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Frost hardiness of Scots pine progenies and some woody horticultural cultivars under different preconditioning

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Academic dissertation

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ABSTRACT

Frost hardiness (FH) is one of the limiting factors for the successful growth of woody plants in the boreal zone. To cultivate the plants in cold conditions, they need to be tested before they are launched to the farmers and forest owners. Appropriate preconditioning for the different progenies of the plus tree of forest seedlings within the same species, and different horticultural woody species and cultivars are not known well. To answer those questions, this study designed and implemented experiments for Scots pine (*Pinus sylvestris* L.) progenies, for three horticultural species, i.e., apple (*Malus domestica* Borkh.), blueberry (*Vaccinium corymbosum* L.), blackcurrant (*Ribes nigrum* L.), and for pear cultivars (*Pyrus communis* L.). The study is composed of three parts with the following aims: i) to determine whether the pollination site affects the FH of the Scots pine progenies, ii) to determine the proper late-autumn preconditioning before running the frost hardiness tests of different apple, blueberry, and blackcurrant cultivars, and iii) to determine the effects of a short warm spell in mid-winter on the FH of pear cultivars. One of the important aims of the thesis was to assess and compare the different FH testing methods with the help of the experiments executed here.

The first part of the study consisted of the progenies of Scots pine plus-tree seed orchards in Finland and Ukraine, in addition to the progenies from natural stands in Finland, with three seed lots from each site. FH was examined twice during cold acclimation in controlled conditions. The second part concerned the effects of different preconditioning temperatures (+3, −3, −7, and −10 °C) and their durations (one or three weeks) on the FH of two apple cultivars, three blueberry cultivars, and three blackcurrant cultivars. The third part concerned the effects of short term warm spells in mid-winter on the FH of three pear cultivars that were preconditioned in natural conditions, then dehardened in a growth chamber at +5 °C for either 3-4 days or 16 days, and then rehardened at −7 °C for 5-7 days.

It was found that the freezing test temperature had a strong effect on the physiology and growth of different organs of the plus-tree progenies of Scots pine, but no consistent differences were found in FH among the progenies. The proper preconditioning temperature for the development of the maximum frost hardiness of the aboveground parts in late autumn is three weeks at −3 °C for apple and blueberry, though a shorter time for blackcurrant would be enough. The frost hardiness of the pear cultivars responded to temperature changes in mid-winter, but less than expected, and the responses were similar in all cultivars. In addition, the FH estimates of the stem by electrical impedance spectroscopy (EIS) and relative electrolyte leakage (REL) were quite similar, but these methods overestimated FH when compared to the FH by visual damage scoring. DTA results had a small variation compared to other methods but the use of DTA is limited due to the low occurrence rate of the low temperature exotherm (LTE) in several species (e.g., blackcurrant).

***Keywords*:** Climate change, cold acclimation, cultivation zone, differential thermal analysis, dormancy, electrical impedance spectroscopy, electrolyte leakage, freezing test, frost hardiness

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Vantaa, 21st June 2021

Dongxia Wu

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on data presented in the following articles, referred to by the Roman Numerals I–III.

I. Wu D., Pulkkinen P., Pappinen A., Neyko I., Zhang G., Di B., Heinonen, J., Repo T., 2021. Frost hardiness of Finnish plus-tree progenies of Scots pine from seed orchards in Finland and Ukraine. (submitted manuscript).

II. Wu D., Kukkonen S., Luoranen J., Pulkkinen P., Heinonen J., Pappinen A., Repo T., 2019. Influence of late autumn preconditioning temperature on frost hardiness of apple, blueberry and blackcurrant saplings. *Scientia Horticulturae*, 258, 1-9. https://doi.org/10.1016/j.scienta.2019.108755.

III. Wu D., Palonen P., Lettojärvi I., Finni S., Luoranen J., Repo T., 2020. Rehardening capacity in the shoots and buds of three cultivars of European pear (*Pyrus communis* [L.]) following a warm spell in midwinter. *Scientia Horticulturae*, 273, 1-8. https://doi.org/10.1016/j.scienta.2020.109638.

The present author was the principal author of all the papers, with the main responsibility for the experimental design and realization, analysis, and reporting of the results. The results were also partly analyzed and reported by the second and seventh authors in *Paper I*, the second, third, and fifth authors in *Paper II*, and the second, fourth, and fifth authors in *Paper III*. The other co-authors participated in the experimental design, data collecting, and writing of the papers.

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LIST OF ABBREVIATIONS

FH	Frost hardiness
EIS	Electrical impedance spectroscopy
Z_{Re}	Real part of impedance
Z_{Im}	Imaginary part of impedance
REL	Relative electrolyte leakage
L1	The first conductivity measurement
L2	The second conductivity measurement
CF	Chlorophyll fluorescence
DTA	Differential thermal analysis
PSII	Photosystem *II*
F_v/F_m	Maximum quantum yield of *PSII* in dark acclimated samples
Y(*II*)	Quantum yield of photochemical quenching in light
RS	Root scanning
VD	Visual damage scoring

1 INTRODUCTION

1.1 Background

The climate is predicted to become warmer in the future and extreme weather events will be increased at the same time (Venäläinen et al. 2020). In the boreal zone, the frost temperatures in winter will not disappear but varying temperatures with high precipitation and freeze-thaw events may occur more frequently. Woody plants growing at their northern distribution areas may be exposed to frost damage (Kishimoto et al. 2014; Muffler et al. 2016). Therefore, trees as long-living organisms must have enough frost hardiness (FH) to withstand the impact of extreme weather events during their long lifespans (Eccel et al. 2009; Jylhä et al 2014). Although there are many studies on the effects of climate change, the impact of tree species distribution and diversity on forest productivity, as well as the frost hardening and the adoption of different species and cultivars of trees is still under discussion (Kellomäki et al. 2008; Nielsen and Rasmussen 2009; Pagter et al. 2011; Pardos et al. 2014; Neuner et al. 2019).

The growth of the trees in the boreal zone is based on the acclimation and adaptation of their annual cycle with the changes of the weather conditions during the four seasons. There are four main development phases in the annual cycle: active growth, lignification, rest, and quiescence (Pulkkinen 1993; Kellomäki et al. 2008; Hänninen 2016). Based on the hardening competence of the plants, the annual cycle can also be divided into four periods, i.e. susceptible, hardening, maximal hardiness, and dehardening (Howe et al. 1999) (Figure 1). In the annual growth cycle, the timing of growth cessation (shoots and needles) and initiation of frost hardening have key roles in the synchronization of the phenology and frost hardiness (FH) of native origins with the weather conditions of the local growing site. After the active growth period, the plants must develop sufficient FH to survive in cold winter conditions (Repo et al. 2008).

When the growth of trees ceases due to shortening days, this usually means the start (first phase) of frost hardening (Leinonen 1996). Many of the injury cases have occurred in this period because the trees are still actively growing with little frost tolerance (Christersson 1978; Howe et al. 2003). In the second phase of frost hardening, the temperature is the main driver of FH and at the end of this phase, the plants potentially reach the genotype specific maximum level of FH. The hardening competence of trees changes during the annual cycle. In autumn, trees are more susceptible to hardening and dehardening but later, during the winter, they become susceptible to dehardening by increasing temperatures (Leinonen 1996). In natural conditions, dehardening occurs usually in early spring and more rapidly than hardening in autumn, depending on the temperature, and the rate changes of the former is calculated by days to weeks, and then later by weeks to months (Chen and Li 1980). However, the fluctuating temperature, such as a warm spell in midwinter or a cold spell in later spring, may increase the risk of frost damage to woody plants. In many overwintering plants, rehardening is a possible process for returning FH to the previous levels during dehardening, but not always, especially if the dehardening has proceeded close to the initiation of new growth (Chen and Li 1980). Many of the injury cases have occurred around the time when the buds start to bloom and when the trees begin to grow actively in spring as well.

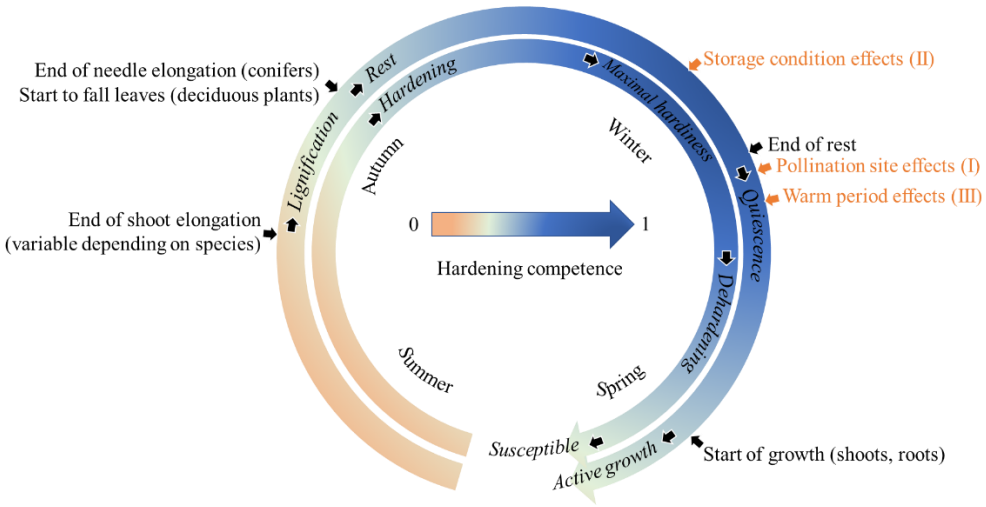

Figure 1. A schematic drawing of the annual growth cycle of trees with some phenological events (active growth, lignification, rest, and quiescence) with the annual cycle of frost hardiness of trees (susceptible, hardening, deep hardiness, and dehardening) in spring, summer, autumn, and winter. The change in the hardening competence from 0 to 1 is indicated schematically with color in the cycle. The timing of the three studies of the thesis is marked on the cycle based on the state of cold acclimation at the sampling time.

In the forest management and silviculture in the boreal zone, tree breeding has become an essential part of forest regeneration since the middle of the twentieth century. Tree breeding is an integral part of modern silviculture to increase economic profit through improved seed quality and wood production (quality and quantity [Haapanen et al. 2016; Lehtinen and Pulkkinen 2017; Egbäck et al. 2018]). Since climate change is expected to cause shifts in the distribution of tree species and affect the productivity of forests, tree breeding and planting can be a way to accelerate the adaptation of the trees to the changing environment. The genes of plus trees are thought to include a coded capability for acclimation and adaptation to high seasonal variations in environmental conditions, especially temperature, photoperiod (which stays stable in a given latitude), and the quality and quantity of light (Andersson et al. 2018; Alakärppä et al. 2019). Therefore, they are thought to be suitable for breeding purposes.

Scots pine (*Pinus sylvestris* L.) is a very wide-spread pine species which is distributed from Spain in the south to northern Scandinavia in the north, and from Scotland in the west to the eastern part of Russia in the east (Mirov 1967; Repo et al. 2001; Bieker and Rust 2010). In Finland, the Scots pine grows between the latitudes of 60°N and 69°N (Hurme et al. 1997; Briceno-Elizondo et al. 2006). To produce high-quality seeds for forest regeneration, seed orchards of grafted (cloned) plus trees of Scots pine have been established in different locations in Finland. In these orchards, surrounding pollination may reduce the genetic quality of the seeds, however. To avoid this effect, Finnish plus-tree seed orchards have been established in Ukraine, i.e., in an area where there is no surrounding pollination (Lehtinen and Pulkkinen 2017, Neyko et al. 2020). However, there are no comparative studies on whether the FH of the seedlings raised using seeds from Finnish and Ukrainian orchards (i.e., with or without surrounding pollination) differ from each other as

well as in comparisons with the seedlings from natural stands of the same plus trees as in the seed orchards.

During recent decades, the cultivation of horticultural plants has increased rapidly in boreal countries. In Finland, the horticultural crops have occupied a big part of the market, for example, apples (*Malus domestica* Borkh.) and berries account for 29% and 4% of total fruit production, respectively, with a total cultivation area of 2 200 ha (Niemi and Väre 2019). In particular, cultivars with good quality and high economic benefits such as apple, blueberry (*Vaccinium corymbosum* L.), and blackcurrant (*Ribes nigrum* L.) are common species in Finnish gardens (Gusta et al. 1983; Lindén 2001; Eccel et al. 2009; Ehlenfeldt et al. 2012). There is even a demand for more new cultivars in the expanding market (Niemi and Väre 2019). However, frequently recurring winter injuries in northern countries set requirements for the selection of proper cultivars for different hardiness zones (Gusta et al. 1983; Ehlenfeldt et al. 2012). The potential frost damage of many woody horticultural species in late autumn and early spring may need more consideration under changing climate conditions (Ehlenfeldt et al. 2006; Eccel et al. 2009). Especially at the border between maritime and continental climates, the fluctuating temperature will be more intensified (Kuwagata et al. 1994; Suomi 2018). Therefore, FH needs to be considered when evaluating the climatic adaptiveness of new cultivars. To avoid growth and production losses, studies are required for evaluating the FH of the different woody horticultural species and cultivars. The FH tests should be projected to the organ that is sensitive to freezing stress and easy to run by the breeders and seedling producers.

Since FH is a complex trait that includes a multitude of physiological and biochemical changes, such information can be achieved utilizing various methods to measure FH (Repo et al. 2008; Di et al. 2019). Reliable methods for the estimation of FH are required for breeding work, as well as in studying the mechanisms of frost injury and cold acclimation. FH is usually measured by exposing plant tissues or organs to controlled freezing temperatures and quantifying tissue damage by one or more methods. It is both interesting and motivating to study if the FH quantification methods are comparable across materials (different species, cultivars) and treatments (different environment conditions).

1.2 Literature review

1.2.1 Frost hardiness of woody plants and its determinants

Frost hardiness is the freezing temperature which a plant can withstand without being permanently damaged (Repo et al. 2000a, b). In natural conditions, the seasonal variation in the FH of woody plants is affected by different external and internal drivers (Fløistad 2002). Low temperature has been considered the most important determiner to the distribution of plant species and limiter to the yield of forest trees and horticulture plants (Parker 1963; Ashworth 1992). The accumulation of temperature sum and increasing night length are the key driving factors for the cessation of shoot elongation and proper bud development, while the first phase of frost hardening takes place by increasing night length and decreasing temperature (Weiser 1970). The threshold temperature for the initiation of frost hardening (the first phase) in the aboveground organs of boreal forest tree species is around 10 °C. According to the dynamic nature of FH, an organ may reach a steady state of FH with some delay corresponding to each new stable environmental condition in the second phase of hardening (Leinonen et al. 1995). The response of FH to temperature varies with the phase of the annual development in trees (Leinonen et al. 1997). In mathematical models, steady-state

FH is assumed to increase in a piece-wise linear relation with a decrease in air temperature (Leinonen 1996). In addition, the internal status of plants affects hardening competence which changes during dormancy, especially between entering rest and the start of new growth in the following growing season (Leinonen et al. 1997). A consequence of the change in hardening competence is that, for example, a short warm and cool period in midwinter can cause changes in FH, i.e., dehardening and rehardening.

The hardening capacity and the rate of hardening and dehardening are dependent on the genetic properties of cultivars, species, and origins, in addition to the phase of the annual development of the trees (Sakai and Larcher 1987; Leinonen et al. 1995 Repo et al. 2000b; Nilsson 2001; Kalberer et al. 2006). The decrease in FH is not only because of the prevailing environmental conditions but also because of the association with substantial changes in internal factors of plants, such as cell-tissue water relations and the carbohydrate status of cells (Stitt and Hurry 2002; Pagter and Arora 2013). In addition, research has found that the FH could also be affected by seed pollination sites, and the nutrition contents of trees (Pulkkinen 1993).

In cold areas, one-year-old young seedlings are the most susceptible to frost damage, because they may not have enough time for establishment and sufficient cold acclimatization to the low temperatures experienced after planting (Chan 2019). Therefore, the completion of growth acquired in the first summer after planting is quite critical to the FH and survival of seedlings (Sakai and Larcher 1987). The study of *Pinus pinea* L. seedlings shows that the FH increased significantly with age, which is linked to the degree of lignification of the cells (Pardos et al. 2014). The content of lignin in the cell wall of plant tissues (shoots and roots) is increased during cold acclimation (Liu et al. 2018). In similar conditions, the older seedlings with a high proportion of lignified xylem were more frost hardy than young seedlings with less lignified xylem (Pulkkinen et al. 1995).

Roots have been considered the most frost susceptible organ, and in cold areas may be damaged during overwintering if the protective snow cover is missing (Sakai and Larcher 1987; Drescher and Thomas 2013; Domisch et al. 2018). In a recent study with short-term freezing tests, the roots of frost-hardened Scots pine seedlings tolerated lower temperatures than previously thought (Di et al. 2019), however. This would mean that root damage would not necessarily be the primary cause for declined shoot growth even though roots are temporarily exposed to low temperatures. Even though clear differences have been found in the FH of above-ground organs between genotypes, there are no previous studies on whether there are differences in the FH of roots. In fluctuating environmental conditions, FH is dependent on tissue and organ as well (Li et al. 2002, Nielsen and Rasmussen 2009; Pagter et al. 2011). Among the aboveground organs, buds are the most frost susceptible and may be injured by frost following a warm spell in winter (Kalberer et al. 2007a, b). The buds of Norway spruce (*Picea abies* [L.] Karst.) have been observed to lose their maximum FH rapidly upon exposure to above-freezing temperatures in winter and to reharden slowly when the temperature cools again (Räisänen et al. 2006b). In addition, the FH of the basal stem of black wattle (*Acacia mearnsii*) seedlings was found to be lower than the higher parts (Chan 2019). Studies of Scots pine show that the needles and buds are more frost tolerant under constant cold temperatures than when subjected to a fluctuating environment, but the shoot performs in a more stable manner than needles and buds under the same condition (Repo et al. 1996).

1.2.2 Development of FH in forest trees with emphasis on Scots pine

In tree breeding, the parental growth environment (latitude, altitude, pollination conditions) of woody plants plays a role in the phenological variation of the first-year seedlings, which may influence FH expression in the following generation (Lehtinen and Pulkkinen 2017). For example,

plastic responses, genetic differentiation, or epigenetic inheritance enable the spread of Scots pine in the diverse environmental condition at different altitudes and latitudes (Johnsen et al. 1996; Skrøppa et al. 2007). Indications of local genetic adaptation are obtained from seed transfer experiments (Eriksson et al. 1980; Persson 1994). For example, when southern provenances of Scots pine were transferred to the north, their survival was reduced, and those northern provenances transferred to the south had an increased survival rate, but their growth was less than that of the southern local provenances (Beuker 1994). Genetic variation of FH and growth cessation as a consequence of tree breeding are good for adaptation to climatic change (Kellomäki et al. 2008). For example, trees time their growth and reproduction to coincide with favorable conditions, depending on the genotype (Weiser 1970). In the juvenile phase, the first-year seedlings of Scots pine have a free growth pattern, and then their bud set and cold hardening are determined by the joint effect of increasing night length and decreasing temperature (Hurme et al. 1997; Repo et al. 2001). However, their growth pattern changes to predetermined by age, whereupon the cessation of shoot elongation takes place when a certain temperature sum has accumulated, with some variation between provenances (Repo et al. 2000a). The northern provenances of both first-year seedlings and older trees start their frost hardening earlier than southern trees (Hurme et al. 1997; Beuker et al. 1998; Repo et al. 2000b; Nilsson 2001). In the greenhouse and field experiments with different Scots pine genotypes, northern populations set their buds and start their frost hardening earlier than southern populations (Hurme et al. 1997). In addition, indications of local genetic adaptation are obtained from seed transfer experiments (Eriksson et al. 1980; Persson 1994).

1.2.3 Development of FH in woody horticultural plants

In the late phase of frost hardening in autumn, plants are potentially close to the genotype-specific maximum level of FH (FH_{max}). The supercooling capacity or the maximum FH ability is one of the most important determinates, which may be related to the cellular structure or water conditions (Ashworth and Abeles 1984; Pearce 2001; Arias et al. 2017). Potential maximum frost hardiness that plants may reach in midwinter is a critical trait for the winter survival of different crop and fruit species and cultivars. Some varieties of woody horticulture species, such as apples, have been found to tolerate short-term exposure to −40 °C, (Gusta et al. 1983; Quamme 1976, 1991), and therefore they could potentially survive harsh winter conditions in northern regions. However, breeders lack a test protocol to assess FH_{max} (including appropriate preconditioning for the test), and accordingly to define proper cultivation areas for different species and varieties, except for inventories in field conditions after planting. In addition to FH_{max}, the ability to maintain FH and/or to reharden in fluctuating temperature conditions is critical for the winter survival of different cultivars of horticulture plants, such as European pear (Eccel et al., 2009; Pagter et al. 2011, 2013; Suomi 2018). Mild winters have been found to accelerate the break of dormancy more rapidly in pear (*Pyrus* [L.]) varieties than in other commonly cultivated fruit species, e.g., apple, whereupon spring development takes place earlier too (Drepper et al. 2020). Therefore, the pear trees may be susceptible to freeze damage if mild weather periods are followed by cold spells in winter (Pagter et al. 2011, 2013). On the other hand, dehardening is significantly faster than rehardening during dormancy in many woody plants (Howell and Weiser 1970; Repo 1991; Leinonen et al. 1997). Despite these studies, the rehardening capacity during dormancy is still poorly known for different woody plants. There are no such comparative studies for different varieties of European pear trees even though their frost hardiness and cultivation ranges are known to be different. In forcing conditions, the time to budburst is considered a measure of the depth of dormancy (Kalberer et al. 2006; Nielsen 2009; Pagter 2011). Even though the phase of dormancy and FH are not directly

linked together, an early budburst refers indirectly to an early decrease in FH, insofar that the chilling requirement for the rest break of bud is fulfilled (Arora et al. 1997; Lindén 2001; Ehlenfeldt et al. 2012; Laapas et al. 2012; Jylhä et al. 2014). However, the rehardening capacity during dormancy still needs to be studied for different cultivars of woody horticulture species.

1.2.4 Methods in estimating frost hardiness

The assessment of the frost hardiness of woody plants is commonly based on controlled freezing tests in a series of frost temperatures covering the critical range for cellular damage, followed by the measurement of damage using different methods. Electrical impedance spectroscopy (EIS) as a non-destructive method has been much used for detecting the physiological changes of cells, tissues, organs, and even whole plants (Ryyppö et al. 1998; Repo et al. 1994, 2000a; Azzarello et al. 2009). EIS is based on measuring the change in the electrolyte balance of cells by freezing damage. In EIS, the alternating electric current of different frequencies is driven into the tissues. Cellular damage can be concluded according to the change in current-carrying properties of the apoplastic space. Relative electrolyte leakage (REL) is based on a change in electrical conductivity of the incubation solution as a result of ion leaching from damaged cells (Dexter et al. 1932; Räisänen et al. 2007). Chlorophyll fluorescence (CF) is also commonly used to evaluate the FH results. CF was used to indicate the viability of plant tissues following freezing stress in horticulture, agriculture, and forestry (Lichtenthaler 1988; Repo et al. 2000a). This method is used to detect changes in the ability of electrons to flow through the two primary photosystems in the chloroplast thylakoid membranes (Burr et al. 2001). It can be applied to shoots, stems, or needles as long as they are green, either in field or laboratory conditions.

The differential thermal analysis (DTA) in frost hardiness assessment is based on the recording of intracellular freezing in critical organs. In stems of species with a ring-porous xylem structure, they are localized in the ray parenchyma cells and reveal freezing injury in xylem tissues and tissue death at very low temperatures (George and Burke 1977; Hong and Sucoff 1980; Ashworth and Abeles 1984; Arias et al. 2017). The freezing of deep-supercooled cells results in a low-temperature exotherm (LTE) which has been suggested as a measure of FH, and to predict the northernmost distribution limits of woody tree species (Wisniewski et al. 2003). LTE has been observed in many different species, such as apricot (Ashworth et al. 1981), blackberry (Warmund et al. 1988), grape (Andrews et al. 1984; Quamme 1991), peach (Quamme et al. 1973), plum, sweet cherry (Salazar-Gutiérrez et al. 2016), and pear (Quamme 1976; Gusta et al. 1983). In many species, it has not been observed what limits its use in FH assessment (Gusta et al. 1983; Fujikawa and Kuroda 2000; Neuner et al. 2019). However, the lack of xylem continuity is an important feature of the buds of some woody plant species. Therefore, DTA is not always an effective method to evaluate flower buds in all species, such as in most of the *Prunus* species (Ashworth and Abeles 1984; Salazar-Gutiérrez et al. 2016).

Visual damage scoring (VD) involves freezing entire or parts of plants in a controlled temperature chamber (Di et al. 2019). VD for the FH assessment of plants is based on color changes in leaves, needles, buds, and in the cambium and phloem of the shoots by cellular damage and it considers their possible recovery too (Repo et al. 1996, 2000b; Burr et al. 2001). This method usually requires a longer time (seven days to 10 days, or even more) than the other FH assessment methods, such as EIS and REL, to evaluate the damages. The accuracy of this method is affected by the incubation and re-growth conditions, as well as the observer. However, visual damage scoring for the FH assessment of plants integrates the effects of the damage and their recovery in different organs (Repo et al. 2000b, Di et al. 2019).

1.3 Research objectives and hypotheses

In the first study (I), a growth chamber experiment was designed and implemented to assess the frost hardiness of different progenies of Scots pine seedlings that were raised using the seeds of Finnish and Ukrainian plus-tree orchards in addition to the seeds of natural stands in Finland. The study aimed to determine the questions if the surrounding pollination affects the cold acclimation of plus-tree progenies. This study hypothesized that the FH of the plus-tree progenies of seed orchards in Finland and Ukraine would not differ.

The second study (II) aimed to study what the most appropriate preconditioning temperature is and its duration for assessing FH_{max} for different apple, blackcurrant, and blueberry cultivars in late autumn. This study hypothesized that for observing the differences in FH_{max} between horticultural species and cultivars there is a need for preconditioning the test material at specific conditions before controlled FH tests. In addition, different methods were compared for assessing the FH of those species.

The third study (III) aimed to determine if pear varieties with different cultivation ranges differ in their susceptibility to dehardening during a warm period in mid-winter and in their ability to reharden during a subsequent cold period. The suitability of DTA was studied to for assessing the FH of different pear varieties. This study hypothesized that the pear varieties differ in their response to warm and cold spells in winter.

Overall, the results of this study offer solutions for a preconditioning strategy to test the frost hardiness of woody plants in late autumn/winter, with the focus on those economically important species in forestry and horticulture.

2 MATERIALS AND METHODS

2.1 Study materials

2.1.1 Seedlings of Scots pine (I)

The study was carried out using 2430 one-year-old Scots pine (*Pinus sylvestris* L.) seedlings of nine lots that were raised in the research nursery at the Haapastensyrjä unit of the Natural Resources Institute Finland (Luke – 60°36' N, 24°25' E, 54 m asl). There were three lots of the seeds gathered from different natural stands (Rautavaara, Jyväskylä, and Rauma, termed as N1, N2, and N3, respectively), i.e., population N; three lots of open-pollinated seeds gathered from the Finnish seed orchard (Viiala [termed as F1, F2, and F3]), i.e., population F; and three lots of open-pollinated seeds gathered from the Ukrainian seed orchard (Vinnitsa [termed as U1, U2, and U3]), i.e., population U (Figure 2). There were 270 seedlings in each lot which are termed as progenies. The mother trees of the progenies from the Finnish and Ukraine seed orchards were the same and they are labeled by the same numbers.

Figure 2. The locations of natural stand seed sources of Scots pine in Finland (N1, N2, N3), and the location of the open-pollinated plus-tree seed orchards in Ukraine (U: U1, U2, U3) and Finland (F: F1, F2, F3). The locations of the mother trees are indicated (M1, M2, M3). The green (native range and isolated populations) and orange (introduced and naturalized area, and isolated populations) colors show the distribution of Scots pine (Caudullo et al. 2017).

2.1.2 Cultivars of horticultural woody plants (II)

The material consisted of two apple (*Malus domestica* Borkh.) cultivars (‘Pirja’ and ‘Lobo’), three blueberry (*Vaccinium corymbosum* L.) cultivars (‘Aino’, ‘Alvar’ and ‘Arto’), and three blackcurrant (*Ribes nigrum* L.) cultivars (‘Marski’, ‘Ben Tron’ and ‘Mortti’) (Figure 3). ‘Pirja’ for apple, ‘Aino’ for the blueberry, and ‘Marski’ for the blackcurrant were assumed to be harder (recommended for the Finnish hardiness zone IV, V) than the others. The apple and blueberry seedlings were two and half years old and the blackcurrants six months old. The blackcurrant saplings were raised from cuttings in the greenhouse of the Natural Resources Institute Finland (Luke), at the Piikkiö unit (60°39´N, 22°55´E, 18 m asl). The Blueberries were micropropagated at the Luke unit in Laukaa (62°28´N, 25°52´E, 95 m asl) and rooted and grown from micro cuttings in a commercial nursery in Raasepori (60°08´N, 23°40´E, 40 m asl) Finland.

2.1.3 Cuttings of different pear cultivars (III)

The material consisted of the previous season’s shoots of three dormant pear (*Pyrus communis* L.) cultivars (‘Conference’, ‘Clara Frijs’ and ‘Pepi’). The cultivars ‘Conference’ and ‘Clara Frijs’ were growing in a commercial orchard in the vicinity of Jomala, Åland, Finland (60°09´N, 19°56´E; 35 m asl), and the variety ‘Pepi’ both in Jomala and in the experimental orchard of the Natural Resources Institute Finland (Luke) in Piikkiö, Kaarina, Finland (60°39’N, 22°55’E, 18 m asl). The trees were 10 years old and were growing in a trellis support system. The selected sample trees were mature, and of normal health and medium vigor. At the time of sampling on February 5, 2019, the air temperature in Piikkiö was between 0 °C and 1 °C in Jomala. The sample representativeness was ensured by defining five separate field blocks for subsampling. Five subsamples (I-V), each consisting of 51 shoots (approx. 30 cm long with at least 10 vegetative axillary buds), were col-

lected from each cultivar. Any branches with mechanical damage were not sampled. Ten-centimeter-long subsamples were cut from the sampled shoots after the samples had been transported to Joensuu, and the 10 cm shoot sections of each cultivar were enclosed in one plastic bag, wrapped inside bubble wrap and stored in a Styrofoam box outside, under the snow at around 0 °C. The dehardening and rehardening treatments and the freezing tests in controlled conditions were carried out at the Luke Joensuu unit (for DTA) and at the University of Helsinki (for REL, VD, and bud dormancy). For the DTA-tests in Joensuu, there were a total of 225 samples of each cultivar, separated into 45 bags for five test times during the dehardening and rehardening treatments (3 samples/bag × 5 bags × 3 cultivars × 5 times of sampling).

2.2 Methods

2.2.1 Freezing test (I, II, and III)

The frost hardiness of Scots pine seedlings was twice assessed by controlled whole plant freezing tests in chambers in the dark (Figure 4, left panel). The first test was started on August 3, 2016 (T1), immediately after the seedlings were transferred to Joensuu, and the second test started one month later on September 3 (T2). In both tests, there were four freezing temperatures (−3, −8, −16, −30 °C) for roots and six freezing temperatures (−3, −8, −16, −30, −48, −80 °C) for needles, with the control (+4 °C) for both roots and needles. There were 27 seedlings from each progeny at each test temperature. The cooling rate was 2 °C·h^{-1} from 5 °C to −3 °C, which was maintained for five hours. Then the cooling continued at the rate of 2 °C·h^{-1} to the target temperature, which was maintained for four hours. In this way it was ensured that the soil in the pots was frozen too. The warming rate back to +5 °C was 5 °C·h^{-1}. After the freezing exposures, the seedlings were thawed at +5 °C for 3-4 days and then moved to room temperature for one day before different measurements on the roots and needles. After the measurements, the seedlings were moved to the greenhouse for regrowth (I).

Figure 3. Two apple cultivars (‘Pirja’ and ‘Lobo’), three blueberry cultivars (‘Aino’, ‘Alvar’, and ‘Arto’), and three blackcurrant cultivars (‘Marski’, ‘Ben Tron’, and ‘Mortti’) at the Luke unit in Suonenjoki (left) before transportation to the Luke unit in Joensuu and storage in four different temperatures (+3, −3, −7, −10 °C) with plastic covers (right), before sampling for the controlled freezing tests.

Figure 4. The chamber for the cold hardening of the progenies of Scots pine (left). The chambers for the freezing tests of Scots pine, apple, blueberry, and blackcurrant, and the same chamber for the DTA test, but with different programs (middle and right).

For apple, blueberry, and blackcurrant, the stem samples (10-15 cm long) of each cultivar and preconditioning temperature were cut from the middle part of the current year shoot and placed in plastic bags. There were three cuttings in each bag (one cutting for each of EIS, REL, and VD) and three replicate bags for each freezing test temperature. Each test consisted of eight temperatures (+3, −3, −15, −25, −35, −42, −50, −70 °C) that were assumed to cover the range from no damage to serious damage. The tests were carried out on five consequent days. The initial and final temperature in the freezing tests was 3 °C, the rate of cooling and warming was 5 °C·h^{-1}, and the duration of the target temperature was four hours (II).

The freezing exposures of the pine seedlings and of the apple, blueberry, and blackcurrant cuttings took place in programmable chambers (ARC 300/−55/+20, Arctest, Espoo, Finland), except for −70 °C (apple, blueberry, and blackcurrant) and −80 °C (Scots pine), which took place in a chamber cooled by liquid nitrogen (GCC−30, Carbolite, UK, with XL−180 liquid N_2-tank, Taylor-Whatron, UK). After the freezing tests, REL, EIS, and VD scorings were used to assess the frost hardiness of the samples (I and II).

For the freezing tests of pear cultivars at the University of Helsinki, 240 shoots of each of the cultivars ‘Clara Frijs’ and ‘Conference’ were sampled from the five blocks of the orchard in Jomala. The ‘Pepi’ samples were collected partly (blocks I-III, 144 shoots) from Jomala and partly (blocks IV and V, 96 shoots) from Piikkiö. An additional three shoots per block for all cultivars were collected for the determination of dormancy using a growing test. The samples were packed in plastic bags maintaining field blocking, wrapped inside bubble wrap and corrugated fiberboard, and transported to the University of Helsinki. The samples were placed in a growth chamber (Weiss 2600/45. +5DU-Pi, Weiss Umwelt Technik, Reiskirchen, Germany) and subjected to a dehardening treatment at +5 °C for 3 days (D1-H where H refers to Helsinki), followed by a rehardening treatment (R1-H). In the latter phase, the temperature was lowered from +5 °C to −7°C in 48 h and then maintained at −7 °C for 5 days. No light was provided during the treatments. The controlled freezing tests were conducted at the end of the dehardening and rehardening period (III).

2.2.2 Relative electrolyte leakage (I, II, and III)

In the first study (I), 32 needles were sampled from three Scots pine seedlings of each freezing temperature and each progeny for the REL test after the freezing tests. Ten-millimeter-long samples were cut in the middle of the needles, set in the test tubes (eight samples/tube), and four tubes of each of three replicates. Ten milliliters of distilled water were added to each test tube.

In the second study (II), four 3 mm thick discs were cut off the apple and blackcurrant shoots, and four 10 mm long pieces cut off the blueberry shoots for the freezing tests and the assessment of damage by REL. The stem discs of apple and blackcurrant were split in half, and the samples (without rinse) were distributed into 50 ml test tubes (four pieces/tube, three tubes/test temperature for apple and blueberry, and one tube/test temperature for blackcurrant), and 15 ml of distilled water was added to the tube of apple and blackcurrant in the first sampling time, whilst 20 ml was added in the second sampling time. The amount of distilled water in each tube of blueberry samples was 10 ml. The tubes were set to a shaker (200rpm) at room temperature for 22 h for Scots pine and 20 h for apple, blueberry, and blackcurrant. After this, the electrical conductivity was measured (L1 [CDM92-conductivity meter with CDC64T-electrode, Radiometer, Copenhagen, Denmark]). Then the samples were heat-killed at +92 °C for 20min and shaken for another 22 h (20 h) before the second conductivity measurement (L2). The relative electrolyte leakage (REL) was calculated as:

$$REL = \left(\frac{L1}{L2}\right) \times 100 \quad [1]$$

In the third study (III), two 5 mm long intermodal sections were cut from the pear shoots of each of the five replicates by test temperatures. The samples were rinsed with ultrapure water (RiOs^{MT} Essential 5 Water Purification System, Merck Millipore Co., Burlington, Massachusetts, USA) and placed into 15 ml plastic test tubes with 5 ml of ultrapure water. There were two samples in each tube and five replicate tubes for each test temperature, The tubes were set in a rotary shaker (SHKE80008CE, Thermo Fisher Scientific, Marietta, USA [130 rpm]) for 22 h at room temperature before the measurement of the first electrical conductivity (L1 [Jenway, Felsted Essex, UK]). The samples were then heat-killed by placing the tubes in a water bath at 95 °C for 1 h and shaken for another 22 h before the second conductivity measurement (L2 [III]). REL was calculated as in Eq. 1.

According to the REL data, frost hardiness was estimated as the inflection point (parameter C) of the following equation:

$$y = \frac{A}{1+e^{B(C-x)}} + D \quad [2]$$

where y refers to REL, x to the exposure temperature, A and D define the asymptotes, and B is the slope at the inflection point (Repo and Lappi 1989).

2.2.3 Differential thermal analysis (II and III)

The assessment of FH by DTA for saplings with deep-supercooling property is based on measuring low-temperature exotherms (LTE) in specific cells that are critical for survival. Five-millimeter-long pieces of apple, blueberry, and blackcurrant (II) and ten-millimeter-long pieces of pear (III) were cut from the middle part of the current year shoots (buds excluded). DTA measurements were

performed in a custom-designed device that consisted of four aluminum blocks with three differentially measuring temperature channels in each block (i.e., 12 samples in one DTA run) and a blank as the reference in each block (Räisänen et al. 2006a). The blocks were in a programmable freezing chamber (ARC 300/−55/+20, Arctest, Finland). The temperature difference between the sample and the reference junction was measured by NiCr/Ni thermocouples (diameter 0.25 mm). The thermocouple was set on the surface (II) or into the pith part (III) of the samples, wrapped with aluminum foil, and then placed into the plastic tube in the block. The temperature of each block was measured by a Pt-100 thermistor. The starting temperature in a DTA run was +3 °C. The rate of cooling to the target temperature (−50 °C) was 5 °C·h^{-1}. Freezing events were detected as exotherms, i.e., a high temperature exotherm (HTE) for apoplastic freezing and LTE for intracellular freezing, if any. In the data analysis, LTE was taken as the temperature where the first indication for initiation (II) and peak (III) of LTE was observed.

2.2.4 Electrical impedance spectroscopy of shoots (II)

After the freezing tests of the apple, blueberry, and blackcurrant stem cuttings, a 15 mm long sample of apple and blackcurrant and a 10 mm long sample of blueberry was cut from the middle portion of the cuttings. The samples were placed between the electrode pastes in an Ag/AgCl cell to measure impedance spectra (see above [Repo et al. 1994; 2000a, b]). The impedance spectra for stems were modeled using the distributed circuit element model (single-DCE). The resulting extracellular resistance was normalized by the cross-sectional area and length of the sample to obtain extracellular resistivity (r_e). The assessment of FH was based on the decrease in r_e due to damage to cell membranes and the consequent leaking of the intracellular ions into the apoplastic space.

2.2.5 Chlorophyll fluorescence of needles (I)

After the freezing tests, 15 needles were sampled from the top of three seedlings of each freezing temperature and each progeny for the measurement of dark-acclimated (20 min) chlorophyll fluorescence (F_v/F_m [PAM-2500, Walz, Heinz Walz Gmbh, Effeltrich, Germany]). The sample needles were attached side by side on the sellotape. The measurement gained information on the change in the potential efficiency of the quantum yield of photosystem II (*PSII*) by freezing damage (Baker 2008; Di et al. 2019).

2.2.6 Shoot growth and biomass (I)

After the freezing tests, 810 Scots pine seedlings of each sampling time were moved to the greenhouse with a temperature of 20 °C, a photoperiod of 18 h/6 h (day/night), a photon flux density of 300 µmol·m^{-2} (HS400W, Philips, Vantaa, Finland), and air humidity of between 70% and 80%. After three weeks of growth in the greenhouse, the lengths of the new shoots and the proportion of the new shoot dry mass from the total aboveground dry mass (including the dry mass of the old shoot) of each seedling were measured. The new and old shoots (including stem and needles) were dried at 40 °C for one week before the dry weight measurement.

2.2.7 Visual damage scoring (II and III)

For visual damage scoring of apple, blueberry, and blackcurrant, three shoot samples (10-15 cm in length including at least three buds) from the three replicate bags were collected into one plastic

bag. The bags were maintained on a laboratory desk at room temperature (+22 °C) under LED lightning (GreenPower LED, DR/B 150, 40 W, Phillips, Amsterdam, The Netherlands) providing a photon flux density of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (PAR) and a photoperiod of 12 h/12 h (day/night) for two weeks before damage scoring (II). The pear samples were incubated in plastic bags at room temperature inside a Styrofoam box for 13 days before visual damage scoring (III). After two weeks, the shoot samples were dissected and scored visually as damaged if the cambium and phloem were brown, and alive if they were green. Buds (dissected) were scored dead if the primordial shoot was brown, and alive if it was green.

2.2.8 Root morphology (I)

In the final harvest, the roots of the Scots pine seedlings of both test times T1 and T2 (a total of 1620 seedlings) were cleaned from the soil. The total root length and the number of root tips of each seedling were determined by scanning (WinRhizo 3.1.2, Quebec, Canada), and analyzed by test temperatures and progenies.

2.2.9 Bud dormancy (III)

For the bud dormancy of pear cultivars, three shoots from each of the five blocks, altogether 15 samples for each cultivar, were used to determine the depth of bud dormancy. After the samples arrived at the University of Helsinki, they were placed in a greenhouse in a mist tent in long-day conditions with a photoperiod of 20 h, a 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon flux density, a temperature of +19 °C, and relative humidity of 95%. A fresh cut was made at the basal ends of the shoots. Then they were placed in plastic test tube racks on plastic trays (VEFI PK050, Vefi Europe, Sklemiewice, Poland) filled with tap water. There were five plastic trays, each of them representing one block, and three shoots per cultivar in one tray. One bag of Broekhof Flower Food, containing 2.97 g glucose, 0.30 g aluminum sulphate, and 0.10 g potassium chloride (Broekhof, Noordwijkerhout, NL), was added to one liter of water in each tray. Fresh cuts were made on the base of the shoots once a week when the water was changed too. Bud break was observed twice a week for five weeks. When approximately 0.5 cm of new growth had emerged from the bud, the bud was considered broken. Upon completing the observations after five weeks, all the unbroken buds were dissected and scored for damage with a microscope, as described by the controlled freezing tests. Dead buds were omitted from the count of the total buds. The percentage of the broken buds was calculated for each shoot. The relative time to bud break was calculated for each bud separately by dividing the time to bud break (days) by the total duration of the experiment (35 days). If the bud remained unbroken, the relative time received the value of 1.

2.3 Statistical analysis

In DTA, the difference of LTE between treatments was tested using one-way ANOVA and the Holm-Bonferroni method (IBM SPSS 25.0, IBM Co., New York, USA). For FH, different measurement methods were compared using the Pearson correlation coefficients (II and III). The frost hardiness by EIS, CF, and REL was assessed by nonlinear regression (parameter C in Eq. 2). The standard error of the parameter was calculated using bootstrapping. The approximate significance for the difference between treatments was computed using a normal distribution and the asymptotic standard errors of the C parameters. In multiple comparisons, the significance values were adjusted

by cultivar and storage time applying the Holm-Bonferroni step-down method (Holm 1979). The significance values were computed using an R script (R version 3.3.2, R Foundation for Statistical Computing, Vienna, Austria [I, II, and III]). The significant values between the two sampling times of FH by REL and CF were computed using paired T-test by SPSS (I). FH based on the VD scoring in buds and stems of each cultivar was analyzed using a generalized linear mixed model (Lappi and Luoranen 2018). The Holm-Bonferroni method was applied in multiple comparisons. In the generalized linear mixed model, the indicator of damage was taken as the response variable. A random block effect was insignificant and was excluded from the final models. The software used was PROC GLIMMIX in SAS for Windows 9.4 (SAS Institute Inc., Cary, NC, USA). The LT_{50} values and the unadjusted significance levels for the pairwise comparisons were computed using SAS macro NLEstimate (II). The degree of visual injury was obtained as a proportion of the damaged part in the shoot (VS) and as the proportion of damaged buds per sample (VB). For each variable, differences between the cultivars in each hardening treatment and differences between the hardening treatments within each cultivar were analyzed separately. Those cultivars or treatments in which the damage level in the control temperature (+5 °C) was > 0.5 were excluded from the analysis. The model used was:

$$y_i = \left[a + \frac{(d-a)}{1+e^{b(c-x_i)}}\right] \quad [3]$$

where y_i is the observed value of the ith case of the dependent variable (VB, VS, REL); x is the temperature of the *i*th case; parameter d (1 for VB and VD) is the upper and a the lower (used in REL and for others in those cases when the proportion of damage in the highest temperatures was between 0 and 0.5 asymptote of the estimated curve); b is the slope, and c is the inflection point of the estimated curve (III).

The study was interested in the temperatures at which the probability of damage was 0.5 (DT_{50}). DT_{50} values and their standard errors were estimated using the equation

$$DT_{50} = c - \frac{log\left[\frac{(d-0.5)}{(0.5-a)}\right]}{b} \quad [4]$$

The statistical significances of the differences between the estimated DT_{50} values among the cultivars in each hardening treatment or among the hardening treatments within the cultivar were calculated using the delta method and the Wald test statistics as described by Lappi and Luoranen (2018 [III]).

3 RESULTS

3.1 Effects of pollination site on Scots pine progenies (I)

3.1.1 Frost hardiness of needles

According to the REL test, the FH of needles varied between −40 °C and −80 °C, depending on the progeny (Figure 5). Among the progenies of natural stands, the FH of N3 was the highest (−70 °C). Among the Finnish and Ukraine seed orchard progenies, the FH of F3 (−77 °C) and U3 (−75 °C) were the highest, respectively. Frost hardiness in two of the progenies from the Ukrainian seed orchards (U1 and U2) was less than their corresponding progenies from the Finnish seed orchards (F1 and F2). The frost hardiness of needles assessed by F_v/F_m was less than by REL (−25 °C to −50 °C) and the variations between the progenies were less than by REL as well. Compared to the FH of the three different locations (by REL), the Finnish population significantly higher (23 °C and 15 °C) than the Ukraine population in both sampling times, and also higher than the natural populations in both sampling times (Table 1). In addition, there are no difference between the two sampling times within the same location populations. The FH has no difference among the different populations by CF, but the FH of all three populations in the T2 were significantly lower than T1.

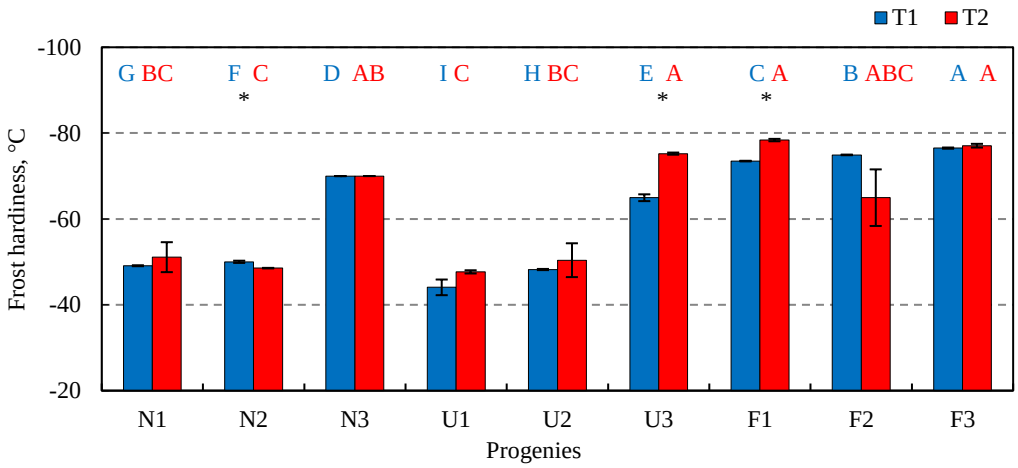

Figure 5. Frost hardiness (FH) of needles of the first-year Scots pine progenies from the open-pollinated natural stands in Finland (N1, N2, N3), and the open-pollinated seed orchard in Ukraine (U1, U2, U3) and Finland (F1, F2, F3) as assessed by relative electrolyte leakage method. FH was tested twice (T1, T2) during the cold acclimation in controlled conditions. Blue letters in the first row indicate significant differences between the progenies by frost hardiness tests in T1, and red letters in T2 ($P \leq 0.05$). In the second row, the difference in FH between T1 and T2 within the same progeny is indicated by '*' ($P \leq 0.05$). No letter means no difference. Bars indicate standard errors.

Table 1. The mean frost hardiness (FH) of the three subpopulations at the natural locations in Finland (N), and in the seed orchards in Ukraine (U) and Finland (F) at two sampling times (T1 and T2) as assessed by the relative electrolyte (REL) and chlorophyll fluorescence (CF) of needles, by shoot length, root length, number of root tips and total shoot dry mass. Different letters indicate the statistically significant difference among the locations at T1 and T2. 'ns' means no difference. The star '*' indicates the difference between two sampling times within the same population (P < 0.05) (n=3). In the lower panel are shown the P-values for the source of variation in the FH estimates by the sampling time and the population with their interactions (P-value ≤ 0.05 in bold).

Sampling time	Population/ location	FH assessment method					
		REL	F_v/F_m	New shoot length	Shoot dry mass	Root length	Root tips
T1	N	−56±12 b	−44±4 ns	−10±1 ns	−11±3 ns	−12±0 ns	−9±0 ns
	U	−52±6 b	−40±8 ns	−12±4 ns	−13±1 ns	−9±2 ns	−8±1 ns
	F	−75±2 a	−45±1 ns	−10±1 ns	−15±2 ns	−10±1 ns	−8±4 ns
T2	N	−57±12 b	−27±3 ns*	−11±1 ns	−12±3 ns	−7±4 ns	−15±0 ns*
	U	−58±15 b	−31±2 ns*	−11±2 ns	−10±2 ns	−6±5 ns	−13±0 ns*
	F	−73±7 a	−31±1 ns*	−10±0 ns	−11±4 ns	−6±2 ns*	−16±1 ns*
Time (T)		0.663	**<0.001**	1	0.116	**0.013**	**<0.001**
Population (P)		**0.01**	0.479	0.418	0.642	0.565	0.316
T*P		0.634	0.249	0.323	0.282	0.839	0.615

3.1.2 Frost hardiness of root and shoot

The new shoot length varied slightly between the progenies within the same temperature at both T1 and T2. A clear threshold for decreases in the new shoot length was found between −8 °C and −16 °C of all progenies (Figure 6). The highest and lowest new shoot lengths of the control samples (at +4 °C) were F1 (20 cm) and N3 (13 cm). In the final harvest, the proportion of the dry mass of the new shoots (needles and stems) of the total shoot dry mass varied from 44% to 67% at +4 °C, −3 °C and −8 °C, depending on the progeny and the sampling time. The dry mass ratio of the new shoot decreased significantly between −8 °C and −16 °C as well, and even no shoot growth was observed at −30 °C in any of the progenies at either sampling time (I). In addition, the shoot length was not affected by the sampling time or the population (Table 2). However, the total shoot dry mass was affected by the sampling time but not by the population. The freezing temperature affected both on the shoot length and the total dry mass (Table 2). There were no differences in FH among the population and between the sampling times if the assessment was based on the new shoot length and the total shoot dry mass (Table 1)

At both sampling times, there were differences in the total root length between the progenies, depending on the exposure temperature. The most significant changes were found between −8 °C and −16 °C, but no or minor changes between −16 °C and −30 °C (Table 1). At T1, root length decreased already between −3 °C and −8 °C in the progenies N1, U1, U2, and U3. The highest and lowest root length in the control samples were in U1 (424 cm) and N1 (242 cm), respectively. At

T2, root length decreased in all progenies except in F1, already between +4 °C and −3 °C, and in N2, U2, and F1 between −3 °C and −8 °C (I). The highest and lowest root length in the control samples were U1 (485 cm) and F1 (330 cm), respectively. In addition, the root length was significantly affected by sampling time, population, temperature, and their interactions, expect by the interaction of sampling time and population. The number of root tips were affected by the freezing temperature and by the interaction of time and test temperature (Table 2). In FH based on root length, there was no difference between the populations, but there was a difference in the Finnish population between two sampling times. There was no difference in FH between the populations as estimated according to the number of root tips. The FH based on the number of toot tips was higher at T2 than T1 in all populations (Table 1).

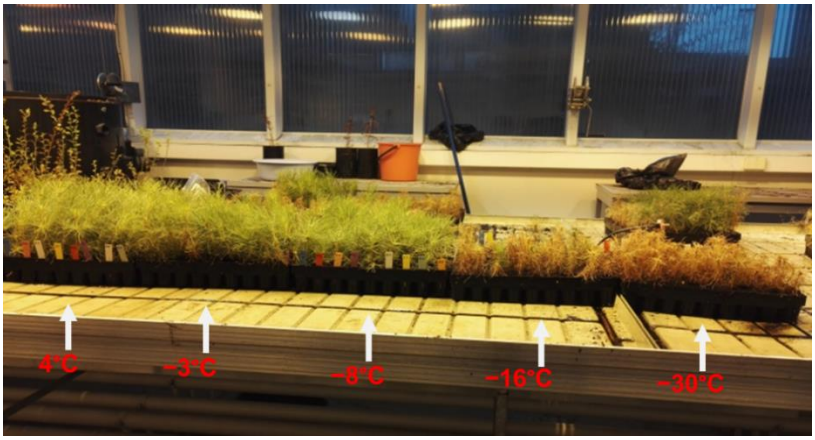

Figure 6. The Scots pine progenies from the natural stands in Finland, and the open-pollinated seed orchards in Finland and Ukraine after the freezing tests (+4, −3, −8, −16, −30 °C) of T2 and three weeks growing in the greenhouse.

Table 2. The P-values for the source of variation of different variables by the sampling times (T1, T2), populations (N, U, F), and test temperatures (+4 °C, −3 °C, −8 °C, −16 °C, −30 °C), with their interactions (P-value ≤ 0.05 in bold).

Sampling time	New shoot length	Total shoot dry mass	Total root length	Number of root tips
Time (T)	0.22	**0.00**	**0.00**	0.90
Population (P)	0.64	0.11	**0.05**	0.80
Temperature (C)	**0.00**	**0.00**	**0.00**	**0.00**
T*P	0.30	0.31	0.56	0.47
T*C	**0.00**	**0.00**	**0.00**	**0.00**
C*P	**0.00**	0.54	**0.00**	0.81

Table 3. The P-values for the source of variation of different variables of the control samples (+4 °C test temperature) by the sampling time (T1, T2), population (N, U, F) with their interactions ($P \leq 0.05$ in bold).

Source of variation	REL	F_v/F_m	Total shoot dry mass	Shoot length	Root length	Root tips
Sampling time (T)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Population (P)	0.230	0.900	0.209	0.925	0.094	0.850
T*P	0.167	0.312	0.573	0.609	0.296	0.468

3.1.3 Variation in the control samples

In the control samples (+4 °C), the effect of the sampling time was significant on all measured variables both in shoots and roots (Table 3). When the mean values were compared between the sampling times T1 and T2, the REL of needles increased from 20% to 27%, the F_v/F_m of needles increased from 0.70 to 0.74, the total shoot dry mass per seedling increased from 0.8 g to 1.0 g, the shoot length per seedling increased from 8cm to 10 cm, the total root length per seedling increased from 349 cm to 400 cm, and the number of root tips per seedling increased from 300 to 430. The effect of the population was not significant in any of the measured variables in shoots and roots. The interaction between the sampling time and the population was not significant in any of the variables either.

3.2 Effects of preconditioning on frost hardiness of horticulture saplings

3.2.1 Occurrence rate of exotherms by DTA (II and III)

The DTA profiles for stems were characterized by two exotherms, HTE and LTE (Figure 7). HTE was observed in all samples of all species. LTE was observed in most of the apple and blueberry samples but more randomly in blackcurrant (II). In apple, the LTE occurrence rate varied from 67% to 100%, depending on the preconditioning temperature and cultivar (Table 3). In pear, the DTA profiles for the shoots were characterized by LTE in all samples of all the cultivars (III). The peak value of the LTE varied between −38 °C and −41 °C in all treatments. Besides HTE and LTE, intermediate exotherms (*i*LTE) were observed in several pear samples and a few samples of apple and blueberry.

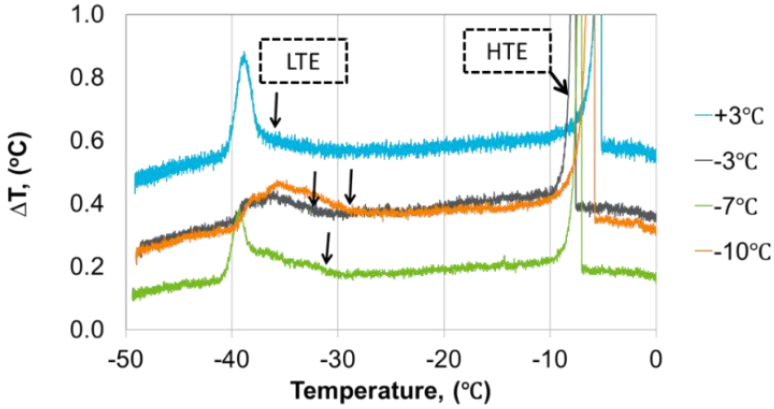

Figure 7. Examples of four DTA curves of current-year stems of blueberry (cultivar 'Aino') kept for three weeks at different preconditioning temperatures (+3, −3, −7, and −10 °C). High (HTE) and low temperature exotherms (LTE) are shown on the right and left of the curves, respectively. The initiation of LTE is indicated by an arrow.

Table 3. LTE occurrence rate (%) in all DTA-tested samples of different species and varieties that were stored at different temperatures (pooled data of two sampling times [II]).

Storage temperature, °C	Apple		Blueberry			Blackcurrant		
	Pirja	Lobo	Aino	Alvar	Arto	Marski	Ben Tron	Mortti
+3	100	84	100	100	100	84	100	100
−3	67	84	67	84	100	50	100	100
−7	84	100	100	84	100	0	0	34
−10	84	100	100	50	100	0	0	0

3.2.2 Frost hardiness assessed by controlled freezing test (II)

In apple, there was no significant difference in FH assessed by LTE between the 'Pirja' and 'Lobo' cultivars ($P = 0.10$). In both cultivars, the lowest LTE was observed at around −40 °C in the samples taken from the freezer of −7 °C. There was no change in LTE with prolonged preconditioning ($P = 0.57$). In apple, the effect of the preconditioning temperature was significant with all methods (DTA, $P = 0.03$; EIS, $P = 0.02$; REL, $P = 0.03$; VD, $P \leq 0.01$). The FH of stems increased during preconditioning between the 1st and 2nd sampling, the rate of hardening for 'Pirja' being 0.6 °C day^{-1} at a temperature of −3 °C by EIS and 0.9 °C day^{-1} at a temperature of +3 °C by REL. The effect of the cultivar on FH was significant by VD only (examples are shown in Figure 8 and 9). The frost hardiness of stem was the highest preconditioning at −7 °C and lowest at +3 °C by both EIS and REL. The frost hardiness of buds and stems of 'Lobo' by VD was highest at a temperature

of −7 °C. A slight decrease in FH was observed in samples taken from −7 °C. Even though the FH by REL was typically somewhat higher than by EIS, there was a high and significant correlation in FH between those methods (r^2 = 0.66**, P < 0.001). Frost hardiness by REL correlated significantly with LTE too (r^2 = 0.38*, P = 0.01 [II]).

In blueberry, the sampling time, preconditioning temperature, and cultivar significantly affected the FH of the stem, as assessed by the EIS and REL methods. The highest FH was typically found at the preconditioning temperature of −3 °C. However, the VD of buds gave the highest FH estimate for 'Aino' and 'Alvar' after storage at −7 or −10 °C. There was a tendency to increase the FH of the stem during preconditioning, the rate depending on the cultivar and temperature. In 'Alvar', the rate of hardening between the two sampling times was 0.8 °C day^{-1} at −3 °C by EIS and 0.6 °C day^{-1} at −7 °C by REL. In 'Arto', FH by EIS was even higher than by REL. The correlation between FH estimated by REL and EIS was r^2 = 0.32**, $P \leq 0.01$.

In blackcurrant, for 'Marski', EIS revealed no changes in FH due to preconditioning temperature or duration. For 'Ben Tron', FH by EIS increased after one week of preconditioning at below zero temperatures, but there were no differences between the preconditioning temperatures after three weeks. For 'Mortti', the preconditioning temperature affected FH by EIS after three weeks of preconditioning only. FH measured by REL revealed differences between the cultivars, but with different gradation as compared to EIS. By REL, the FH of 'Marski' was highest at a preconditioning of −7 °C and −10 °C, whereas, for 'Mortti', FH decreased at those temperatures compared with FH at −3 °C. Between the sampling times, the FH of 'Marski' increased at the rate of 0.7 °C day^{-1} at +3 °C, but decreased at the rate of −0.9 °C day^{-1} at −10 °C (by EIS) and the rate of −1.9 °C day^{-1} at −7 °C (by REL). According to the VD method, the buds of 'Marski' tolerated temperatures even below −70 °C.

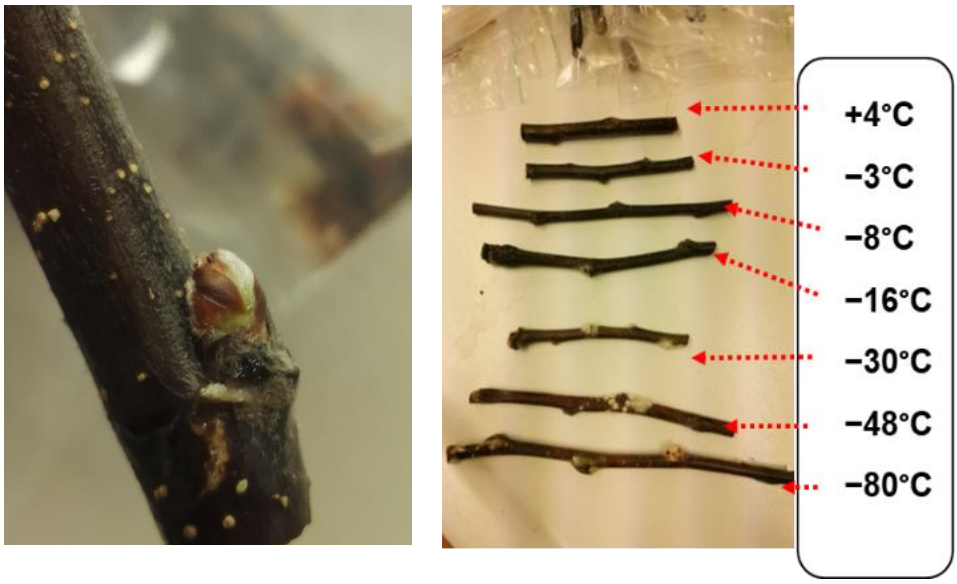

Figure 8. An example of a growing bud (left), and the apple ('Pirja') stems were kept at (+3 °C) for two weeks after the freezing tests (+4, −3, −8, −16, −30, −48, −80 °C) and maintenance in plastic bags on the laboratory desk for two weeks (right) by visual damage scoring. Some stems are even moldy at low temperatures (−30, −48, −80 °C) during the observing period.

Figure 9. The examples of dead (left) and live (right) stems of apple ('Pirja') by visual damage scoring.

3.3 Dehardening and rehardening of pear cultivars

3.3.1 Frost hardiness by differential thermal analysis

The peak value of the LTE varied between −38 °C and −41 °C in all the cultivars and treatments (Table 4). Accordingly, 'Conference' had the lowest FH (−38 °C) in the second (16 days) dehardening (D2) and 'Pepi' the highest FH (−41 °C) in the second rehardening (R2, seven days rehardening after D2) and among all the cultivars and conditions. In all the conditions, there were significant differences between the cultivars, except in the first (four days) dehardening (D1). Among the different conditions, all the cultivars had the lowest FH in D2 ($P < 0.001$), and the highest in the first ($P < 0.001$). All the cultivars were able to reharden during either the first rehardening (R1, seven days rehardening after D1) or R2. Besides HTE and LTE, intermediate exotherms (*i*LTE) were observed by DTA in many shoot samples of all the pear cultivars (data not shown).

3.3.2 Frost hardiness assessed by REL and VD

Following the three-day dehardening treatment, the FH varied between −26 °C and −30 °C by REL, but there were no significant differences between the cultivars ($P = 0.10$ [Table 5]). During the seven-day rehardening treatment, the FH increased significantly in 'Conference' ($P = 0.049$) but not in 'Clara Frijs' ($P = 0.217$) or 'Pepi' ($P = 0.375$). The daily rate of rehardening was 0.6 °C·day^{-1} in 'Conference'. After the rehardening, the FH was different between the cultivars ($P < 0.001$), with 'Pepi' being significantly harder than 'Conference'.

Table 4. The mean low-temperature exotherm (± standard error) of three pear cultivars ('Conference', 'Clara Frijs', 'Pepi') sampled from outside on February 5th and 6th, 2019, and tested by DTA after different dehardening and rehardening treatments: Outside (N), first dehardening (four days at +5 °C [D1]) followed by the first rehardening (seven days at −7 °C [R1]), or second dehardening (16 days at +5 °C [D2]) followed by second rehardening (seven days at −7 °C [R2]). Different capital letters indicate the differences ($P \leq 0.05$) between the cultivars within the same condition, and different small letters indicate the differences ($P \leq 0.05$) between the conditions within the same cultivar (n= 15). No letters mean no difference.

	Low temperature exotherm (temperature, °C)					*P-value*
Cultivar	N	D1	R1	D2	R2	
Conference	−38.9±0.2 C b	−39.2±0.2 ab	−39.6±0.1 B a	−38.4±0.2 C c	−39.2±0.1 C ab	*<0.001*
Clara Frijs	−39.6±0.2 B bc	−40.1±0.3 b	−40.8±0.1 A a	−39.3±0.3 B c	−40.0±0.2 B bc	*0.001*
Pepi	−40.4±0.3 A bc	−40.3±0.4 c	−41.2±0.2 A ab	−40.4±0.4 A bc	−41.4±0.3 A a	*0.027*
P-value	*<0.001*	*0.018*	*<0.001*	*<0.001*	*<0.001*	

Table 5. Frost hardiness (± standard error) of three pear cultivars ('Conference', 'Clara Frijs', 'Pepi') sampled from outside on February 5th, 2019, as assessed by the relative electrolyte leakage method (REL) after three-day dehardening at 5 °C (D1), followed by five-day rehardening at −7 °C (R1), and visual damage scoring (VD). The different capital letters indicate the statistical differences ($P \leq 0.05$) between the cultivars within the same treatment, and the small letters the differences ($P \leq 0.05$) between the treatments within the same cultivar. No letters mean no difference.

Treatment	Cultivars	REL	VD Shoots	VD Buds
D1	Conference	−26.0±1.9 b	−28.3±0.3	−27.0±1.6
	Clara Frijs	−29.2±2.0	−27.9±0.3 b	−27.3±1.3 a
	Pepi	−30.1±1.6	−30.3±2.3	−25.9±1.6
	p-value	*0.100*	*<0.001*	*0.529*
R1	Conference	−30.0±0.6 B a	−28.8±0.7 B	−24.7±1.0
	Clara Frijs	−33.0±2.1 AB	−29.7±0.6 B a	−24.4±1.0 b
	Pepi	−34.0±1.3 A	−32.0±0.7 A	−24.5±0.8
	p-value	*<0.001*	*<0.001*	*0.972*

3.3.3 Bud dormancy

In all pear cultivars, bud dormancy was broken (rest break) quite comprehensively, as more than 80% of the buds broke in five weeks, with even 99% in 'Clara Frijs', in the forcing conditions. Dormancy was relatively weak, as the first bud break took place after one week in the forcing conditions only: first in the cultivar 'Conference' and last in 'Clara Frijs' (Table 6). The relative time to bud break was also shorter in 'Conference' than in 'Pepi' and 'Clara Frijs'.

Table 6. The mean percentage (± standard error) of broken buds of three pear cultivars after five weeks in the forcing conditions, and the mean relative time to bud break. The samples were collected on February 5th, 2019. Values followed by different letters are significantly different ($P \leq 0.05$) by Tukey's test. (n=15).

Cultivar	The proportion of broken buds (%)	Relative time to bud break
Conference	96 ± 3 A	0.53 ± 0.03 B
Clara Frijs	99 ± 1 A	0.64 ± 0.02 A
Pepi	81 ± 5 B	0.69 ± 0.04 A
p-value	*0.001*	*0.003*

4 DISCUSSION

4.1 Frost hardiness of plus tree progenies of Scots pine (I)

4.1.1 Frost hardiness of needles

At the population level, the FH of the needles by REL was higher in the Finnish than in the Ukrainian seed orchards. This is opposite to our hypothesis and could be an indication of the epigenetic effect in seed development. In addition, the FH of the needles in the Finnish plus-tree seed orchard progenies was higher than in the Finnish natural progenies indicating that the plus-tree progenies are superior for frost hardening as compared to the natural progenies. It is known that growth in diverse environmental conditions is possible through plastic responses, genetic differentiation, or epigenetic inheritance (Johnsen et al. 1996; Skrøppa et al. 2007) which are supported by our results on FH too. In the seed orchards in Finland, southern effect may lead to a decrease in FH due to the southern winds at the time of pollination (Johnsen et al. 1996; Lehtinen and Pulkkinen 2017; Chan 2019) but that kind of effect was not found here, especially in FH of needles of the Finnish plus-tree seed orchard progenies, that was higher than in the progenies of the natural stands. There were no or small changes in the FH of needles with prolonged cold acclimation in constant conditions. Since the cold acclimation conditions (e.g. temperature, moisture, light, etc.) were similar for all the progenies during the two sampling times, the differences in FH among the progenies were due to the genetic factors and the conditions at the pollination sites (Lehtinen and Pulkkinen 2017; Andersson et al. 2018; Neimane et al. 2018; Alakärppä et al. 2019; Chan 2019). In this study, the same plus-tree genotypes were cultivated from the grafts in two locations more than 1600 km apart from each other. The light and temperature conditions differed greatly between these seed orchards, but due to the grafts, there was no chance for local adaptation. In a sense, as the epigenetic effect there is a possibility for local adaptation in seed development, such as in the development of pollen and embryos or both, in the new environmental conditions leading to different FH, thus giving a possible explanation for the results (Eriksson et al. 1980; Beuker 1994; Persson 1994; Lehtinen and Pulkkinen 2017; Alakärppä et al. 2019).

4.1.2 Frost hardiness of root and shoot growth (I)

The growth measurements of shoots and roots (e.g., new shoot elongation, dry mass ratios, root length, root tips) suggested that the threshold of freezing tolerance at a whole-plant level is between −8 °C and −16 °C. The FH varied from −10 °C to −15 °C for shoots and from −6 °C to −16 °C for roots, depending on the trait used in the FH assessment, without differences between the populations for either shoots or roots. The results differ much from FH of the needles that were obtained immediately after the freezing tests. The whole-plant freezing test followed by raising of the seedlings in the favorable conditions integrates the injuries in different organs. Then the most sensitive plant part determines the overall frost hardiness of the plant. According to our results, roots or root collar was the critical part in the whole-plant freezing tests, and therefore determines the overall FH of the seedlings. In accordance with our hypothesis, there were no differences in FH of roots among the populations, however. The progenies were presumably genetically close enough to each other, and therefore, roots did not differ in their response to freezing stress. Roots are considered as the most frost sensitive part of trees (Bigras et al. 2001). Our results based on root growth and root tip formation support that too. In a recent study was found, however, that new root tip formation in containerized seedlings was observed even after exposed to −30 °C (Di et al. 2019). This indicated that the roots, or some parts of the root system, may tolerate lower temperatures than previously thought if properly acclimated but this seemed not to have happened in the current study.

4.2 Frost hardiness of the woody horticultural plants tested

4.2.1 Effects of preconditioning temperature on frost hardiness (II)

The frost hardiness after one week of preconditioning at different temperatures indicated that different apple, blueberry, and blackcurrant cultivars were already quite frost tolerant in the first test. The FH may have increased because the saplings were exposed to cool temperatures in the field and stored for several days or even weeks in cool temperatures before being moved to different preconditioning conditions. However, one-week preconditioning seemed not to be long enough to reach a steady state of FH in each preconditioning temperature. Instead, there was more hardening in apple and blueberry but dehardening in blackcurrant with prolonged preconditioning, especially in −7 °C and −10 °C storage of all cultivars. Frost hardening of most woody plants takes place in phases where the first phase is driven by the increase of night-length and decrease in temperature (< 10 °C) and the second phase by low temperatures (Weiser 1970; Leinonen et al. 1995). However, apple and some other Rosaceae (e.g., pear) family trees are insensitive to photoperiod, but their growth cessation and dormancy induction are particularly affected by decreasing temperature (< 12 °C), not by increasing night-length (Heide and Prestrud 2005). The results from apple, blackcurrant, and blueberry cultivars which were kept at cold precondition in darkness revealed that the first phase of frost hardening had passed, and the plants were in the second phase of hardening at the beginning of preconditioning.

4.2.2 FH_{max} of apple, blueberry, and blackcurrant (II)

The preconditioning temperature range to reach the maximum FH of stems was between −3 °C and −7 °C, depending on species and cultivars. Since there was hardening, and in some cases even dehardening, between the first and the second sampling time, this study is not able to conclude whether the steady states at each preconditioning temperature were reached after three weeks or not. As shown in the study, the optimum depends on the plant species and the origin/cultivar. In forest trees, a steady state of FH was found to depend nonlinearly on air temperature. This was considered in the mathematical models as a piece-wise linear function where the maximum frost hardiness of Scots pine needles was reached at an air temperature of −16 °C (Leinonen et al. 1996; 1997). This is a much lower temperature than that observed in this study where FH turned to decrease already at temperatures below −7 °C but may be explained by different species and organs. Because there is much uncertainty about the optimum temperature for frost hardening, more studies are required for different species, cultivars/origins and organs such as roots, and even different tissues in stems as well (Takeuchi and Kasuga 2018; Di et al. 2019).

4.2.3 FH_{max} of apple, blueberry, and blackcurrant concerning their cultivation zones (II)

In general, at the northern distribution limit, the FH of xylem tissue of native species parallels the average annual minimum temperature isotherm. If winter temperatures drop below the threshold, the site is not considered suitable for the cultivation of the species (Quamme 1976; Becwar et al. 1981; Kreyling et al. 2015). In the cultivation of horticultural woody plants, Finland is divided into nine cultivation zones according to annual minimum temperatures ranging from −18 °C to −40 °C (Finland plant success zone map 2015). According to the 71-year historical records of winter injury in Finnish apple orchards, the temperatures for apple trees (without specifying the cultivars) in field conditions are possible at −22 °C in December and −29 °C in February (Lindén 2001). According to the results, it may be concluded that FH_{max} based on deep-supercooling would not be a critical factor for extending the cultivation areas of apple and blueberry beyond those current areas, but it is rather the question of other frost tolerance mechanisms, for example, lack of parenchyma cells in stems (Quamme 1976, 1991; Repo et al. 2008). In apple, the results suggest that the hardiness zone for ‘Pirja’ would be somewhat wider than for ‘Lobo’. In blueberry, the FH_{max} of ‘Arto’ was higher and more stable than that of ‘Aino’ and ‘Alvar’. This is contrary to the known hardiness zones of these varieties. Even though the potential hardiness zone for blackcurrant cultivar ‘Ben Tron’ is not known, the results by EIS and REL for FH_{max} suggest that all cultivars would have the potential to be used in most hardiness zones in Finland. The most tolerant blackcurrant cultivar could not be determined because of the high variation in FH between different methods (Takeda et al. 1993; Rowland and Ogden, 2005; Ehlenfeldt et al. 2006, 2012; Pramsohler et al. 2012; Salazar-Gutiérrez et al. 2016).

4.2.4 Susceptibility of different pear cultivars to deharden and reharden (III)

Based on the DTA data of shoots of the three pear cultivars, the FH of ‘Pepi’ was the highest and of ‘Conference’ the lowest at the beginning of the experiment. The same grading between the cultivars was observed after dehardening and rehardening treatments too. A relatively small change in LTE took place by dehardening and rehardening. This indicates that the cold-hardened xylem ray parenchyma cells are quite conservative in their FH changes in the temperature range between +5 °C and −7 °C. Therefore, longer exposures and/or higher temperatures than +5 °C would be

required for a more significant change in LTE, such as a change in LTE with cold acclimation/hardening in oak (Repo et al. 2008). The same grading between cultivars as by DTA was observed by the REL of the shoot, with 'Pepi' being the most frost hardy and 'Conference' the least frost hardy.

There was a larger difference in FH between the first dehardening and the following rehardening by REL than by DTA, but the variation in FH by REL was also higher than DTA. VD scoring yielded a somewhat different result since the FH of 'Conference' and 'Clara Frijs' by VD was the same after both treatments even though the FH of 'Clara Frijs' by VD increased during the first rehardening. Some differences in FH between VD and REL are probably due to biases or different premises of the methods (Luoranen et al. 2004). To conclude, the grading of the cultivars according to the FH of shoots fits well with the observations in field conditions where 'Pepi' has been found to have the best overwintering capacity.

In contrast to the FH of the shoot, there was a tendency for the FH of the buds to decrease in the rehardening conditions, without differences between cultivars. It is possible that an irreversible cell division was initiated in the buds in the dehardening condition (Nuotio et al. 1990), after which they lost their capacity to reharden and even continued to deharden. Similar results have been reported for many other woody plants (Pagter et al. 2011). Commonly, buds are more susceptible to freezing than shoots in most woody species (Kalberer et al. 2007a; Salazar-Gutiérrez et al. 2016). In deciduous trees, a short-term warm spell in winter may initiate cell divisions leading even to bud burst and therewith increase susceptibility to damage, e.g., apple, and cherry plum (*Prunus avium* [L.]). It has been suggested that late spring frosts occurring at the time of budburst are more destructive than midwinter frost in determining tree fitness (Lenz et al. 2013; Vitra et al. 2017).

4.2.5 Frost hardiness in bud dormancy of pear cultivars (III)

Even though bud rest (endodormancy) was broken quite comprehensively in all the cultivars at the start of the experiment (the proportion of the broken buds was between 81% and 99%), bud dormancy was released at different rates, depending on the cultivar. Bud break occurred most rapidly in the cultivar 'Conference', which was found to be the least frost tolerant. On the other hand, the proportion of broken buds was the lowest (81%) in the most frost-tolerant cultivar 'Pepi'. This indicates that in 'Pepi' the chilling requirement was not fulfilled at the time of sampling. The frost hardening and dehardening potential change during the dormancy, such that the plants are more susceptible to harden in endodormancy but to deharden in ecodormancy (Leinonen 1996). With a decrease in FH in the latter phase, there is a strong decrease in the rehardening capacity, being almost nil at the time of budburst (Leinonen et al. 1997; Vitra et al. 2017). Therefore, the timing of budburst has been considered a critical component of tree fitness because warm spells will promote irreversible deacclimation especially in buds in mid-winter (Arora and Taulavuori 2016; Vitra et al. 2017).

4.3 Comparison of frost hardiness assessment methods

4.3.1 Differential thermal analysis (II and III)

The low-temperature exotherm was observed at the lowest at −41 °C in apple and pear, and −38 °C in blueberry. In theory, LTE should not occur below −50 °C because homogeneous ice crystallization of pure water is −38.1 °C which is further decreased due to dissolved electrolytes in plant cells (Sakai and Larcher 1987). However, in this study LTE was occasionally higher than −38.1

°C indicating that the freezing in those samples took place by heterogeneous nucleation. Several studies on the structure and function of xylem ray parenchyma cells of woody plants have pointed out that the freezing events in these cells are related to the embolism, cavitation, ion contents of xylem sap, vessel size, cell wall rigidity, and the degree of lignification and cell maturation (Hacke and Sperry 2001; Alves et al. 2004; Ishikawa et al. 2009; Guillaume et al. 2014; Arias et al. 2015; Zhang et al. 2016). These may explain variability in the LTE occurrence rate and dehardening of blackcurrant after three weeks of preconditioning too. However, blackcurrant floral buds have multiple LTE as has been identified by Takeda et al. (1993). Even though the LTE occurrence rate in the stem was low in this study, DTA may have the potential to be used for blackcurrant, especially for buds. Therefore, further research on the deep undercooling of shoots and buds of the blackcurrant is required.

The LTE was observed in all pear stem samples, and the LTE occurrence rate was high in stems of apple and blueberry but low in blackcurrant. For apple, this is consistent with other studies where LTE was incidentally missing (Hong and Sucoff 1980). As LTE is considered critical for survival and has been found to define the distribution limit of several tree species, including apple, pear, blueberry, and blackcurrant (Quamme 1976; Pramsohler and Neuner 2013), this would be a potential measure of the FH of different cultivars of these. The lack of LTE in apples may be due to the small amount of or missing deep-supercooled parenchyma cells in stems, or they were dehydrated effectively by apoplastic freezing. Then no intracellular ice crystal formation occurred, or their freezing was not recorded with the temperature sensor set on the surface of the stem. The seasonal changes, the maturity of xylem, and the initial location of ice nucleation activity linked to primary freeze initiation, and the adaptive freezing behavior of the stem bark and flower buds of blueberry have been reported in previous studies (Flinn and Ashworth, 1994a, b; Kishimoto et al. 2014).

In blackcurrant, the second exotherm was found at higher temperatures (between −14 °C to −18 °C) than expected for LTE, similarly as in the DTA-profile of apical buds of Norway spruce with a 1 cm long piece of the stem (Räisänen et al. 2006b). The origin of this exotherm is not known. Furthermore, LTE was quite low in all cultivars at +3 °C for apple and blueberry, and typically there was not much difference between preconditioning temperatures. However, the intermediate exotherms (*i*LTE) were observed by DTA in many shoot samples of all the pear cultivars and some of the apple and blueberry samples, as in the previous studies on several woody species, e.g., pear, apple, blueberry and Norway spruce (Kaku and Iwaya 1978; Rajashekar and Burke 1978; Räisänen et al. 2006a). These may be due to the secondary xylem tissue in the shoots, e.g., the large number of or multiplex of deep-supercooled parenchyma/pith cells (Ashworth and Abeles 1984; Ketchie and Kammereck 1987; Takeda et al. 1993). Furthermore, the seasonal changes, and the initial location of ice nucleation activity, the intercellular spaces, water retention in the cell wall and organelles, and cell wall microcapillaries of tissues may affect supercooling and the occurrence of multiple exotherms (Kishimoto et al. 2014).

4.3.2 Comparison of frost hardiness of needles by REL and CF

In the study with Scots pine, the FH of needles by REL varied between −40 °C and −80 °C, depending on the plus-tree progenies, but much less by CF. At the first sampling time, the seedlings were already quite frost hardy, and there was no additional hardening in most of the progenies after four weeks at 5 °C in the growth chamber (T2) by REL. The frost hardiness of needles by REL is based on ion leaching from damaged cells. In chlorophyll fluorescence, F_v/F_m is a measure of the efficiency of the electron transfer chain in PSII (Ivanov et al. 2001), which is affected, for example,

by seasonal rhythms and chlorophyll content (Luoranen et al. 2004; Repo et al. 2006; Linkosalo et al. 2014). In addition, the curve estimation of FH may also affect the evaluation of different test methods (Sutinen et al. 2000; Repo et al. 2006). Although the correlation between REL and CF was significant, FH by REL was typically much higher than by CF. The FH differences varied between 3 °C and 34 °C, depending on the progenies. The electron transfer chain in PSII may be more sensitive to freezing stress than the cell membranes, mostly plasma membrane, thus explaining the different results (Sutinen et al. 2000; Rizza et al. 2001). In the test of Scots pine, measurements of needles suggest that most progenies may tolerate very low temperatures.

4.3.3 Regrowth and visual damage scoring (I, II, and III)

The growth of shoots and roots and the morphology of roots of Scots pine seedlings indicated that the threshold of freezing tolerance at the whole-plant level was between −8 °C and −16 °C. Although some differences were observed among the progenies, they were not consistent throughout the test temperatures and were not supported by the FH of needles by REL and CF or the growth of new shoots either. The threshold is defined by the organ with the lowest FH which may differ due to different physiological mechanisms (Domisch et al. 2018). For example, the stem was found to be less frost hardy than the needles as in some previous studies (Ryyppö et al. 1998; Repo et al. 2000a). The frost hardiness based on the root growth and the number of root tips supports the results of shoot growth, i.e., the threshold between −8 °C and −16 °C. Therefore, it may be concluded that in the whole-plant freezing tests, the damage took place either at root collar or in roots. Their damage impeded water and nutrient uptake and resource transportation between roots and shoots, thus inhibiting their growth.

In buds of horticulture woody species, the regrowth test indicates damage of the primordial shoot and therefore is not comparable with the FH of stems. However, as in the case of stems, differences were found in FH among four preconditioning temperatures within the same apple, blueberry, and blackcurrant cultivars. The temperature range to reach the maximum FH of buds was related to the preconditioning and species. There was no or minor additional hardening in buds between one and three weeks of preconditioning for apple and blueberry, but it was the opposite case in all the blackcurrant cultivars. The highest FH was even close to −80 °C in the stem of one blackcurrant cultivar after three weeks of preconditioning. However, there was high variability in the FH of buds estimated by visual observation. Buds have been noted to be very sensitive to environmental changes (Salazar-Gutiérrez et al. 2016). Therefore, the previous season condition or the short-term rapid changes in the storage temperature may have caused damage or increased FH before the start of this experiment. In addition, factors such as water content, subjective effects by the observer, sample size, and estimation method may affect the results too (Takeda et al. 1993; Lindén et al. 1996; Rowland and Ogden 2005; Ehlenfeldt et al. 2006, 2012). However, together with the other methods, visual damage scoring can be quite a useful and comparable way for the FH assessment of woody species.

4.3.4 Comparability of different methods (I, II, and III)

Control freezing tests have been widely used in the FH assessment of woody plants for many years and they form the basis for the frost hardiness assessment by different methods too (Weiser 1970; Leinonen et al. 1995; Repo et al. 2006). In the study with Scots pine seedlings, the FH of the needles by chlorophyll fluorescence (CF) among the progenies differed from the results by REL. In the study with different apple, blueberry, and blackcurrant cultivars, high variability was found

in FH by different methods, but quite consistent results were obtained for different species concerning the preconditioning temperatures. For the pear cultivars, the FH of the shoot by DTA (based on LTE) was much higher (between −38 and −41 °C) than by REL (−26 and −34 °C) and VD (−28 and −32 °C), or of buds by VD (−24 and −27 °C).

The highest correlation was typically found in FH by EIS and REL in accordance with previous studies (Ryyppö et al. 1998; Repo et al. 2000b; Li et al. 2009). The difference and the variation of the FH among cultivars and treatments was small by DTA in comparison to FH by REL and VD respectively, as has been found in other studies too (Quamme et al. 1973; Quamme 1991; Carter et al. 2001). The variability in FH by different methods can be explained by their different bases (Luoranen et al. 2004; Repo et al. 2008). In EIS and REL, this is a question of the integrated effect of cellular damage in different tissues (phloem, cambium, xylem), whereas VD of the stem is based on the color change of phloem by damage from green to brown during incubation. In addition, samples for EIS and REL were taken immediately after exposure, but in VD, damage scoring took place after a certain period (e.g., two weeks) of regrowth of samples. On the other hand, FH according to LTE of DTA in stems is based on deep-supercooling and consequent ice crystal formation in xylem ray parenchyma cells in species with a ring-porous xylem structure (Quamme 1991; Lindén et al. 2000; Carter et al. 2001; Vitra et al. 2017).

In addition, the relatively large difference in FH between DTA and other methods can also be explained by the differences in the pretreatment conditions. In the pear test, the samples were kept at 0 °C for 10 to 13 days before the start of the DTA-tests in the Luke Joensuu unit laboratory, whereas the dehardening treatment (D1-H), followed by the freezing tests for REL and VD, was started immediately after the samples arrived at the University of Helsinki laboratory. It is possible that the FH increased during the pretreatment in Joensuu compared to the treatment in Helsinki. DTA measures deep supercooling and consequent ice crystal formation in xylem ray parenchyma cells. In isolated cells, LTE is defined by the homogenous ice nucleation of water (−38.1 °C), with some additional decrease by diluted ions (Sakai and Larcher 1987). The low LTE-values in this study indicate that the shoots were close to their maximum FH, as previously observed in oak (Repo 2008).

5 CONCLUSIONS

5.1 Effects of pollination sites

The study aimed to determine the differences in the FH of different organs during cold acclimation in controlled conditions among the plus-tree progenies of Scots pine to explore the effects of the growing sites and conditions of the seed orchards. The frost hardiness of needles was higher in the Finnish seed orchard population than in the Ukrainian one or in the Finnish natural populations. These differences disappeared when the seedlings were raised in the greenhouse after the whole-plant freezing tests and then their FH was assessed according to the growth of shoots and roots. Based on the relatively small differences in the FH in the whole-plant freezing tests between Ukrainian and Finnish populations, the location of both of these seed orchards are suitable for seed production for northern conditions.

5.2 Preconditioning effects on horticulture saplings

The study aimed to calculate the most appropriate preconditioning temperature and its duration for assessing FH_{max} for different apple, blueberry, and blackcurrant cultivars in late autumn. The results reveal that the precondition temperature affected the FH of stems in all species and varieties during acclimation. The proper precondition temperature to reach the maximum FH varied between −3 °C and −7 °C, depending on the species and variety. For the species of apple and blueberry, the proper precondition for the development of the FH_{max} of aboveground parts in late autumn is three weeks between −3 °C, though a shorter time for blackcurrant would be enough. This study did not consider the FH of roots.

5.3 Dehardening and rehardening of pear cultivars

This study aimed to determine if pear cultivars differ in the depth of dormancy and to determine their susceptibility to deharden during mild weather periods in winter and reharden when the temperature decreases again. A clear relationship could not be found between the dormancy status and the extent of dehardening and rehardening in these cultivars. However, ‘Conference’ was found to be the least hardy cultivar in all conditions, and it had the most rapid bud development in forcing conditions. The hardiest cultivar was typical ‘Pepi’, which had the lowest proportion of bud break in the forcing conditions. Following a short warm period, some rehardening was found in the shoots but not in the buds. The frost hardiness of shoots by DTA was much higher than by REL and VD, which is explained by the different bases of the methods.

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