embryos (Park et al. 1993; Bonga 2016). However, using immature embryos has the drawback of a narrow initiation window each year (Salaj et al. 2019), which is particularly limiting for species with irregular seed production like *P. abies*. In the case of *P. abies*, optimal cone collection for explants occurs around 800 degree days of temperature sum (Varis et al. 2023).

Examples of SE initiation from other types of explants than seed embryos have been reported, for example with white spruce (*Picea glauca* (Moench) Voss) and *P. abies* (Klimaszewska et al. 2011; Varis et al. 2018). However, in most cases, the initiation frequency remains low, and the success is highly dependent on the genotype (Klimaszewska et al. 2011; Rutledge et al. 2017; Varis et al. 2018). Using adult trees with proven

characteristics as explant donors would be most efficient for capturing genetic gain (Klimaszewska and Cyr 2002). However, practical applications of conifer SE are currently limited to cell lines initiated from zygotic embryos.

The initiations are carried out aseptically on artificial media with salts, sugar, vitamins, and a solidifying agent, e.g., agar or gellan gum. Exogenous auxin, typically in combination with cytokinin, is often used to induce SE in the explant (Vondráková et al. 2011; Garcia et al. 2019), although there are some exceptions like *Juniperus communis* L. SE initiation for which exogenous auxin is not necessary (Helmersson and von Arnold 2009). The auxin most often used to induce SE for conifers is 2,4-D (Fehér 2008; Vondráková et al. 2011; Garcia et al. 2019), but alternative auxins like 1-naphthaleneacetic acid (NAA) or picloram can also be used (Hazubska-Przybył et al. 2020). 2,4-D, a potent auxin, is also used as an herbicide due to its ability to cause abnormal growth and subsequent death of susceptible plants (Song 2014). The application of exogenous auxin increases the endogenous natural auxin indole-3-acetic acid (IAA) (Vondráková et al. 2011). An increase in endogenous IAA is considered a key factor in triggering the embryogenic response (Wójcik et al. 2020). A high concentration of exogenous auxin in SE induction is also thought to act as a stressor, which promotes SE (Fehér 2008; Karami and Saidi 2010). Other stress factors like lowered water availability or heavy metals can also induce SE (Zavattieri et al. 2010).

Cytokinins are involved in many aspects of growth and dedifferentiation in plant cells (Asghar et al. 2023). Exogenous cytokinins are widely applied in SE protocols to induce SE and promote culture proliferation. Cytokinins are adenine derivatives with either aromatic or isoprenoid side chains (Vondráková et al. 2016). Additionally, there are synthetic compounds derived from phenylurea with cytokinin-like action, like thidiazuron (TDZ). The cytokinin most used with the auxin of choice to promote conifer SE is a synthetic aromatic cytokinin 6-benzylaminopurine (BA) (Vondráková et al. 2016). However, other cytokinins are used in protocols as well, like zeatin and isopentenyl adenine (Hakman and von Arnold 1985).

In the presence of auxin and cytokinin, the embryogenic cultures proliferate as proembryogenic masses (PEM). PEMs consist of two cell types, small meristematic cells with dense cytoplasm and elongated and highly vacuolated cells, which develop into suspensors (Stasolla and Yeung 2003; Larsson et al. 2008). Suspensors support the growth and the establishment of polarity of the embryo. Eventually, the meristematic cells continue development into mature somatic embryos, and the suspensor cells are eliminated through programmed cell death (Filonova et al. 2000b). According to Filonova et al. (2000a), these aggregates can be divided into three stages from PEM I to III based on their size and morphology. Under the effect of auxin and cytokinin, PEMs III can develop into early somatic embryos but they disperse back into PEMs I (Filonova et al. 2000a; Larsson et al. 2008). Early somatic embryos can form from PEMs III and they are responsive to maturation stimuli by abscisic acid (ABA). The embryogenic cultures are heterogeneous, and all stages of PEM are present in a proliferating culture in varying proportions (Klimaszewska et al. 2016). To transition the cultures from proliferation to formation of early somatic embryos, prematuration on PGR-free media is sometimes included in protocols before transfer to maturation media containing ABA.

The proliferation of embryogenic masses exhibits flexibility, offering various options for cultivation. *P. abies* ET can be propagated as clumps placed on semisolid media. During subculture, the fresh growth of the clumps is picked using forceps and placed as clumps on new media. The clumps contain PEMs, early somatic embryos, nonembryogenic callus cells, and dead cells (Klimaszewska et al. 2016). The dead cells are predominantly located inside the clumps and the newly formed embryonic growth is on the periphery. Filter disc culture

can also be applied in the proliferation stage for a faster proliferation rate (Lelu-Walter et al. 2006; Aronen et al. 2009). With *P. sylvestris*, the utilization of filter disc culture results in fewer embryos but of higher quality in comparison to culturing as clumps (Aronen et al. 2009). For stone pine (*Pinus pinea* L.), filter disc cultures grow faster than small clumps, which in turn grow faster than large clumps (Carneros et al. 2009).

Liquid media is generally thought to be better for the growth of embryogenic tissue (ET) than semisolid media (Boulay et al. 1988; von Arnold et al. 2002; Denchev and Grossnickle 2019). Nutrient gradients can form in semisolid media due to uptake and enzymatic modification of nutrients by ET (Kubeš et al. 2014), whereas in suspension the nutrients are dispersed more evenly. This leads to better and more even access to media components, but also compounds exuded by the ET. Better access to the media might also lead to some substances being present in excess if the same media formula is used for liquid as for semisolid media. In addition, suspension cultures need to be grown under agitation to maintain gas exchange, and this can lead to shear stress which hinders embryo development (Sun et al. 2010, 2011).

Bioreactors are defined as systems that are developed to facilitate the growth of organisms, tissues, or cells (Valdiani et al. 2019). Bioreactors have large variation in size and the model of operation depending on their purpose. The different types of bioreactors used for plant tissue cultures include stirred tank, rocker, airlift, and temporary immersion system (TIS) bioreactors (Mamun et al. 2015). Bioreactors can be used to facilitate and automate the normally labor-intensive plant tissue culture protocols.

TIS bioreactors are commercially used for the SE of coffee (*Coffea arabica* and *Coffea canephora*) (Etienne et al. 2018). The use of TIS bioreactors has been proposed to enhance and scale up SE of conifer species (Welander et al. 2014; Egertsdotter et al. 2019). In TIS bioreactors, the access of the plant material to the culture medium is regulated by raising the medium to the tissue periodically. The irrigation intervals can be optimized for the plant material in question, and with some TIS bioreactor models, aeration can be provided without shaking through ventilation, reducing the harmful shear stress. In principle, in comparison to suspension cultures, TIS bioreactors provide the benefits of liquid media with the solid support that is necessary for the maturation process.

Somatic embryo development and maturation

The maturation of PEMs into somatic embryos is triggered by transferring the tissues onto media that has no auxin or cytokinin. Maturation is often carried out in two stages, prematuration on hormone-free media and maturation on media supplemented with ABA (Dunstan et al. 1993; Bozhkov et al. 2002). Prematuration is applied to increase the number of ABA-responsive PEMs III and early somatic embryos and to synchronize the development of the cultures (Filonova et al. 2000a, b; Högberg et al. 2001). However, *P. abies* SE can be efficiently carried out without applying prematuration and by directly transferring the proliferating cultures onto media with ABA (Tikkinen et al. 2018a). Reduced water availability in the form of higher concentration of cell membrane penetrating (e.g. sucrose) or non-cell membrane penetrating (e.g. polyethylene glycol [PEG]) agents or increased gel strength facilitate maturation (Attree et al. 1991; Tikkinen et al. 2018a).

The removal or gradual depletion of cytokinin and auxin stimulates the formation of early somatic embryos with distinct morphology from PEMs III and the elimination of PEMs through programmed cell death (Filonova et al. 2000b). The second round of programmed cell death is the elimination of suspensor cells and it takes place during early embryogeny

(Filonova et al. 2000b). As demonstrated by studies carried out using auxin transport inhibitors, the formation of endogenous auxin gradient is important for the formation of basal-apical polarity and the development of embryos (Larsson et al. 2008; Hakman et al. 2009). The accumulation of 2,4-D into the embryos during proliferation can disrupt the endogenous auxin gradient formation and cause morphological aberrations (Garcia et al. 2019; Asghar et al. 2023). However, the effect of 2,4-D during proliferation on maturation is not solely negative, for example it has been reported to increase embryo yield in European silver fir (*Abies alba* Mill.) (Vondráková et al. 2011).

For zygotic embryos, ABA is produced by the seed coat (Senaratna et al. 1995; Hoekstra et al. 2001), which is obviously not there for somatic embryos. The application of exogenous ABA is generally required for continuous maturation, and it promotes the development of desiccation tolerance and accumulation of storage compounds (Hazubska-Przybył et al. 2016). Optimizing the ABA concentration in the media can improve the maturation results (Tikkinen et al. 2018a). ABA and the high osmolarity of the maturation media also prevent detrimental precocious germination during maturation (Stasolla et al. 2002; Hazubska-Przybył et al. 2016).

For maturation, the tissue can be spread on a filter disc for more uniform exposure to the maturation media. However, it is also possible to mature ET as clumps that are in direct contact with the media. The media is often semisolid, but bioreactors can be used to propagate somatic embryos as they can provide mechanical support which promotes the formation of apical-basal polarity in conifer somatic embryos (Egertsdotter et al. 2019). The maturation of somatic embryos is routinely carried out in suspension for some species like carrot (*Daucus carota* L.) (de Vries et al. 1988). There are reports on successful maturation in suspension for *P. abies* and *P. glauca*, but only with a few genotypes (Hakman and von Arnold 1985; Gorbatenko and Hakman 2001).

The germination of somatic embryos

After the maturation period, the embryos can be germinated, but post-maturation treatments are often applied to improve their further survival. The post-maturation treatments promote the acquisition of desiccation tolerance which is necessary for the ex vitro survival of the embryos. The post-maturation treatments described are either partial desiccation which is done in high humidity, or cold storage of embryos on their original maturation media (Högberg et al. 2001; Konrádová et al. 2003; Tikkinen et al. 2018b). Cold storage also brings flexibility to the process as the embryos do not need to be germinated immediately after the maturation period, allowing their year-round production (Varis et al. 2017).

The germination of somatic embryos is carried out on hormone-free media under lights in contrast to the earlier stages of the process that take place in darkness (Tikkinen et al. 2018b). The germination process utilizes the storage reserves of the embryo and the nutrients from the media. Using a germination period as short as possible makes the transplantation to the nursery easier as the roots are easier to handle if they are not too long. In germination, the embryos need to be handled individually, which makes it the most expensive part of the process (Park et al. 2016; Tikkinen 2018).

Organogenesis and axillary budding in elm species

In organogenesis, plant organs like shoots and roots are formed from explants from a donor plant. Whole individual plants can be propagated through manipulation of the culture

conditions and media composition. In indirect de novo organogenesis, new meristems are formed from less organized callus tissue that develops into adventitious buds and shoots (Monteuuis 2016). In axillary budding, the pre-existing axillary meristems are stimulated to activate and form shoots, which can be excised and grown into plants. Axillary budding is often stimulated by excising the connection to the apical meristem to break its dominance. With axillary budding, there is a lower chance of somaclonal variation and a better subsequent conversion rate than with de novo organogenesis (Monteuuis 2016).

With elm species the efforts to develop micropropagation methods have been related to conservation (Harvengt et al. 2004), or propagation of either DED-resistant old trees or hybrid cultivars (Biroščíková et al. 2004; Thakur and Karnosky 2007). Research has also been done on elm transformation to potentially produce more resistant genotypes (Gartland et al. 2000). The initiation in organogenesis is the introduction of the plant material into in vitro conditions. Shoots (Corchete et al. 1993; Fenning et al. 1993; Cheng and Shi 1995; Anna et al. 1997; Thakur and Karnosky 2007; Shukla et al. 2012), leaves (Kapaun and Cheng 1997) seeds (Beck et al. 2018) and buds (Paques et al. 1997; Biroščíková et al. 2004; Diez and Gil 2004; Harvengt et al. 2004; Malá et al. 2007; Conde et al. 2008) have been used as explants for elm tissue culture initiation. The first and critical step is the surface sterilization of the introduced material. While numerous chemicals and protocols are available, achieving surface sterilization requires striking a balance between sterilization efficacy and preserving explant viability during the treatment, as sterilizing agents are often toxic to the explant as well. Explants like shoots and seeds are generally easier to sterilize than dormant buds, especially those that have been collected from the field instead of a greenhouse (Tanner et al. 2020a).

For introduction to in vitro culture, meristems are excised from the donor plant and placed on media. Alternatively, for de novo organogenesis many types of explants can be used, e.g. shoot formation from English elm (*Ulmus procera* Salisb.) leaves (Fenning et al. 1993). When dormant buds are used, only the meristem with possibly some leaf primordia is transferred to the media (Harvengt et al. 2004; Malá et al. 2007). Especially for explants collected from outdoors, it is beneficial to keep the explants initially in individual culture vessels to avoid losing multiple explants to endogenous contaminations. The most common basal media used are Murashige and Skoog (MS) (Murashige and Skoog 1962; Harvengt et al. 2004; Malá et al. 2007) and Driver and Kuniyuki walnut medium (DKW) (Driver and Kuniyuki 1984; Fenning et al. 1993), although woody plant medium (WPM) has also been used (Lloyd and McCown 1980; Thakur and Karnosky 2007). In many cases, tissue culture laboratories make their own modifications to the basal media.

The propagation media most often uses BA as the cytokinin to promote explant growth (Harvengt et al. 2004; Malá et al. 2007). However, meta-topolin (mT) has been proposed as an alternative cytokinin for *U. glabra* (Malá et al. 2013). BA can have negative effects on the long-term subculture and the rooting capacity of the cultures (Werbrouck et al. 1996), and alternative cytokinins like mT can give better multiplication rates long-term (Malá et al. 2013). Synthetic cytokinins like BA tend to accumulate in tissue more as their degradation or inactivation through conjugation is less efficient (Werbrouck et al. 1996). The culture media can also be supplemented with auxin, usually indole-3-butyric acid (IBA) (Fenning et al. 1993; Malá et al. 2007). However, successful propagation has been achieved also using BA as the sole PGR during propagation (Harvengt et al. 2004). Additionally, media containing BA, gibberellin A3 (GA_3), and antiauxin has been reported to be most suitable for American elm (*Ulmus americana* L.) micropropagation (Shukla et al. 2012).

The established cultures are subcultured regularly to support multiplication. Subculturing can be carried out in a number of ways, by transferring individual nodal segments (Anna et al. 1997) or cutting the shoot into the apical and basal parts and transferring both to new media (Malá et al. 2007). Shoots can also develop from basal callus, which can elongate after transferring the larger shoots (Fenning et al. 1993). Adventitious shoots are often not used for subculture, as they can have somaclonal variation and poorer true-to-type plant conversion rate than shoots originating from axillary meristems (Monteuuis 2016). On the other hand, more shoots can often be produced de novo, than with axillary budding. In most protocols, the multiplication media is the same as initiation media, as in many protocols either shoots are used as explants, or the shoot elongation is forced already ex vitro. The elm in vitro cultures are maintained under relatively low light at 30–80 µmol m^{-2} s^{-1} at around +25 °C temperature (Fenning et al. 1993; Malá et al. 2007).

Rooting of the elm shoots is done using separate rooting media, from which cytokinin is omitted and auxin is either added or its concentration is increased. Auxins most often used in rooting are IBA (Harvengt et al. 2004; Malá et al. 2007) and NAA (Fenning et al. 1993). However, based on results by Conde et al. (2008) auxin is not necessary for elm rooting but improves it. Rooting of elms without separate rooting media has also been reported, using induction in high auxin concentration prior to transplantation (Conde et al. 2008). Fenning and coworkers (1993) also reported that with 1-year-old cultures NAA or IBA treatment did not markedly improve rooting success further, but auxin treatment was necessary for shoots from younger 5-month-old cultures. Also, according to Conde et al. (2008), elm material that has been propagated in vitro longer is more successfully transplanted. The response to rooting treatment with exogenous auxin is genotype-dependent and could be related to the efficiency of conversion of IBA to IAA (Kreiser et al. 2016).

Cryopreservation of plant material

Cryopreservation is storing living material in ultralow temperatures, where biochemical activity is minimal or halted, usually in liquid nitrogen or liquid nitrogen vapor (Engelmann 2004). This enables space-efficient long-term storage of valuable plant material (Forsline et al. 1998). Many different plant cryopreservation techniques for in vitro and ex vitro plant material have been developed since 1960 when the first successful cryopreservation and regeneration of poplar and willow twigs was reported (Sakai 1960). During freezing, most of the damage to the cells is caused by the formation of ice crystals that disrupt and break the cell structure. The goal of different cryopreservation techniques is to keep the cells alive during freezing by reducing the formation of intracellular ice crystals.

In classical cryopreservation methods, cryodamage is mainly avoided by reducing the intracellular water through slow cooling. When successfully applied, controlled rate slow cooling causes the extracellular ice to form first, as the plasma membrane functions as a physical barrier preventing the nucleation of ice within the cell (Engelmann 2004). During the cooling, the water gradient between the supercooled liquid cytoplasm and the freezing water outside the cell increases, and the cell loses water and becomes dehydrated (Benson 2008). In many cases, the material needs to be pregrown or cryopreserved together with cryoprotectants. Cryoprotectants are solutes that improve post-thaw recovery of the plant material (Elliot et al. 2017). For cold-acclimated ex vitro material such as dormant buds, slow cooling alone can be sufficient to reach satisfactory sample regeneration rates (Ryynänen 1996; Harvengt et al. 2004).

In more recently developed cryopreservation techniques, dehydration is achieved before freezing and the samples are placed directly into liquid vaporous nitrogen. The main techniques used are vitrification, dehydration, and encapsulation. In vitrification, the desiccation of the plant material is achieved with a highly concentrated vitrification solution, and with dehydration through exposure to laminar airflow or with silica gel (Engelmann 2004). For example, the PVS2 vitrification solution contains glycerol, ethylene glycol, sucrose, and dimethyl sulfoxide (Sakai and Engelmann 2007). The plant material can also be encapsulated in alginate beads, which increases the ability of the explants to withstand more drastic dehydration or vitrification techniques (Gonzalez-Arnao and Engelmann 2006). These methods allow cryopreservation of species with very low or no natural cold tolerance.

The ability to cryopreserve ET is pivotal for the utilization of SE for forestry purposes, as it allows the preservation of the juvenility of the material during the lengthy field testing (Park et al. 2016). In addition, continuous subculture of the lines is laborious, their embryogenic capacity may reduce over time and the likelihood of somaclonal variation can increase (Breton et al. 2006; Klimaszewska et al. 2016).

The embryonal heads of ET are small and uniform cells with few vacuoles, making them more suitable for cryopreservation than differentiated adult tissues (Engelmann 2004). The suspensor cells of *P. abies* ET die during the application of cryoprotectants and the freezing, but the remaining embryonal heads can regenerate after thawing (Vondráková et al. 2010). As a sidenote, cryopreservation could even improve the embryogenic potential of the tissue, as nonembryogenic callus cells are less tolerant of freezing (Bercetche et al. 1990). The ET of *P. abies* can be cryopreserved after pregrowth on media with increasing sucrose concentrations (Varis et al. 2017). During the preculture, water is drawn from the cells osmotically and sucrose accumulates in them, improving cryotolerance (Santos et al. 2003). After the pregrowth, ET is suspended to cryovials in liquid media with high sucrose concentration and cryoprotectants and frozen gradually using a programmable freezer (Varis et al. 2017, 2022). The choice of cryopreserved material is also important as a vigorous growth rate is associated with better cryotolerance (Vondráková et al. 2010).

For the conservation of genetic diversity, dormant buds are a good option as they are easy to cryopreserve without extensive pretreatments allowing the storage of large numbers of genotypes. In cryostorage, the material is safe from pests and pathogens, including the DED which threatens field collections. Efforts in Europe have led to the collection of dormant buds from 444 elm clones representing *U. laevis*, *U. glabra*, *U. minor* (Mill.), and their hybrids, from nine different countries, establishing a cryocollection located in France and Germany to preserve the genetic diversity of elms (Harvengt et al. 2004). Most dormant bud protocols have been developed for valuable fruit trees as a backup for field collections and for forest trees there are fewer protocols.

Slow cooling can be sufficient for the buds of most cold-hardy species, but many species require dehydration before freezing (Tanner et al. 2021). The dehydration is carried out in a cold room to preserve natural cold acclimatization and the moisture content needs to be monitored throughout the drying. The freezing is carried out with slow cooling often without the addition of cryoprotectants. The regeneration of dormant buds after thawing is often done by grafting the buds directly to a stock plant or forcing the budding of the twigs before transferring the shoots to in vitro where they can be multiplied (Forsline et al. 1998; Tanner et al. 2020b). The material can also be directly regenerated in vitro (Ryynänen 1996; Harvengt et al. 2004), which may allow cryopreservation of smaller explants and multiplication of the plant material through micropropagation.

OBJECTIVES

The research hypothesis in the present work were the following:

1. The proliferation method (tissue clumps or tissue suspended on filter disk on semisolid medium, suspension culture, or TIS bioreactor) affects not only the SE proliferation rate, but also the later phases of SE (embryo maturation, germination, and embling performance).

   Research questions:
   - Are Plantform TIS bioreactors more suitable than semisolid media on plates for *P. abies* SE in terms of embryo yield, greenhouse survival, and reduction of manual labor?
   - Is ET proliferation in suspension culture more efficient than on semisolid plates and does it have adverse effects on embryo quality?
   - Does spreading the ET on filter discs on semisolid media improve proliferation rate and embryo quality?

2. The methodology used during SE propagation affects biochemistry of SE cultures.

   Research questions:
   - Are suspension cultures more conducive to oxidative stress than cultures on semisolid media?
   - Are there differences in storage compound profile of the somatic embryos and zygotic embryos?
   - How does cold storage of somatic embryos affect their storage compound profile, and based on that what is the optimal cold storage time?

3. Dormant elm buds cryopreserved using slow cooling are regenerable using organogenesis.

   Research questions:
   - Can cryopreservation be utilized in the conservation of elm genetic resources, i.e., can a high enough proportion of genotypes be recovered from cryostorage?
   - Does dehydration of *U. glabra* buds improve the regeneration success after thawing?

The aim of this work was to develop the tissue culture methods for *P. abies* forest regeneration material production, and tissue culture and cryopreservation methods for the conservation of elm (*U. laevis*, *U. glabra*) genetic resources. Different *P. abies* ET proliferation and maturation methods were evaluated to improve embryo yield and quality, the production efficacy and to reduce costs and manual labor. With elm cryopreservation experiments, the objective was to enable the use of cryopreservation in the conservation of *U. laevis* and *U. glabra* genetic resources. The elms could be regenerated from the cryocollection when required to re-establish lost trees into the outdoor ex situ gene reserve collections.

MATERIALS AND METHODS

Evaluation of different *P. abies* SE proliferation and maturation methods (I–III)

Different proliferation and maturation methods for *P. abies* SE were evaluated in Papers I-III. The control treatment for these experiments was the method currently in use, where ET is grown as clumps on semisolid media. The current method was compared to ET spread on filter paper discs on semisolid media (III), and culture in liquid media suspensions (II). ET growth, cotyledonary embryo yield, and survival in the nursery were considered (I-III). ET growth was monitored by weighing the ET before and after the culture period, and by dividing the FW after the culture period with the initial FW (II-III). The embryo yield was assessed by counting the cotyledonary embryos that develop from ET proportional to the amount of ET put into maturation (II-III), or into bioreactor as an inoculum (I). The plant quality was assessed by inventorying the survival of the transplanted plants in a greenhouse (I-III). For some suspension culture experiments, embryo yield per plate was counted as ET suspension was dispensed into maturation making weighing it impossible (II). ET growth was studied in commercially available Plantform temporary immersion system (TIS) bioreactors (I). The suitability of bioreactors for spruce SE was assessed based on the ease of handling of the bioreactors, cotyledonary embryo yield, and greenhouse survival of the resulting plants. Additional treatments, i.e., prematuration on semisolid media (III) and post-maturation dehydration treatments for embryos produced in bioreactors were evaluated (I).

Plant material

Altogether 48 SE lines derived from seeds obtained from the crossings of elite trees in the Finnish tree breeding programme were utilized in the experiments. The *P. abies* SE lines were initiated according to Klimaszewska et al. (2001) and Varis (2018). Most of these lines were initiated in 2014 and cryopreserved and thawed according to Varis et al. (2017) (I-III). However, the SE lines initiated in 2019 were used in suspension culture experiments without undergoing cryopreservation (II). In Papers I-III, cell lines were initially maintained as clumps on semisolid Litvay's modified media (mLM) (Litvay et al. 1985; Varis 2018). Regular subculturing by selecting newly grown tissue was carried out every two weeks until the lines were used in the experiments. The media were solidified with 4 g l^{-1} gellan gum (Phytagel) supplemented with 1% (w/v) sucrose, and the PGRs were 10 µM 2,4-D and 5 µM BA. The cultures were maintained at a temperature of +23 °C (±2 °C) in darkness.

Control treatments

For proliferation experiments (II, III), ET cultures grown on semisolid media as clumps were used as controls. Control maturations were done according to Tikkinen et al. (2018a) by suspending 140–200 mg of ET into 3 ml of hormone-free liquid maturation media and pouring the suspension onto a filter paper in a Büchner funnel (I-III). The filter paper was dried with a low-pressure pulse and placed on a semi-solid maturation medium with 60 g/l sucrose, 30 µM ABA, and solidified with 6 g l^{-1} Phytagel. Embryo yield was evaluated by counting the good quality cotyledonary embryos (straight and intact stem, at least four

cotyledons), either from all plates or a sample of plates after 7–8 weeks of maturation, after which the plates were moved into the dark cold storage room (+2 °C).

TIS bioreactors (I)

TIS bioreactors (Plantform) (Welander et al. 2014) were assessed for their suitability for *P. abies* SE (I). ET (1,200 mg) was placed into the bioreactor and nourished with liquid mLM proliferation media (260 ml) for one week (same composition as semisolid media but without Phytagel). Based on the protocol by Egertsdotter and Clapham (2015), the media was then changed to a prematuration medium containing PEG 4,000 and a higher concentration of sucrose (30 g/l). After a week, the prematuration media was changed into maturation media containing 24 µM of racemic (±)-ABA, which was refreshed after two weeks. Post-maturation media with the same composition as maturation media, except for additional myo-inositol (45 g l^{-1}), was used for the final three weeks of culture.

Plastic netting or polyurethane foam with filter paper, and metal netting were tested for support pad materials. Aquarium pumps were used to provide filtered airflow for feeding and aeration cycles. Different aeration cycles were assessed: 20 min every 4 h, 2 h every 4 h, first 20 min/4 h, and then increased to 2 h /4 h from the second week, or constant aeration. The separation of the embryos from the cell mass by rinsing with autoclaved distilled water was tested for bioreactor embryos.

Post-maturation desiccation treatments for embryos placed in nested plates with or without a moistened filter paper and cold storage inside bioreactors were tested to improve the quality and greenhouse survival of bioreactor embryos. The desiccation treatments were carried out in a cold room (+2 °C) for five weeks.

Suspension culture (II)

Suspension cultures (II) were established by collecting 900–1,200 mg of ET (FW) from plates into either 500 ml or 250 ml laboratory bottles with 145 ml or 125 ml of liquid mLM media respectively. The cultures were grown in darkness on a shaker (120 rpm) for one week. In the first experiment of Paper II, suspension culture was compared with semisolid culture using the same composition in both media, except suspension media did not have Phytagel. In the second and third experiments, media containing halved (0.5x) PGR concentration was applied. In the fourth experiment, an even lower 0.1x PGR concentration was tested for suspension culture. The ET growth in different treatments was assessed as mFW (end of culture) / mFW (start of culture).

The maturations were done by dispensing 2.5–3 ml of suspension onto a filter paper disc in a Büchner funnel and drying it with a low-pressure pulse. Rinsing the ET with hormone-free liquid maturation medium before plating was tested in the second and third experiments. In the fourth experiment, all of the suspension was first poured from the culture bottle into a Büchner funnel with a filter paper in it and dried with a low vacuum pulse. Instead of pipetting, the maturations were then carried out by weighing individual samples from the dried ET the same way as if from the plate-grown tissue.

Filter disc culture (III)

The proliferation of the ET as clumps and spread on filter discs on semisolid media were compared in three experiments presented in Paper III. The filter disc cultures were carried out according to Lelu-Walter et al. (2006). In the first experiment, ca. 200 mg of fresh ET was picked from the clump cultures and suspended in 5 ml of PGR- and L-glutamine-free liquid proliferation medium. After manual shaking, the suspension was poured onto a filter paper which was dried with a low-pressure pulse, and the paper was placed onto a fresh media. Additionally, a one-week prematuration on hormone-free maturation media was tested in the first experiment. In the second experiment, the amount of ET put onto filter discs was increased to around 300 mg and the necessity of selecting fresh ET both for subculture and maturation was evaluated for both filter disc and clump cultures. The third experiment involved a practical comparison between filter disc and clump proliferation. This comparison was based on the number of subcultures required to achieve 40 maturations from each of the 20 lines in the experiment, as well as the resulting maturation yield.

Germination

All available embryos (I, III) or a sample of up to 27 embryos from each line and treatment (II) were germinated from the experiments. The germination was carried out under photosynthetic photon flux of 130 µmol m^{-2} s^{-1} (Valoya L14 spectrum AP67 Milky LED, Valoya Oy, Helsinki, Finland), (I, II) or under 190–210 µmol m^{-2} s^{-1} (Valoya L35 spectrum AP673L Clear LED) (III) lights with an 18 h/6 h day/night cycle for two weeks (I, II) or one to two weeks (III). In Paper I, embryos were photographed for measuring the embryo dimensions and shoot and root lengths before and after germination. The germination media with all experiments was PGR-free mLM supplemented with 2% (w/v), sucrose, solidified with 6 g l^{-1} Phytagel. In comparison to proliferation and maturation media, inorganic nitrogen was reduced by omitting NH_4NO_3. (Tikkinen et al 2018b).

Transplantation and greenhouse conditions

The embryos from bioreactor experiments (I) were first transplanted into miniplugs in Vivi trays (ViviPak) and cultured in high (over 90%) humidity under laser perforated covers for three weeks in 130 µmol m^{-2} s^{-1} (Valoya L14 spectrum AP67 Milky LED, Valoya Oy, Helsinki, Finland) with an 18 h/6 h day/night cycle. The covers were removed, and the plants were grown for 35 days and irrigated daily by misting with Taimi-Superex fertilizer (0.1% w/v, Kekkilä Oy). After that, they were transplanted into peat in 81F containers (Plantek). From the suspension and filter disc culture experiments, the embryos were directly transplanted after germination into peat in 81F containers in a commercial nursery, with conditions set for growing *P. abies* seedlings.

The survival (dead or alive) was evaluated in bioreactor experiments by observing the plants directly (I), and in suspension and filter disc culture experiments the survival was evaluated from photographs taken 41 d after transplantation (II, III).

Biochemistry of SE

Storage compounds (III)

Mature somatic embryos are cold stored in darkness at +2 °C. To determine the optimal cold storage time, the carbohydrate and protein content over time was determined from somatic embryos and compared with zygotic embryos. Somatic embryos were matured for 7 weeks and snap-frozen in liquid nitrogen after maturation, and after 4, 8, 31, and 61 weeks of cold storage, they were stored in a –80 °C freezer. Zygotic embryos from *P. abies* seeds were also frozen and analyzed as pooled samples. Soluble proteins were extracted from the samples according to Teyssier et al. (2014). Protein content was assessed using the Bradford assay with bovine albumin as a standard (Bradford 1976). The protein profile for different treatments was analyzed with SDS-PAGE. Carbohydrates and starch were analyzed using an ethanol-based method with a high-performance liquid chromatography system according to Gautier et al. (2018). Carbohydrates were quantified using an evaporative light scattering detector and identified by co-elution with standards. Starch content was determined using a glucose assay kit after amyloglucosidase hydrolysis (Gautier et al. 2019).

Oxidative stress (II)

Indicators of oxidative stress, i.e., H_2O_2 content and guaiacol peroxidase (POX) activity in suspension and semisolid cultures were measured from snap-frozen ET samples. In the fourth suspension culture experiment, samples (80–100 mg, FW) were collected after a week of suspension culture or a week from the previous subculture on semisolid media. Three samples from each treatment and line were collected from suspension cultures and two from the control plates. The samples were snap-frozen in liquid nitrogen and stored at –80 °C until shipping with dry ice. To determine the hydrogen peroxide (H_2O_2) content, samples were finely ground in liquid nitrogen, homogenized with 5% trichloroacetic acid (TCA) containing 10 mM ethylenediaminetetraacetic acid (EDTA), and analyzed for H_2O_2 using the Sagisaka method (Sagisaka 1976). A reaction mixture with supernatant, TCA, iron-ammonium sulfate, and potassium thiocyanate was used, with 5% TCA as a control. Absorbance at 480 nm was measured, and results were expressed as nmol g^{-1} FW.

To determine the POX activity, proteins were extracted in 50 mM sodium phosphate buffer (pH 7.0) with EDTA and polyvinylpolypyrrolidone at 4°C. Extracts were centrifuged at 20,000 × g for 20 min at 4°C. POX activity was measured using the Chance and Maehly method (Chance and Maehly 1955). The guaiacol oxidation reaction was performed at 470 nm for 1 min at +20 °C. The reaction mixture contained phosphate buffer, guaiacol, H_2O_2, and enzyme extract, and only the buffer in the control. POX activity was presented as nkat min^{-1} mg^{-1} of protein. Protein content was estimated using the Bradford method with bovine serum albumin as a standard (Bradford 1976).

The development of elm cryopreservation and tissue culture methods (IV, V)

The developed tissue culture and cryopreservation methods are presented in Papers IV and V. In Paper IV, cryopreservation and micropropagation methods were developed and tested for *U. laevis* and *U. glabra*, and in Paper V the cryopreservation method improved for *U. glabra* by including a dehydration treatment before freezing.

Plant material

U. laevis (36 genotypes) and *U. glabra* (13 genotypes) twigs for dormant bud cryopreservation method development were collected between September and March in multiple years from Preitilä and Solböle genetic reserve collections in Southwestern Finland and Punkaharju research forest in Eastern Finland. The buds from the collections were transported to the laboratory as 5–20 cm twigs with cold packs. Buds from the nearby research forest were brought in branches up to 1 m long, and placed in a bucket with snow. Both fresh and cryopreserved buds were used in the experiments. The fresh buds were stored in water at +2 °C in darkness until used in the experiment (1–3 weeks).

Pre-treatments before freezing

For *U. glabra*, a dehydration treatment was tested to improve the survival from liquid nitrogen (V). The twigs were dried on trays in darkness in a cold room (–5 °C) for 19 days to decrease the sample moisture content from an initial 50% to around 30% (Forsline et al. 1998; Tanner et al. 2020a). In all experiments (IV, V), the buds were cut from the twigs in a cold room (+2 °C) and placed into 1.8 ml cryotubes, which were then kept on ice in the same cold room overnight.

Cryopreservation

The buds were cryopreserved using Planer programmable freezer with –0.17 °C min^{-1} until the samples reached –38 °C (Ryynänen 1996). Then the samples were plunged into liquid nitrogen. The thawing was done in a water bath at +38 °C for 2 min and then the samples were kept on ice for 2 min before surface sterilization.

Micropropagation

The cultures were initiated the same way from fresh and cryopreserved buds. Multiple surface sterilization methods were tested to decrease the contamination rate of initiations, including various exposure times and combinations of ethanol, H_2O_2, and sodium dichloroisocyanurate (NaDCC) (Table 1). After surface sterilization, the buds were aseptically prepared by removing the outer bud scales and most of the wood at the stem and placed onto initiation media. Multiple basal media (MS, WPM, DKW) and PGRs (1 mg l^{-1} or 0.5 g l^{-1} BA, 0.1 mg l^{-1} GA_{4+7} + 0.5 mg l^{-1} BA, 0.02 mg l^{-1} TDZ) were evaluated for *U. laevis*. For *U. glabra*, DKW was found to be adequately suitable, and no additional initiation media testing was done. The cultures were grown at +25 °C (± 2°C) in photosynthetic photon flux density around 150 µmol m^{-2} s^{-1}. Since the end of 2017, the cultures were grown under cloths to reduce the light intensity to under 50 µmol m^{-2} s^{-1}.

The initiations were monitored for contaminations and shoot formation. The contamination-free cultures that formed shoots were moved from the culture tubes into jars with 27 ml of media from which GA_{4+7} was omitted. For multiplication, no additional media testing was done, except 0,5 mg l^{-1} mT was tested as an alternative for BA in the shoot multiplication of *U. glabra*. For subculture, the cultures were transferred onto new media at 2–3 weeks intervals. The subculture was carried out by transferring shoots with or without callus, nodal segments, or shoots from which the apex was excised.

For rooting the shoots, methods utilizing semisolid media and liquid were tested. Induction in 3 mg l^{-1} IBA and longer exposure to 0.5 mg l^{-1} IBA were compared with hormone-free media. In 2020, induction in 5 mg l^{-1} IBA solution was tested (3 d, in darkness). After the induction, the shoots were directly transplanted into peat in 81f Plantek containers and grown in a commercial greenhouse, in germination conditions for *P. abies*.

Table **1** Information on the different experiments for regeneration of elm buds, either with or without cryopreservation

Exp.	1	2	3	4	5	6
Species	*U. laevis*	*U. laevis*	*U. laevis*	*U. laevis, U. glabra*	*U. laevis, U. glabra*	*U. glabra*
Purpose	Media testing, cryo	Cryo testing	Testing DKW, reduced light	*U. l.* and *U.g.* cryo and microprop. techniques	Improving cryo survival	Dehydration of *U. g.* buds
Year	2016	2017	2017	2020	2021	2022
Surface sterilization agents	EtOH	EtOH	H_2O_2	EtoH, H_2O_2	EtoH, H_2O_2	NaDCC
Media	MS and WPM	MS	DKW	DKW	DKW	DKW
PGRs	GA_{4+7}, BA, TDZ	GA_{4+7}, BA	GA_{4+7}, BA	GA_{4+7}, BA (mT)	GA_{4+7}, BA	GA_{4+7}, BA
No. trees	5	20	5	20	12	5

Data analysis

The data analysis was carried out using IBM SPSS software (versions 25–28), except for storage compound analysis (III) which was carried out with R (version 4.1.3). Significant differences between treatments regarding embryo yield or ET growth were determined by analysis of variance (ANOVA) with Bonferroni correction (II, III). If the variances were not equal, Welch ANOVA with Games-Howell correction (III) for pairwise corrections was applied. If the normality requirements of ANOVA were not met, a non-parametric Kruskal-Wallis test or Mann-Whitney U-test was used (I-III). For the indicators of oxidative stress -experiments, a non-parametric Kruskal-Wallis test was used as the data were not normally distributed (II). For storage compound analysis, one-way ANOVA with Tukey correction was used, and hierarchical ascendant cluster analysis with Ward's minimum variance clustering and Euclidian distance as a measure for similarity (III). Greenhouse survival of emblings was analyzed with logistic regression with relevant cofactors (I-III) or with a chi-squared test (II). The differences between the treatments on successful elm shoot elongation, rooting, and greenhouse survival were evaluated with chi-squared test or Fischer's exact test (IV). In Paper V, the differences between the treatments on successful regeneration after cryopreservation were evaluated with logistic regression. In all experiments, a p-value < 0.05 was considered statistically significant.

RESULTS

Scaling up SE via alternative propagation methods

Bioreactors (I)

The production of *P. abies* somatic embryos using Plantform TIS bioreactors was successful but required multiple media changes. Three lines were used in each experiment, and the lines showed inconsistent responses to the tested culture conditions. Alterations to aeration duration and different support pad materials were tested in hopes of improving embryo development.

Increasing aeration duration from 20 min to 2 h decreased embryo yield with two out of three lines, and one of the lines was not significantly affected. Constant aeration of the bioreactors resulted either in contaminations or the development of no cotyledonary embryos. Increasing the length of aeration beginning from the second week of maturation was tested, with a decrease in the embryo yield from 311 (± 42, SEM) to 258 (± 52) embryos / g FW. Additionally, it decreased the survival after transplantation from 47% to 35%.

For the different support pad materials, the highest average embryo yield with the three lines combined was with plastic netting with a filter paper on top (474 ± 86 embryos / g FW). Plastic netting was also the easiest support pad material to handle. On metal netting (control) 311 (± 42) embryos on average / g FW were produced, and the metal netting was difficult to spread flat to the bioreactor basket. Maturation on filter paper placed on polyurethane foam resulted in 238 (± 76) embryos / g FW. There were however contradictory results from individual lines, e.g., 653 developed on average 533 (± 13) embryos / g FW on polyurethane foam, and with the lines 645 and 1606, the results were 142 (± 13) and 38 (± 11) respectively. The highest survival (with the three lines combined) after transplantation was using plastic

netting with filter paper at 52%, and the lowest on polyurethane foam with filter paper at 44%.

After maturation, the embryos were germinated directly, cold-stored inside a bioreactor, or desiccated with or without filter paper. Rinsing to separate embryos from proliferating tissue was tested with embryos from all the treatments except cold storage. Rinsing the embryos and drying them in the Büchner funnel prior to germination decreased greenhouse survival by between 28–34 percentage points. Rinsed embryos had shorter shoots than their non-rinsed counterparts after germination. Desiccation, on the other hand, increased survival by 16 percentage points on average. The best survival (66%) was with non-rinsed embryos desiccated on moistened filter discs. Desiccated embryos that were not rinsed were significantly longer (2.5 mm) than those that were directly germinated (2.3 mm).

Suspension cultures (II)

The growth rate was measured as FW after one week of culture, divided by the initial inoculum FW from semisolid plates (Table 2). The growth rate in suspension culture was not higher than on semisolid plates. In suspension cultures, there was more variation: the lowest growth was only 1.6 times the original mass, and the highest was 5.0 times. In contrast, with semisolid media, the variation was between 2.9 and 3.4 times. However, in subsequent experiments, higher growth rates were achieved in suspension culture and on semisolid media when the FW of clumps put onto semisolid media was adjusted. The highest suspension growth rate was achieved with line 4146 reaching 8.6x the inoculum FW. Decreasing the PGR concentrations initially to half and then to one-tenth of that in the semisolid media did not negatively affect the ET growth.

Table 2 Proliferation rate of the ET with different suspension culture PGR (BA and 2,4-D) concentrations in the suspension culture experiments of Paper 2 as mean ± SEM. The different methods are compared with 1x PGR suspension, which was used in each trial.

		ET growth (FW at 1 week / inoculum) ± SEM		
Trial	Treatment		1x PGR suspension	no. lines
1	1x PGR semisolid	3.2 ± 0.08	3.0 ± 0.40	5
2	0.5x PGR suspension	6.0 ± 0.76	5.2 ± 0.69	4
4	0.1x PGR suspension	4.1 ± 0.60	3.8 ± 0.32	5

The embryo yield was very low when the suspension was directly dispensed from the bottles into maturation without rinsing. The maturation plates prepared without rinsing had only 1.7 (± 0.38, SEM) cotyledonary embryos on average, whereas maturation plates prepared with ET grown on semisolid media had on average 30.8 (± 4.1) embryos per plate. In the subsequent experiment, the directly matured suspension ET produced on average 6.6 (± 0.75) embryos per maturation plate, and control plates had 30.3 (± 5.1) embryos. Rinsing the ET with hormone-free maturation media before maturation improved the maturation result from the suspension-cultured ET and it was 22.9 (± 2.2) embryos per plate, which was not significantly different from semisolid-grown ET. Halving the PGR concentration also improved the embryo yield to 14.2 (± 1.2) embryos per plate. Rinsing the ET from 0.5x PGR suspension did not significantly further improve the embryo yield compared to rinsing the 1x PGR suspension tissue, nor did the reduction of the PGR concentration further to 0.1x.

Oxidative stress levels were analyzed from suspension- and semisolid-grown tissue. H_2O_2 content was increased in suspension cultures in comparison to semisolid cultures. However, some lines in the suspension cultures also had higher POX activity to cope with oxidative stress by H_2O_2. As neither H_2O_2 content nor POX activity correlated with embryo yield, it was not possible to interpret that the suspension cultures were dealing with excessive oxidative stress.

The greenhouse survival of the suspension-cultured embryos was not lower than the survival of embryos cultured on semisolid media. However, the overall survival was lower for Trials 1, 2, and 3, which were transplanted in July 2020 (51%). This was likely due to harsh summer conditions during transplanting. For Trial 4 transplanted in July 2021, the success was better (81%).

Filter disc cultures (III)

Utilizing filter discs for proliferation improved the proliferation rate, but the proliferation rate was highly dependent on the inoculum size. In the first experiment, when around 200 mg (FW) of ET was put onto filters, it grew to on average 7.8 times the inoculum mass. Increasing the inoculum size did not translate to a higher growth rate, as with the inoculum size of 300 – 350 mg the tissue proliferated to only 4.8 times the original inoculum.

Embryo yield was slightly higher with filter disc cultured ET than with ET cultured as clumps. Selection of the fresh ET significantly improved the proliferation rate and embryo yield of filter disc cultures, but for clump cultures, the difference was not significant. Prematuration did not significantly improve the embryo yield for either filter disc or clump cultures. However, the highest average embryo yield (99 ± 13 embryos / g FW) was achieved with prematured filter disc cultured tissue and was significantly higher than clump cultures on average matured without prematuration (58 ± 7 embryos / g FW).

No clear differences were found in the greenhouse survival of the plants with ET originating from filter discs or clumps, or with or without selection. The greenhouse survival was somewhat improved when embryos matured from filter disc cultured ET were also prematured. However, this was not the case for all lines but mainly due to the line 3492 performing better with than without prematuration.

Cold storage (III)

In the filter disc culture experiments (III) the embryos were counted before and after cold storage. The number of cotyledonary embryos was higher after cold storage than before it. In

Trial 1, 2049 embryos were placed into cold storage and 2804 embryos were available for germination after six months (37% increase). In Trial 2, the embryo count was 3918 before and 4622 after one month of cold storage (18% increase). For some lines individually, the effect of cold storage was negative, mainly due to precocious germination taking place during cold storage.

The storage compound profile of the embryos was assessed from four genotypes after different times (0, 4, 8, 26, and 61 weeks) in cold storage and compared to that of a sample pooled from zygotic embryos (III). Raffinose family oligosaccharides (RFO) stachyose and raffinose were present in very low quantities before cold storage in somatic embryos in comparison to zygotic embryos. After 4 weeks in cold storage, they had increased to almost the same level as in zygotic embryos. Sucrose and starch contents were higher in somatic than in zygotic embryos, and the difference got more pronounced between 8 to 26 weeks in cold storage. Simple carbohydrates (fructose, glucose) did not change substantially during the cold storage but were generally present at higher levels than in zygotic embryos. The protein content in the somatic embryos increased until 26 weeks but then dropped somewhere between 26 and 61 weeks. Based on SDS-PAGE, the protein profile of somatic embryos was similar to zygotic embryos until 8 weeks, and then shifted between 8 and 26 weeks.

Cryopreservation and tissue culture of *Ulmus laevis* and *U. glabra (*IV and V*)*

Dormant bud cryopreservation through slow cooling worked well for *U. laevis* and had minimal impact on the initiation success rate (IV). In a 2017 experiment, cryopreserved buds exhibited slightly better regeneration (64%) than fresh buds (57%), although the difference was not statistically significant. In the 2020 experiment, *U. glabra* cryopreservation was also included. *U. laevis* regeneration rates were similar to before (64% for fresh and 63% for cryopreserved), but for *U. glabra* the regeneration from cryopreservation was only 13%, whereas 44% of fresh *U. glabra* buds were recovered. The regeneration of *U. glabra* from cryopreservation was finally improved by dehydrating the buds before cryopreservation (V). The dehydration of the buds to around 30% water content from the initial 50% improved the *U. glabra* regeneration from cryostorage from 11% to 43%.

Contaminations were a major issue, especially with *U. glabra*. In the 2020 experiment (IV), contamination rates were 18% and 51% for fresh and 25% and 46% for cryopreserved *U. laevis* and *U. glabra* buds, respectively. For cryopreserved *U. glabra* buds, the contaminations were reduced from 42% to 10% with the combination of pre-cryo dehydration treatment and utilization of long NaDCC sterilization (V).

The initiation rate for *U. laevis* was improved by the inclusion of GA_{4+7} in the initiation media. In the first 2016 experiments, the addition of GA_{4+7} improved the initiation success of fresh buds on MS media from 20% to 78%, in contrast to only using 0.5 mg l^{-1} BA as the sole PGR. Despite the good initiation results, the shoots kept dying after the initial elongation. Lowering the photosynthetic photon flux density from the initial 150 to around 50 $\mu mol\ m^{-2}\ s^{-1}$ was needed to sustain the multiplication rate. In trying to solve the issue of dying shoots, MS basal media was switched to DKW base, which was found to be suitable and was used in all later experiments. It was also found suitable for *U. glabra* based on the fresh initiations in 2020 and no further media testing was carried out specifically for *U. glabra*.

The rooting of the shoots required IBA, as the rooting on semisolid media with 3 mg l^{-1} IBA induction for 3 days resulted in 67% of the plants surviving and growing roots. With

constant 0.1 mg l^{-1} IBA media 45% and on hormone-free media only 7% of the shoots stayed alive and produced roots. However, the IBA pulse treatment also had the highest mortality, followed by the continuous culture and the hormone-free media. A pulse treatment in 5 mg l^{-1} IBA solution supplemented with sucrose and direct transplantation into peat-based substrate was then tested on a small scale, and it gave the best results. On a larger scale, rooting induction with IBA-solution was tested together with spruce seedlings in a large commercial greenhouse. In the first year, the success rate for *U. laevis* was only 18%, and in the second year 20%, and for *U. glabra* 64% and 9% respectively.

DISCUSSION

P. abies SE propagation techniques

Proliferation rate (II and III)

The proliferation of *P. abies* ET can be carried out on semisolid media or in suspension. Suspension cultures are viewed as faster in growth and thus more suitable for bulking up material for commercial propagation (Denchev and Grossnickle, 2019). Additionally, they are inherently more scalable than cultures on semisolid media in individual Petri dishes. However, when suspension and semisolid cultures were compared regarding proliferation rate, no significant differences were found (II). With Plantform bioreactors, the proliferation rate was not assessed in bioreactor experiments (I), but it has been reported to be higher than on semisolid plates (Salonen et al. 2017; Le et al. 2021) The proliferation step progressed without issues in the bioreactors and the challenges were met at the maturation stage.

In the suspension culture experiments, different PGR concentrations were tested to improve the further performance of the embryos (II). The PGR concentrations of 0.5x and 0.1x with respect to the PGR concentrations used in semisolid media were tested for suspension cultures. The components of the liquid media such as nutrients and PGRs are more readily available than those in semisolid media (von Arnold et al. 2002). They could be in excess if applied in the same concentration as in semisolid media (Carneros et al. 2009). Lowering the PGR concentration did not reduce the proliferation rate in suspensions, indicating that it is not a limiting factor for the ET growth measured in FW.

Proliferation rate measurements using different propagation methods are difficult to carry out in a comparable and nondestructive manner when comparisons are made between ET grown in suspension and on semisolid media. Dry weight, FW, and settled cell volume can be used to assess the growth of ET (Tautorus et al. 1992). FW was used for all proliferation rate comparisons in Papers II and III. Settled cell volume was not operationally feasible as there were many cultures, and it took a long time for the ET to settle in a graduated cylinder (II). Additionally, the comparability of settled cell volume between suspension-grown tissue and semisolid clumps is poor, as suspension-grown tissue packs more tightly depending on culture rotation speed (González-Cabrero et al. 2018). For the determination of the dry weight of the tissue, it needs to be dried and cannot therefore be used to further study embryo yield.

The proliferation rate depends on inoculum size in suspension culture (González-Cabrero et al. 2018). Based on the results, this is also the case for semisolid media (II, III). An ideal inoculum size would be the least amount of ET possible that still reaches its maximum growth at the end of the subculture period. With the filter disc cultures, the proliferation rate was

faster, but the final FW of the ET was lower than with cultures grown as clumps (III). For clumps on semisolid plates, the upper limit was around 3 g of tissue per plate, and for filter discs around 1.5 g. With filter discs, no improvement in final ET mass was achieved by increasing the inoculum size from 200 mg to 300 mg per plate.

One often neglected aspect is the selectivity of subculture. According to Denchev and Grossnickle (2019), subculture should be carried out meticulously under a stereo microscope. The importance of selecting fresh growth from the periphery of the clumps or filter discs was evaluated (III). The selection was done with the naked eye. The proliferation rate was higher for selected tissue for both clumps and for filter disc cultures. However, the difference was significant only for filter disc cultures. As subculturing is carried out manually, the skill level of the person carrying out the subculture plays an important role. However, subculture without selection would be significantly faster and save labor, but this must be balanced in the light of reduced proliferation rate. Other factors in the subculture also have an effect, such as the size of the clumps (Carneros et al. 2009). Some genotypes might also require a more rigorous subculture than others.

The results of Papers II and III showed considerable variation in proliferation rate between the experiments. This can be partly explained by different sets of lines being used in different experiments. In many studies on SE, only a few or even a single line is used in the experiments. Based on our results, there is considerable variation in how lines respond to different conditions. The protocol development should be carried out with as many lines as possible to ensure that the protocols are suitable for most lines and that the loss of usable lines due to laboratory proliferation performance is minimized.

Maturation yield (I-III)

ET cultures have many types of structures during proliferation, PEMs I, II, and III, early somatic embryos, nonembryogenic cells, and dead cells. In many protocols, hormone-free prematuration media is used between the proliferation and maturation stages (Bozhkov et al. 2002; Denchev and Grossnickle 2019). The reasoning behind prematuration is to synchronize the development of the tissue and reduce the amount of exogenous PGRs accumulated in the tissue. In proliferating tissue, there are PEMs in all developmental stages, but only early somatic embryos are considered responsive to maturation stimuli (Filonova et al. 2000a). Additionally, cytokinin and auxin, especially 2,4-D, act against embryo development (Garcia et al. 2019), as they promote tissue growth and proliferation. A prematuration period on hormone-free media can help reduce the auxin and cytokinin concentrations within the tissue.

According to the results of Paper III, prematuration improved embryo yield for both ET grown as clumps and filter discs, but the difference was not statistically significant. However, the combination of prematuration and filter disc culture had significantly higher embryo yield than clump culture and no prematuration. This suggests at least a slight positive effect from implementing prematuration.

The proliferation of ET on filter discs placed on semisolid media has been reported to improve the embryo yield in comparison to culturing as clumps, e.g., for *Pinus sylvestris* and *Pinus pinaster* (Lelu-Walter et al. 2006; Aronen et al. 2009). Based on the results of Paper III, filter disc proliferation improved the embryo yield, and the results were statistically significant in two out of three trials of Paper III. Improved embryo yield was also indicated by unpublished earlier pre-trials. The better embryo yield from filter disc cultured tissue has been attributed to more even access to the media.

Different cell lines responded in different ways with some lines exhibiting undesired precocious germination when prematuration was implemented. On the other hand, for some lines, it helped to reduce tissue proliferation after transfer to maturation, which is beneficial for embryo development and processing of the material in subsequent steps. Some lines performed better on filter discs and some as clumps. Whether the protocol can be adjusted to the preferences of individual cell lines depends on the number of cell lines in production. This would also require studying and recording individual lines and their preferences, which is a laborious undertaking.

In Paper II, the suspension culture of ET was tested. The suspension was dispensed into maturation by pipetting it onto a filter paper, which was subsequently dried with a low vacuum pulse. This resulted in a dramatic drop in cotyledonary embryo yield in comparison to tissue proliferated on semisolid media. Rinsing the ET with hormone-free media before it was transferred to maturation improved the embryo yield.

The hypothesis was that the reduction of PGRs would improve the embryo yield and morphology. The reduction was tested both through rinsing the tissue between proliferation and maturation, and by reducing the PGR concentration in the proliferation media. Both using 0.5x PGR proliferation medium and rinsing the tissue before maturation significantly improved the maturation yield (II). By rinsing the tissue, the effect was more pronounced. However, implementing PGR reduction together with rinsing did not significantly further improve the embryo yield. In addition to removing the PGRs, rinsing could also remove maturation-inhibiting proteins that some lines have been reported to secrete into the media (Egertsdotter et al. 1993; Egertsdotter and von Arnold 1998). In liquid media, the effect of these proteins could be more pronounced than in semisolid media, due to better availability of the media components. Additionally, when semisolid-grown tissue is matured onto filter discs, the tissue is made into a suspension that can be spread onto the filter discs (Klimaszewska et al. 2001). Therefore, ET grown on semisolid media is also inadvertently rinsed if it is carried out on filter discs.

One of the indicators of oxidative stress is the amount of H_2O_2 in the tissue. H_2O_2 was higher in suspension-grown tissue than in ET cultured on semisolid media (II). However, higher H_2O_2 was not connected to the subsequent embryo yield. For some cell lines, POX activity was also higher in suspension-grown tissue, indicating a response by the antioxidant system. However, no clear indication of increased oxidative stress in suspension culture was observed, as H_2O_2 also has a signaling role in SE (Hazubska-Przybył et al. 2020).

The Plantform TIS bioreactors were suitable *P. abies* SE, when the protocol was optimized (I). However, the cons were higher than the pros for bioreactors to be implemented into *P. abies* SE production, in comparison to the current method utilizing semisolid media. To achieve the production of cotyledonary embryos, following the steps similar to the protocol described in a patent by Egertsdotter and Clapham (2015) was necessary, which added many media changes to the protocol.

Post-maturation cold storage & desiccation treatments (I and III)

After maturation, the cotyledonary embryos can be directly germinated and transplanted. The acquisition of embryo desiccation tolerance is promoted by ABA in the maturation media (Hoekstra et al. 2001). However, somatic embryos can benefit from various post-maturation treatments which prime them for germination. During late maturation, seed embryos acquire storage compounds and lose water. For somatic embryos, desiccation in cold temperatures and in high relative humidity has been found beneficial for embryo quality (Eliášová et al.

2022; Fischerová et al. 2022). One of the markers of improved desiccation tolerance is an increase in RFO compounds, raffinose, and stachyose, and their proportion to sucrose (Konrádová et al. 2003).

Cold storage has similar effects to desiccation and can at least partly replicate the benefits of desiccation (Konrádová et al. 2003). Cold storage improves the germination of *P. abies* SE plants (Varis et al. 2017). It is less laborious to carry out than desiccation, where embryos need to be transferred from the original cultures into the desiccation plates (Högberg et al. 2001; Konrádová et al. 2003). The ability to cold store embryos provides flexibility as embryos can be produced year-round, instead of just before germination before the growing season.

In somatic embryos, RFOs were very low at the end of maturation before applying cold storage (III). However, after four weeks of cold storage, RFOs had markedly increased and were close to the levels in zygotic embryos. This agrees with Konrádová et al. (2003) who report an increase in raffinose after 5 days and stachyose after 10 days of cold treatment. Sucrose was always higher in somatic embryos than in their zygotic counterparts, and starch increased throughout the cold storage. This is not necessarily a bad thing, as noted by Konrádová et al. (2003), as somatic embryos need to be more self-sufficient than zygotic embryos, as they are not nursed by the megagametophyte. Sucrose was also abundantly available in culture media, although according to Kubeš et al. (2014), it is not imported as such, but first hydrolyzed into glucose and fructose and then produced endogenously.

In terms of storage proteins, the cotyledonary embryos after maturation are quite similar to zygotic embryos (III). However, already between 4 and 8 weeks of cold storage the protein profile starts to shift from that of zygotic embryos. In Paper III, the effects of longer cold storage were studied, and between 26 and 61 weeks of cold storage the protein content of the embryos decreased. The carbohydrate profile did not exhibit major shifts after the initial increase in RFOs, except for the decrease of stachyose between 8 and 26 weeks. These results, especially the shift in protein content, suggest that the optimal cold storage length would not exceed 8 weeks. In practice, however, somatic embryos need to be often stored for a longer time.

The bioreactor embryos benefitted from desiccation on nested plates instead of just applying cold storage (I). Desiccation is considered especially important if PEG is used in the maturation media (Klimaszewska et al. 2016; Maruyama and Hosoi 2019), which was the case in the bioreactor experiments. The usage of PEG can improve maturation but can have negative effects on germination (von Arnold et al. 2002). Additionally, when the bioreactor experiments were carried out, the germination regime was less refined, and better results could be achieved following the protocol by Tikkinen et al. (2018b).

Greenhouse survival (I-III)

The germination success of *P. abies* somatic embryos was high, as presented in Table 1 of Paper I (84% germination success for the worst treatment). In the experiments of Papers II and III, short germination according to Tikkinen et al. (2018b) was applied, after which the germinated embryos were directly transplanted into containers in a commercial greenhouse with conditions adjusted for *P. abies* seedlings. Whether the ET was propagated as clumps, spread on filter disc paper, or as suspension did not affect the greenhouse survival of the resulting plants (II, III). The effect of the genotype was always more prominent than the effect of the proliferation treatment.

Prematuration between proliferation and maturation was applied in Paper III, and it improved the greenhouse survival of the embryos. However, the effect was restricted to only some lines, which seemed to benefit from prematuration more than others. As discussed above, the lines develop at different paces and prematuration could be beneficial for rapidly growing lines, whereas for some lines it could lead to increased precocious germination.

The greenhouse results were complicated by the fact that different amounts of embryos were available from different treatments and lines. In Papers I and II, a sample of the embryos was germinated and transplanted. In Paper III, all available embryos were germinated. With sampling, more equal sample sixes can be achieved except when there are fewer embryos available from a single treatment than the desired sample size. Sampling can lead to inadvertent selection of better-quality embryos from lines where more embryos are available, and picking embryos of borderline quality from the lines from which few are available. By germinating all the available embryos, this effect can be reduced, but cotyledonary embryos are still selected by humans and the sample sizes vary more.

The differences between earlier treatments (proliferation, maturation) can be affected by the screening of embryos of proper morphology when embryos are picked for germination. The lack of differences between the proliferation treatments in the greenhouse survival could point to the screening criteria for the cotyledonary embryos being a good indicator of the actual embryo quality. Even if there are more embryos of abnormal morphology in a given treatment, these are omitted at the germination stage. However, a high number of poor-quality embryos makes picking and screening more laborious, even if the number of embryos with proper morphology is the same. In other words, to make picking embryos easier, it could be more desirable to have fewer good-quality embryos if there are also fewer poor-quality embryos.

As discussed above, for bioreactor-matured embryos high-humidity desiccation was needed to improve survival after transplantation (I). Rinsing the cotyledonary embryos from bioreactors before germination or desiccation treatment to remove nonmatured cell mass decreased the subsequent survival of the embryos in greenhouse. However, the embryos were also dried on a filter paper placed on a Büchner funnel and this might have caused mechanical damage to the embryos more than the rinsing itself. Desiccation improved the survival of the embryos in all treatments, as well as when rinsing was conducted.

The results of Paper I highlight the inherent difficulties of using TIS bioreactors in SE. The multistage nature of the process demands multiple media changes, and this should be made as easy and contamination-proof as possible for a TIS model specifically designed for SE. Additionally, the bulking up of the tissue in the bioreactor means that the fresh tissue is not selected for maturation, which could have a negative effect. Plantform bioreactor is designed with micropropagation of shoots, rather than SE. Using bioreactors for the proliferation stage could be successfully implemented, but the production volume would have to be very high to justify it, and contamination in a large bioreactor is a larger risk than in one of many Petri dishes.

Elm cryopreservation and tissue culture through organogenesis (IV and V)

Cryopreservation (IV and V)

The cryopreservation of dormant in vivo buds through slow cooling has been applied to many temperate deciduous tree species (Jenderek et al. 2013). Cryopreservation with minimal pre-

treatments is generally limited to species that need to be able to withstand low winter temperatures. The cryoprotocol for *Betula pendula* developed by Ryynänen (1996) is the basis for the method described in Paper IV for elms. For *B. pendula*, the protocol works well, and there is no difference in regeneration success with fresh and cryopreserved buds (Ryynänen 1996). Similar results were achieved for *U. laevis*, and no other cooling rates or freezing methods were tested (IV). However, for *U. glabra* the same protocol gave poor results when applied without pre-cryopreservation dehydration treatment.

The slow cooling rates presented in protocols are often around 1 to 2 °C h^{-1} (Tanner et al. 2020a). With *U. laevis*, *U. glabra*, and *U. minor*, Harvengt et al. (2004) successfully used both cooling rate of −0.3 °C·min^{-1} from +5 °C to –40 °C, and stepwise freezing in temperatures of 0, −5, –10, –20, and −30 °C with the buds kept in each chamber for 24 h. Therefore, the requirements for the pre-cryo cooling rate of dormant elm buds appear rather flexible, and the cooling rate of –0.17 °C·min^{-1} used in the experiments of Papers IV and V falls between the cooling rates in protocols presented by Harvengt et al. (2004). However, according to Tanner et al. (2020a), a rate of −1°C to −2 °C h^{-1} is commonly used and the rate of −0.17 °C min^{-1} (-10,2 °C h^{-1}) is considerably faster than that. A slower cooling rate could also improve the cryorecovery of *U. glabra* buds, as with a slower cooling rate dehydration step can be omitted for some species, like cold hardy *Malus* taxa (Towill and Bonnart 2005). Adding a holding period of 24 h at the temperature reached after slow cooling could also improve regrowth (Tyler and Stushnoff 1988; Tanner et al. 2020a).

According to Tanner et al. (2020a), a dehydration step is necessary for all but the most cold-hardy species, and in many dormant bud protocols a dehydration step is implemented (Forsline et al. 1998; Volk et al. 2009). For *U. glabra*, dehydration improved the regeneration rate after cryopreservation (V). *U. glabra* buds are generally larger than *U. laevis* buds, which might lead to more uneven freezing. The protocol could be further optimized by testing dehydration levels, as only one treatment was tested for *U. glabra*. The optimal moisture content could be higher or lower than around 32% described in Paper V, as it varies between different species (Tanner et al. 2021). Dehydration treatment also decreased the contaminations which plagued the *U. glabra* buds after introduction to in vitro. The buds of *U. glabra*, being larger and having more and larger trichomes than the buds of *U. laevis*, offer more surface area and refuge for microbes while making successful sterilization more difficult. However, sterilizing agents might penetrate dehydrated buds more effectively, which could account for their higher sterilization success rate (V).

The timing of collecting the dormant buds affects the success rate for cryorecovery. The level of cold tolerance and optimal collection timing depends heavily on many abiotic and biotic factors (Forsline et al. 1998), and of course geographical location. For hybrid aspen in Finland, the optimal timing for collection is from November to January (Aronen and Ryynänen 2014). According to Forsline et al. (1998), collection should be carried out in midwinter, when the trees have been exposed to at least 72 hours of subzero temperatures. Deacclimatizing conditions like winter thaws can be overcome by storing the budwood in freezing temperatures before cryopreservation as demonstrated by Forsline et al (1996) for *Malus.* The dehydration treatment applied in Paper V also involved storing the twigs at −5 °C and the subzero desiccation temperature could have contributed to better regeneration success. The collections from which the elm buds were collected are mainly located in Southwestern Finland, where winter temperatures vary, and long periods of subzero temperatures are not necessarily guaranteed in early winter.

In most dormant bud cryopreservation protocols, thawed buds are regenerated by grafting them directly instead of in vitro regeneration (Volk et al. 2009; Jenderek et al. 2011). This

eliminates the need for a clean laboratory and staff who are adept in micropropagation. On the other hand, in vitro cultures can be initiated throughout the year and grafting also needs specially trained and skilled staff. Regeneration through in vitro culture enables the multiplication of the plant material through tissue culture before it is again introduced ex vitro into the greenhouse, potentially reducing the requirements for the number and size of cryo samples. One option is also forcing the buds to sprout after thawing, and the newly grown shoots are easier to sterilize for introduction to in vitro culture than buds (Tanner et al. 2020b).

Regeneration through tissue culture (IV)

The main benefit of utilizing tissue culture for the recovery of cryopreserved buds is the ability to propagate the buds into multiple identical plants. This can reduce the number of samples that need to be stored. However, some genotypes are always unresponsive to tissue culture. The protocol for conservation of genetic resources needs to work for most genotypes and it is not practical to tailor the protocol for individual genotypes.

To initiate tissue culture after thawing, buds are surface sterilized. Surface sterilization was the most difficult step in the process and also limited testing of the subsequent steps to an extent. Many surface sterilization protocols were tested especially for *U. glabra,* for which there were more difficulties than for *U. laevis.* Multiple different sterilizing agents and protocols were tested (antibiotics, H_2O_2, ethanol, hypochlorite, mercury(II) chloride). Instead of any single sterilizing agent, the dehydration of the buds before cryopreservation was found the most efficient way to prevent contaminations. NaDCC was, however, found to be an efficient sterilizing agent, but it did not improve the contamination-free regeneration rate when buds cryopreserved without dehydration were used (V). Fresh *U. glabra* buds also had contaminations, but without the mortality caused by the cryopreservation, clean and vigorous cultures were obtained regularly and those could be multiplicated further to achieve shoot production when the buds were sterilized with NaDCC.

After the sterilization procedure, buds are prepared for in vitro culture by removing the bud scales and the part of the twig left for ease of handling and to protect the meristem from cryodamage (Yakuwa and Oka 1988). In many protocols, only the meristem and a few leaf primordia are left intact (Harvengt et al. 2004). However, for *U. laevis* and *U. glabra*, it was only necessary to remove the hard bud scales, some of the outer soft bud scales, and the woody basal shoot tissue during explant preparation. Browning was observed especially with cryopreserved *U. glabra* buds, indicating cryopreservation damage. Browning of the explant in tissue culture is caused by the oxidation of phenolic compounds into quinone derivatives, which are harmful to the plant (Bhat and Chandel 1991). The exudating of phenolic compounds is a wounding response aimed to combat pathogen growth (Bhat and Chandel 1991). Their production can be more pronounced in cryopreserved buds, as they contain more dead tissue than buds prepared for tissue culture without cryopreservation. The exudation of phenolic compounds can be reduced by applying antioxidants or compounds that bind the harmful secondary metabolites, such as activated charcoal, polyvinylpyrrolidone (PVP), and glutamine (Akram and Aftab 2009; Varis et al. 2018). No difference in browning was observed for applying PVP in preparation of cryopreserved *U. glabra* buds (IV).

Different PGRs were initially tested for *U. laevis*, and 0.5 mg l^{-1} with 0.1 mg l^{-1} GA_{4+7} resulted in the best initial shoot growth (IV). The utilization of GA_{4+7} in the initiation media improved the shoot production rate of *U. laevis* in comparison to using BA as the sole PGR. GA_3 instead of GA_{4+7} has been previously successfully used to improve *U. americana* tissue

culture (Shukla et al. 2012, Uchendu et al. 2013). GAs are endogenous PGRs with a complex biosynthesis pathway which play roles in many aspects of plant growth and development (Yamaguchi 2008). Even though there are over a hundred identified GAs, the major bioactive GAs are considered to be GA_1, GA_3, GA_4, and GA_7 (Hedden 2020). The rest are either precursors or metabolites of the bioactive GAs with reduced or no activity (Yamaguchi 2008). For *U. americana*, GA_3 was included in the media throughout the culture. For *U. glabra* and *U. laevis*, GA_{4+7} was omitted after the first subculture due to some reports of GA being harmful to long-term in vitro propagation (Nickell and Tulecke 1959), and as an effort to reduce mortality after initial shoot growth. However, as other changes to the protocols were also made, most notably correcting the light conditions, GA_{4+7} could be tested also for the multiplication of *U. glabra* and *U. laevis*.

The multiplication of *U. laevis* was generally easier than the multiplication of *U. glabra*. Axillary budding through excising the apical meristem was the most successful method for *U. glabra*, and nodal segments containing one bud were often sufficient explants for subculturing *U. laevis*. With *U. glabra*, it was common for the nodal segments to produce callus instead of a new shoot. However, there were differences between the genotypes of both species in how easy they were to propagate in vitro. *U. laevis* cultures tended to also produce adventitious shoots from the basal callus, which were avoided for subculture due to potentially lower genetic fidelity (Monteuuis 2016).

As basal media, WPM performed worse than MS for *U. laevis* initiations (IV). WPM is generally poorer in nutrients than MS (Phillips and Garda 2019). *U. procera* is propagated on DKW media, which is richer in calcium than magnesium than MS or WPM. This can improve explant survival, as noted by Fenning et al. (1993) and Shukla et al. (2012). DKW also has lower total nitrates, which can reduce potential ammonium toxicity (Phillips and Garda 2019). DKW media prepared according to Fenning et al. (1993) was suitable for both *U. glabra* and *U. laevis* tissue culture (IV). The switch from MS to DKW was done to reduce shoot mortality after the first subculture, but that was likely affected by the unsuitable light conditions. Therefore, MS could be suitable for both *U. laevis* and *U. glabra*. MS basal media, and media with Quoirin and Lepoivre macronutrients and MS micronutrients have been successfully used for *U. minor*, *U. laevis* and *U. glabra* (Quoirin and Lepoivre 1977; Harvengt et al. 2004).

For multiplication, 0.5 mg l^{-1} BA was used as the sole PGR for both *U. laevis* and *U. glabra*. Meta-topolin (mT) has been reported to improve shoot production for *U. glabra* and *U. parvifolia* Jacq. and to have lower root inhibition on MS media (Malá et al. 2013; Beck et al. 2018). The propagation of *U. glabra* on mT-supplemented media was tested, but no significant differences were found between mT and BA in shoot production. However, the cultures used in the experiment were originally cultured on BA-supplemented media, which could have affected the results. The trial was also rather short, so no clear conclusions could be drawn. For *U. americana*, the application of antiauxin is necessary for long-term culture success on DKW media (Shukla et al. 2012). Some reduction in culture vigor was observed over time with some genotypes for *U. laevis* and *U. glabra* as well, and the application of antiauxin could be tested as a potential solution.

The rooting of elms was greatly improved by applying auxin IBA into the semisolid half-strength DKW rooting media (IV). Out of the two auxin treatments tested, short 3-day induction with IBA was superior to longer exposure to low concentration (0.1 mg l^{-1}) of IBA, although it led to higher shoot mortality. Media with lower salt concentrations supplemented with auxin, either IBA or 1-naphthaleneacetic acid, are nearly universally used for rooting in the published elm micropropagation protocols (e,g. Fenning et al. 1993; Biroščíková et al.

2004; Harvengt et al. 2004; Beck et al. 2018). However, the auxin concentrations and the length of the exposure vary. In induction treatments, the shoots are moved from the induction media onto hormone-free media where the adventitious rooting takes place. Transplanting without the root formation into the hormone-free media was tested and it appeared more successful than rooting on semisolid media in small-scale tests (IV). The results from the rooting induction in IBA-solution varied, with *U. glabra* sometimes performing better than *U. laevis*. Opting not to induce in vitro root growth would eliminate the need to prepare solidified rooting media. Additionally, as no roots are formed in vitro, roots do not need to be washed from the media and potential damage to them is avoided. Using induction in liquid also saves labor in media preparation, but semisolid induction media for rooting elm shoots could also be tested to achieve more consistent results. High rooting and transplantation success have been reported for elms when using semisolid media (Fenning et al. 1993; Harvengt et al. 2004). However, if higher numbers of plants need to be produced, in vitro root propagation this way is more time-consuming and laborious.

CONCLUSIONS AND FUTURE PROSPECTS

For *P. abies* SE, for small to mid-scale production, semisolid media is the best option, especially with automatic media preparation devices commercially available (Table 3). However, the amount of produced plastic waste can be high when using individual plates. Compartmentalization is a benefit when avoiding contaminations. Suspension cultures are more scalable and can be used when necessary. ET produced in suspension cultures should be rinsed before maturation to improve cotyledonary embryo development. Improvements to the suspension media can be made by adjusting the media to better nutrient and PGR availability in a liquid environment. Scaling SE up with TIS bioreactors is a difficult task, as they proved to be surprisingly laborious to operate.

In Finland, the approach to the utilization of SE has been to use a high number of lines to maintain genetic diversity. Additionally, lines can be selected for production based on both laboratory and field performance. With a high number of lines, it is impossible to tailor the propagation methods for individual lines. However, the flexibility in the proliferation of *P. abies* ET allows the utilization of both semisolid and liquid media. Therefore, if the proliferation step becomes the limiting step, suspension cultures can be used to bulk up the ET for maturation, and otherwise, the lines are maintained on semisolid media. Cold storage of somatic embryos improves their quality and should be implemented, but for optimal results, it should be limited to up to 8 weeks, when possible. In practice, it is however often necessary to store the embryos for longer periods of time. Currently, the costliest stage of the process is germination (Tikkinen 2018), for which robotics are being implemented. Germination is expensive as it requires individual handling of the embryos. However, with germination robotics, the bottleneck could move to another stage of the process and more scalable proliferation methods become more lucrative.

Table **3** Qualitative assessment of different proliferation and maturation methods of SE. Scale: liability (–), neutral (+), advantage (++).

	Semisolid clumps	Semisolid filters	Suspension	TIS bioreactors
Scalability	–	–	++	–
Contamination risk	++	++	–	–
Potential for automation	–	–	++	+
Embryo yield	+	++	+	+
Ease of handling	+	+	–	–
Suitability for high number of genotyopes	++	++	–	–

Dormant buds of both native elm species in Finland, *U. laevis* and *U. glabra*, can be cryopreserved and regenerated in vitro through micropropagation, which allows the backup cryostorage of the field collections. Buds of both species can now in a worthwhile manner contribute to the cryobank of stored elm germplasm. Currently, the cryocollection houses 117 *U. laevis* and 43 *U. glabra* genotypes and will be expanded in the following winters. Based on the results, without dehydration, approximately 80% of the *U. laevis* genotypes could be regenerated from cryopreservation. Without dehydration for *U. glabra*, the recovery was successful for 40% of the genotypes. To what extent exactly this is improved by dehydration would require further testing, but while testing dehydration, five out of five genotypes were recovered from cryopreservation. The rooting success is still poor and the process is inefficient. With enough resources to maintain the cultures for successive rounds of rooting most genotypes can be recovered. Alternative rooting methods could still be tested to improve the rooting success and reduce the labor needed.

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