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Rehardening capacity in the shoots and buds of three European pear (*Pyrus communis* [L.]) cultivars following a warm spell in midwinter

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ABSTRACT

The rates of dehardening and rehardening in response to rapid temperature changes in winter are important traits that affect the survival, growth and productivity of the European pear (*Pyrus communis* [L.]) cultivars in northern countries. The frost hardiness (FH) of shoots of three pear cultivars were studied by a series of freezing tests, after sampling in natural conditions, after dehardening in a growth chamber at 5 °C for 3–4 days (D1) and 16 days (D2), and then after rehardening at −7 °C for 5–7 days (R1 and R2). The FH was assessed by a differential thermal analysis (DTA) to measure the low temperature exotherm (LTE) of shoots, by relative electrolyte leakage (REL) of shoots and by visual damage scoring (VD) of shoots and buds.

According to the DTA, the FH of the cultivars varied between −38 °C ('Conference' in D2) and −41 °C ('Pepi' in R2). The shoots of the cultivar 'Pepi' and 'Conference' had the highest and the lowest FH, respectively, in all conditions and methods. All the cultivars had the lowest shoot FH after dehardening in either D1 (between −26 °C and −30 °C by REL and between −28 °C and −30 °C by VD) or D2 (between −38 °C and −40 °C by DTA), and the highest FH after rehardening (R1) preceded by D1 (between −30 °C and −34 °C by REL, and between −29 °C and −32 °C by VD). After the dehardening in D1, the buds did not reharden but continued to deharden (the average FH by VD − 24.5 °C). In the forcing conditions, bud growth was resumed most rapidly in 'Conference', indicating a shallower dormancy in this cultivar than in 'Pepi' or 'Clara Frijs'. We conclude that the pear cultivars responded to temperature changes in mid-winter, but less than expected, and the responses were similar in all cultivars.

1. Introduction

The pear (*Pyrus* [L.]) is one of the most economically important fruits worldwide. In northern regions, the cultivation of the European pear (*Pyrus communis* [L.]) is limited by the short growing season and harsh winter conditions (Ehlenfeldt et al., 2006; Eccel et al., 2009). In addition, especially at the border between maritime and continental climates, such as in Finland, the fluctuation of winter temperatures is predicted to increase by climate change (Kuwagata et al., 1994; Suomi, 2018). Therefore, the potential for frost damage of the European pear and many other woody horticultural species may increase in the future (Lindén, 2001; Laapas et al., 2012; Jylhä et al., 2014). Some pear varieties have been found to tolerate short-term exposure to −40 °C (Gusta et al., 1983; Quamme, 1976, 1991), and therefore could potentially survive harsh winter conditions in northern regions. However, mild winters accelerate the break of dormancy more in pear varieties than in other commonly cultivated fruit species, e.g., apple (*Malus domestica* [L.]), whereupon spring development takes place earlier too (Drepper et al., 2020). Accordingly, the ability to maintain frost hardiness (FH) and/or to reharden in fluctuating temperature conditions is critical for the winter survival of pear cultivars.

Temperature is a major external driver of frost hardening and dehardening of woody plants in mid-winter. In addition to temperature and its duration, the rate of hardening and dehardening are dependent

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upon the genotype (Repo et al., 2000; Nilsson, 2001; Kalberer et al., 2006). In general, FH is synchronized with changes in ambient temperature, and FH is supposed to reach a steady state at stable temperature conditions with some delay (Repo and Pelkonen, 1986; Leinonen, 1996). However, the response of FH to temperature varies with the phase of annual development (Leinonen et al., 1997). The hardening competence, i.e., the capacity of environmental factors to induce or retain FH (deharden/reharden), changes gradually during dormancy such that the rehardening competence is gradually lost during dehardening (Gusta and Fowler, 1976; Repo, 1991; Leinonen, 1996). Therefore, the rate of change between the states, and the change in the internal status during dormancy, will determine whether the transient warm spell will damage trees or not (Kalberer et al., 2006; Nielsen and Rasmussen, 2009). The time to budburst in forcing conditions is considered as a measure of depth of dormancy (Pagter et al., 2011). Even though the phase of dormancy and FH are not directly linked together, an early budburst refers indirectly to an early decrease of FH, insofar that the chilling requirement for the rest break of bud is fulfilled.

The mechanisms of dehardening and rehardening are important determinants for the survival of woody plants in mid-winter and early spring (Kalberer et al., 2006; Nielsen and Rasmussen, 2009). Loss of FH is associated with substantial changes in internal factors of plants, such as cell/tissue-water relations and carbohydrates affected by altered respiratory metabolism (Stitt and Hurry, 2002; Pagter and Arora, 2013). In fluctuating environmental conditions, FH is dependent on tissue and organ as well (Repo, 1991; Nielsen and Rasmussen, 2009; Pagter et al., 2011). The bud is the most frost susceptible aboveground organ and may be injured by frost after a warm spell in winter (Kalberer et al., 2007a). 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 they reharden slowly when the temperature cools again (Räisänen et al., 2006b). Studies of Scots pine (*Pinus sylvestris* [L.]) show that the needles and buds are more frost tolerant under constant cold temperature than when subjected to a fluctuating environment, but the shoot performs more stable than needles and buds under the same condition (Repo, 1991; Repo et al., 1996). On the other hand, dehardening is significantly faster than rehardening during dormancy in apple (*Malus domestica* [L.]) (Howell and Weiser, 1970). In spite of 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.

The assessment of FH of woody plants is commonly based on controlled freezing tests in a series of frost temperatures. Following exposure, cellular damage can be measured by relative electrolyte leakage (REL), electrical impedance, by scoring of the visual damage of leaves, needles and buds, or according to the color changes in the cambium and phloem in the shoots (Dexter et al., 1932; Repo et al., 2000; Burr et al., 2001). The differential thermal analysis (DTA) in FH assessment is based on the recording of intracellular freezing in critical cells that are localized in the ray parenchyma cells of shoots in species with a ring-porous xylem structure (Ashworth and Abeles, 1984; Arias et al., 2017). The freezing of deep-supercooled cells results in a low temperature exotherm (LTE) that has been suggested to predict the northernmost distribution limit of woody tree species (Quamme et al., 1982; Wisniewski et al., 2003). LTE has been observed in pear varieties (Quamme, 1976; Gusta et al., 1983) but many species miss LTE, which limits its use in FH assessment, however (Gusta et al., 1983; Fujikawa and Kuroda, 2000; Neuner et al., 2019). Therefore, we may assume that a DTA may discriminate the pear varieties in their response to dehardening and rehardening conditions.

In this paper, we aim 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. In addition, we studied the suitability of DTA for assessing the FH of different pear varieties. We hypothesized that the pear varieties differ in their response to warm and cold spells in winter.

Table 1
The country of breeding and the recommended hardiness zone for cultivation in Finland of the three pear cultivars in the experiment. The average calendar week and the temperature sum (in growing degree days, GDD, with a daily mean temperature of 5 °C as the threshold) for harvest maturity in Åland (2016-2019) are indicated.

Cultivar	Country of breeding	Hardiness zone[x]	Harvest maturity, wk (GDD, °C)
Conference	Great Britain	Ia	40 (1405)
Clara Frijs	Denmark	I (II)	37 (1251)
Pepi	Estonia	IV	38 (1302)

[x] Finnish Meteorological Institute (2015).

2. Materials and methods

2.1. Plant material, treatments and experimental design

The material consisted of the previous season's shoots of three dormant pear (*Pyrus communis* [L.]) cultivars ('Conference', 'Clara Frijs' and 'Pepi') (Table 1). The varieties '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 sampled trees were mature, of even age, and of normal health and medium vigor. The training system for trees in Jomala is central leader tall spindle with tree spacing 3.6 × 1.25 m; and in Piikkiö, modifed central leader with tree spacing 3.5 × 1 m. At the time of sampling on February 5, 2019, the air temperature in Piikkiö was 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 collected from each cultivar. Branches were sampled from different parts and sides of the trees, excluding top branches and the very lowest branches. Any branches with mechanical damage were not sampled. The subsamples from Jomala ('Conference' I–V, 'Clara Frijs' I–V, and 'Pepi' I-III) were excised from trees grafted on quince (*Cydonia oblonga* [L.]) clonal rootstock. While there were not enough shoots in 'Pepi' trees on the quince rootstock, the 'Pepi' subsamples (IV and V) from Piikkiö were excised from trees grafted on clonal *P. communis* Pyrodwarf rootstock or on a seedling rootstock of *P. communis*, respectively. The dehardening and rehardening treatments and the freezing tests in controlled conditions were carried out at the Luke Joensuu unit and at the University of Helsinki.

The measurements of the LTE by DTA at the Luke Joensuu unit were carried out on samples collected in Jomala. A total of 45 shoots per cultivar were randomly selected, maintaining their division in the field blocks, and 10 cm long subsamples were cut from the basal parts of the sampled shoots. The 10 cm shoot sections of each cultivar were enclosed in one plastic bag, and all the bags were wrapped inside bubble wrap and stored in a cold Styrofoam box with an ice bag. The samples were transported to Joensuu and stored in a Styrofoam box outside under the snow at around 0 °C. Due to practical reasons, the time under the snow varied from 10–13 days, because the freezing tests for all cultivars were not possible to run at the same time but in the subsequent days and nights. Before the start of the DTA tests, the shoot samples were randomly divided into new plastic bags by cultivars. There were a total of 225 samples of three cultivars, separated into 45

bags for the DTA-tests five times during the dehardening and re-hardening treatments (3 samples/bag × 5 bags × 3 cultivars × 5 times of sampling).

The experimental design and the sampling times were as follows: i) The first DTA-test was carried out after the samples were maintained for 10–13 days in a Styrofoam box outside under the snow (natural conditions, N); ii) After the first sampling, the rest of the samples were moved to the growth chambers (PGW36, Conviron Ltd., Winnipeg, MB, Canada) to deharden for four days at 5 °C (short dehardening, D1); iii) At the end of the D1 period, some of the samples were moved to a growth chamber at −7 °C to reharden for 7 days (R1); iv) At the end of the D1 period, some of the samples were left in the same dehardening conditions for an additional 12 days (long dehardening, D2); v) At the end of the D2 period, the samples were moved to the growth chamber at −7 °C to reharden for seven days (R2). The DTA-tests were carried out at the end of each period. In order to avoid rapid temperature changes between 5 °C (D1, D2) and −7 °C (R1, R2), the move of the samples took place in two phases. In the first phase, the sample bags were set in a Styrofoam box at a temperature of −3 °C for one day and after that at −7 °C for seven days. There was no light in the box or in the growth chambers in any of the conditions.

For the freezing tests 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 by 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 Umwelttechnik, Reiskirchen, Germany) and subjected to a dehardening treatment at 5 °C for 3 days (D1-H where H refers to Helsinki), followed by a re-hardening treatment (R1-H). In the latter phase, the temperature was lowered from 5 °C to −7 °C during 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 re-hardening period.

2.2. Differential thermal analysis (DTA)

At each sampling time, the 75 samples (five samples/bag) in 15 bags (5 bags × 3 cultivars,) were measured. For DTA, ten millimeter long piece without buds was cut from the middle part of the shoot, three replicate pieces of each shoot, i.e., 45 samples (including three cultivars) in total, at each time. There were 12 samples in one DTA run. Therefore, four runs were needed to measure all the samples at each sampling time. Following the last sampling at each time, the bags were moved to the subsequent conditions. The DTA samples were placed 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 pieces and the reference junction was measured with NiCr/Ni thermocouples (diameter 0.25 mm). The thermocouple was set into the pith part of the sample pieces, wrapped with an aluminum foil, and then placed into the plastic tube in the block. The temperature of each block was measured with 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 °Ch^{-1}. Freezing events were detected as exotherms, i.e., a high temperature exotherm (HTE) for apoplastic freezing and an LTE for intracellular freezing.

2.3. Frost hardiness by REL and visual damage scoring

At the University of Helsinki, the frost hardiness of all three cultivars was tested both at the end of the dehardening (D1-H) and re-hardening (R1-H) phases by controlled freezing tests in darkness, followed by the assessment of frost damage by relative electrolyte leakage (REL) and visual damage of scoring (VD). At both times, a total of 120 shoot samples from each cultivar were subjected to eight test temperatures, 24 samples from each of the five blocks. For the tests, three samples per temperature were packed in plastic bags by cultivars, with the exception of 'Pepi', where the sample number was two at some of the test temperatures. A small amount of water was sprayed in the bags to prevent drying of the samples and for the ice nucleation centers during the freezing. The bags were placed in a controlled-climate chamber (Weiss 2600/45, +5DU-Pi, Weiss Umwelttechnik, Reiskirchen, Germany). At the end of the dehardening treatment, the chamber temperature was first decreased from the initial dehardening temperature of +5 °C to −5 °C, which was maintained for 10 h to ensure ice nucleation in the samples. Then the temperature was lowered at the rate of 5 °Ch^{-1} to each target temperature that was maintained for 30 min. At each target temperature, five replicate sample bags of each cultivar were moved to 5 °C to thaw overnight. At the end of R1-H, the freezing test continued from the temperature prevailing at R1-H without thawing of the samples. Based on the expected hardiness level of the samples, the test temperatures were selected at 5 °C intervals between −5 °C to −35 °C in D1-H and between −10 °C to −40 °C in R1-H. The control samples were kept at +5 °C continuously. Temperatures during the treatments were recorded using a data logger (EL-USB-2-LCD+, Lascar Electronics, Wiltshire, UK).

After thawing, two 0.5 cm long internodal sections were cut off the shoot from each of the five replicates by test temperatures. The samples were rinsed with ultrapure water (RiOs™ 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 shaken on a rotary shaker (SHKE8000-8CE, Thermo Fisher Scientific, Marietta, USA [130 rpm]) for 22 h at room temperature (22 °C) before the measurement of the first electrical conductivity (L1), (Jenway, Felsted, Essex, UK.). The samples were heat killed by placing the tubes in a water bath at 95 °C for 1 h and shaken for another 22 h before the measurement of the second electrical conductivity (L2). Relative electrolyte leakage (REL) was calculated as:

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

The remaining samples were incubated in plastic bags at room temperature (+22 °C) inside a Styrofoam box for 13 days and then the shoots and buds were visually scored as either dead or alive. The shoots were scored after removing cortical tissue from the full length of a sample with a surgical knife. Live samples had green or greenish phloem tissues. Ten axillary buds from each replicate sample were cut longitudinally and injuries were assessed using a dissecting microscope. A green or greenish color indicated a living bud. In the case of green leaf primordia but brown vascular tissues and floral primordia, the buds were classified dead.

2.4. Bud dormancy

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 μmol m^{-2}s^{-1} photon flux density, a temperature of 19 °C and a 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 placed on plastic trays (VEFI PK050, Vefi Europa, Skierniewice, 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 as 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.

2.5. Statistical

The differences in LTE (from DTA), and FH accordingly, between the dehardening and rehardening conditions of cultivars, were tested using one-way ANOVA and the Holm-Bonferroni method (IBM SPSS 25.0, IBM Co., New York, USA). 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). FH was also estimated according to the change in relative electrolyte leakage (REL) in shoots. The FH estimates were analyzed for each cultivar and hardening treatment using the nonlinear mixed model (NLMIXED; Usage Note 56992, SAS) in SAS for Windows 9.4 (SAS Institute Inc., Cary, NC, USA). 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:

$$y_i = \left[a + \frac{(d - a)}{1 + e^{b(c - x_i)}} \right] \quad (2)$$

where y_i is the observed value of the ith case of the dependent variable (VB, VS, REL); x is the temperature of the ith 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.

We were 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

$$DT_{50} = c - \frac{log\left[\frac{(d - 0.5)}{(0.5 - a)}\right]}{b} \quad (3)$$

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 described by Lappi and Luoranen (2018). Two of the five sample blocks of 'Pepi' for REL and visual scoring were collected from different rootstocks. Therefore, for 'Pepi', we analyzed the DT_{50} values both for the whole data and separately for only three blocks with same rootstock. Because there were no statistically significant differences among those three blocks the estimates for the whole data are presented only. The differences between cultivars in bud dormancy were tested by ANOVA (Tukey test).

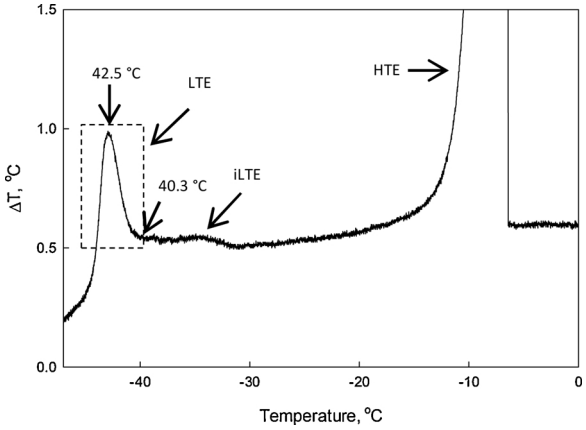

Fig. 1. An example of a DTA curve of the previous season's pear shoots (cv. 'Pepi') after seven days in a rehardening condition (−7 °C), preceded by 16 days in a dehardening condition (5 °C). High and low temperature exotherms (HTE and LTE), and intermediate exotherms (iLTE) are shown on the right, left and middle of the curves, respectively. The initiation and peak of the LTE are indicated with arrows.

3. Results

3.1. Frost hardiness by DTA

The DTA profiles for the shoots were characterized by two exotherms, i.e., a high temperature exotherm (HTE) and a low temperature exotherm (LTE), in all samples of all the cultivars (Fig. 1). The peak value of the LTE varied between −38 °C and −41 °C in all the cultivars and treatments (Fig. 2). Accordingly, 'Pepi' had the highest FH (−41 °C in R2) and 'Conference' the lowest FH (−38 °C in D2) among all the cultivars and conditions. In all the conditions, there were significant differences between the cultivars, except in D1 (Fig. 2). Among the different conditions, all the cultivars had the lowest FH in D2 ($P < 0.001$), and the highest in R1 ($P < 0.001$) (Fig. 2). 'Conference' was the only cultivar that dehardened significantly (during D2). All the cultivars were able to reharden during either R1 or R2, or both rehardening treatments. Besides HTE and LTE, intermediate exotherms (iLTE) were observed by DTA in many shoot samples of all the pear cultivars.

3.2. Frost hardiness of shoots by REL

Following the three-day dehardening treatment, the FH varied between −26 °C and −30 °C, but there were no significant differences between the cultivars ($P = 0.10$) (Fig. 3). During the seven-day rehardening treatment, the FH increased significantly in cv. 'Conference' ($P = 0.049$) but not in 'Clara Frijs' ($P = 0.217$) and '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 hardier than 'Conference'.

3.3. Frost hardiness by visual damage scoring

Following the three-day dehardening treatment, the FH of shoots was between −28 °C and −30 °C, but no significant differences were observed between the cultivars (Fig. 4). During the seven-day rehardening, the FH increased significantly in 'Clara Frijs' ($P = 0.012$) but the increase of FH in other cultivars was not statistically significant. After the rehardening treatment, the FH was significantly different between the cultivars ($P < 0.001$), with 'Pepi' being hardier than the other two cultivars.

After the three-day dehardening treatment, the FH of buds was between −24 °C and −27 °C, but there were no differences between the

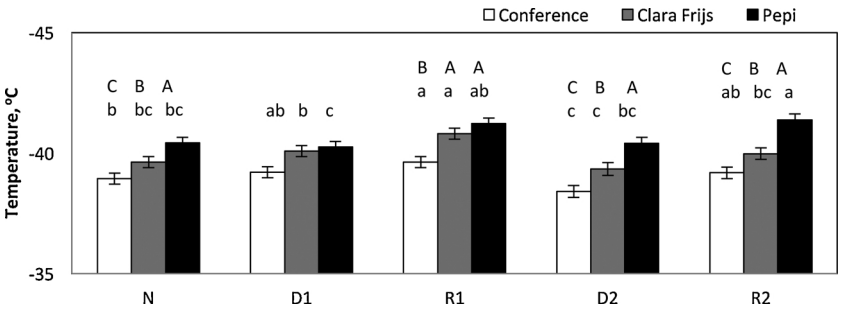

Fig. 2. The mean low temperature exotherm (± standard error) of three pear cultivars ('Conference', 'Clara Frijs', 'Pepi') sampled from outside on February 5th and 6th, 2019, and tested by DTA after different dehardening and rehardening treatments: Outside (N), short dehardening (four days at 5 °C) (D1) followed by rehardening (seven days at −7 °C) (R1), or long dehardening (16 days at 5 °C) (D2), followed by rehardening (seven days at −7 °C) (R2). Different capital letters indicate the differences ($P < 0.05$) between the cultivars within the same condition, and different small case letters indicate the differences ($P < 0.05$) between the conditions within the same cultivar (no letters indicate no differences, n = 15).

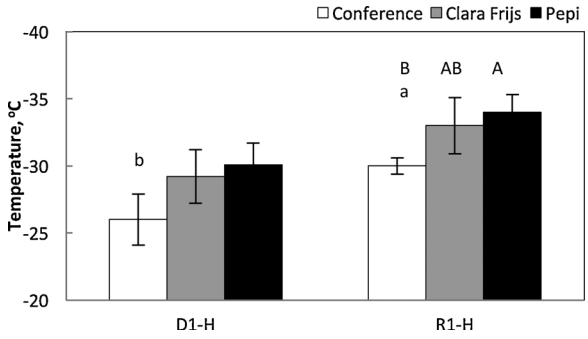

Fig. 3. Frost hardiness (± asymptotic standard error) of three pear cultivars ('Conference', 'Clara Frijs', 'Pepi') sampled from outside on February 5th, 2019, as assessed by relative electrolyte leakage method (REL) after three-day dehardening at 5 °C (D1-H) followed by five-day rehardening at −7 °C (R1-H). The different capital letters indicate the statistical differences ($P < 0.05$) between the cultivars within the same treatment, and the small case letters the differences ($P < 0.05$) between the treatments within the same cultivar (no letters indicate no differences).

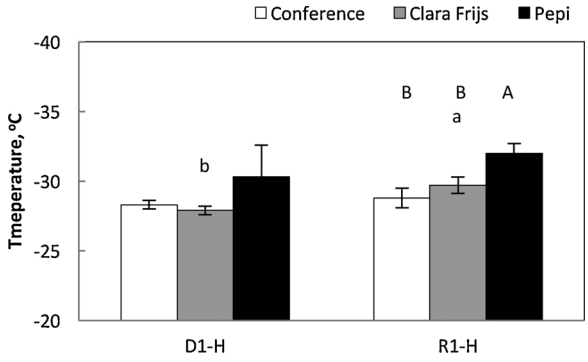

Fig. 4. Frost hardiness (± asymptotic standard error) of shoots of three pear cultivars ('Conference', 'Clara Frijs', 'Pepi') assessed by visual rating of injury, sampled from outside on February 5th, 2019, and dehardened for three days at 5 °C (D1-H), followed by five-day rehardening at −7 °C (R1-H). The different capital letters indicate the statistical differences ($P < 0.05$) between the cultivars within the same treatment, and the small case letters the differences ($P < 0.05$) between the treatments within the same cultivar (no letters indicate no differences).

cultivars ($P = 0.53$) (Fig. 5). FH decreased in cv. 'Clara Frijs' by 3 °C ($P < 0.001$) during rehardening, but there was no significant change in the FH of 'Conference' ($P = 0.48$) and 'Pepi' ($P = 0.08$). There were no significant differences between the cultivars after rehardening either.

3.4. Depth of bud dormancy

In all pear cultivars, bud dormancy was broken quite comprehensively, as more than 80 % of the buds broke in five weeks, with even 99 % in 'Clara Frijs', in the forcing conditions (Table 2). Dormancy was

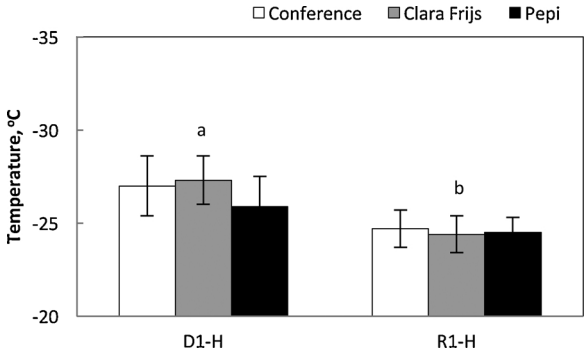

Fig. 5. Frost hardiness (± asymptotic standard error) of buds of three pear cultivars ('Conference', 'Clara Frijs', 'Pepi') by visual damage scoring, sampled from outside on February 5th, 2019, and tested for FH after dehardening for three days at 5 °C (D1-H), and then after five-day rehardening at −7 °C (R1-H). The different small case letters indicate the differences ($P < 0.05$) between the treatments within the same cultivar (note: no differences between the cultivars) (no letters indicate no differences).

Table 2
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. Samples were collected on February 5th, 2019. Values followed by different letters are significantly different ($P < 0.05$) by Tukey's test. (n = 15).

Cultivar	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

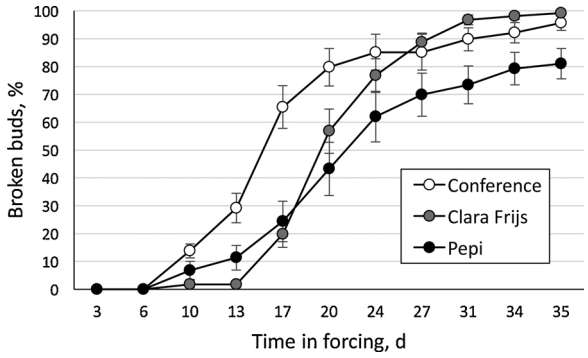

Fig. 6. The mean percentage (± standard error) of broken buds of three pear cultivars in the forcing conditions. The samples were collected on February 5th, 2019, (n = 15).

relatively weak, as the first bud break took place after only one week in the forcing conditions, with the first in the cultivar 'Conference' and the last in 'Clara Frijs' (Fig. 6). The relative time to bud break was also

shorter in ‘Conference’ than in ‘Pepi’ and ‘Clara Frijs’.

4. Discussion

We studied changes in the FH of pear cultivars under fluctuating temperatures (dehardening and rehardening) in winter using different FH assessment methods. Because the cultivars are recommended for cultivation at different hardiness zones in Finland, they were expected to differ in their response to warm and cold spells in winter. By changing the air temperature to induce dehardening and rehardening, we aimed to explore the potential adaptability of cultivars to fluctuating winter temperatures.

4.1. Comparison of FH assessment methods

In all cultivars, the FH of the shoot by DTA (based on LTE) was much higher (between −38 °C and −41 °C) than by REL (−26 to −34 °C) and VD (−28 °C and −32 °C), or of buds by VD (−24 °C and −27 °C). The LTE was observed in all pear samples. As LTE is considered critical for survival and has been found to define the distribution limit of several tree species, including pear (Quamme, 1976), it would be a potential measure of the FH of different pear cultivars. The difference and the variation of the FH by DTA among cultivars and treatments was small in comparison to FH by REL and VD, as has been found in other studies too (Quamme et al., 1973; Quamme, 1991; Carter et al., 2001; Wu et al., 2019). The explanation for the relatively large difference in FH between DTA and other methods can be explained by the different bases of the methods and by the differences in the pretreatment conditions. Before the start of the DTA-tests at the Luke Joensuu unit, the samples were kept at 0 °C for 10–13 days, whereas the dehardening treatment (D1-H), followed by the freezing tests for REL and VD, was started immediately after the samples arrived at University of Helsinki. 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 et al., 2008). On the other hand, REL measured immediately after the exposure is based on the ion leakage out of the cells from damaged tissues, i.e., the phloem, cambium and xylem. Therefore, REL is an integrated measure of damage in those tissues. The VD-method is based on the color change of the phloem and primordial shoot of shoots and buds from green to brown following 13 days of incubation, respectively. Because the VD- and REL-methods for shoots measure the properties of the same tissues, the corresponding FH were close to each other too (Lindén et al., 2000; Vitra et al., 2017).

In addition, the intermediate exotherms (iLTE) were observed by DTA in many shoot samples of all the pear cultivars, as in the previous studies on several woody species, e.g., pear, apple, blueberry (*Vaccinium corymbosum* [L.]) and Norway spruce (Kaku and Iwaya, 1978; Rajashekar and Burke, 1978; Räisänen et al., 2006a; Wu et al., 2019). The origin of these additional or multiple exotherms is not known. They 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; Takeda et al., 1993; Ketchie and Kammerech, 1987). Furthermore, the seasonal changes, and the initial location of ice nucleation activity, the intercellular spaces, water retaining in the cell wall and organelles, wall microcapillaries of tissues, etc. may affect supercooling and the occurrence of multiple exotherms (Kishimoto et al., 2014).

4.2. Susceptibility of different cultivars to dehardening and rehardening

Only ‘Conference’ shoots dehardened significantly when exposed to +5 °C, as measured by DTA, but the difference in FH was only 0.5 °C. All the cultivars were able to reharden in either one of the rehardening treatments, and ‘Pepi’ in both treatments. However, when measured by REL, a significant rehardening was observed only in ‘Conference’, and by VD only in ‘Clara Frijs’.

In the beginning of the experiment, the FH in shoots of the cultivar ‘Pepi’ by DTA was the highest and the cultivar ‘Conference’ the lowest. The same grading between the cultivars was observed after dehardening and rehardening treatments too. Even though the highest FH was observed after rehardening treatments, 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 response in the temperature range between 5 °C and −7 °C, and longer exposures and/or higher temperatures than 5 °C would be needed for a more significant change in LTE. The same grading between cultivars as by DTA, was observed by REL of shoot, ‘Pepi’ being the most frost hardy and ‘Conference’ the least frost hardy. There was a larger difference in FH between D1-H and R1-H by REL than by DTA but the variation in FH was higher, too. VD scoring yielded a somewhat different picture, since the FH of ‘Conference’ and ‘Clara Frijs’ by VD was the same after both treatments even though FH of ‘Clara Frijs’ by VD increased during R1-H. Some differences in FH between VD and REL are probably due to biases or differences in the premises of the methods (Luoranen et al., 2004; Wu et al., 2019). In addition, the rootstock effect, despite not being statistically significant, may have caused additional variance in the results regarding cultivar ‘Pepi’, and reduced the overall significance and resolution. To conclude, the grading of the cultivars according to FH of shoots fits well with the observations in field conditions where ‘Pepi’ has been found to have the best overwintering capacity.

As opposite to FH of shoot, there was a tendency for 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 conditions (Nuotio et al., 1990). Then they lost their capacity to reharden and even continued to deharden. Similar results have been reported for many other woody plants (Repo, 1991; Pagter et al., 2011). Commonly, buds are more susceptible to freezing than shoots (Kalberer et al., 2007a; Salazar-Gutiérrez et al., 2016). In deciduous trees, a short-term warm spell in winter may lead to budburst and therewith damage, e.g., apple, and cherry plum (*Prunus avium* [L.]) (Vitra et al., 2017). However, late spring frosts occurring at the time of budburst seem to be more destructive than midwinter frost in determining tree fitness (Lenz et al., 2013; Vitra et al., 2017).

The pear samples were collected during the dormancy when temperature is the main driver of FH (Repo, 1992; Leinonen, 1996). The initial FH of the shoot before start of D1-H treatment was not assessed by REL and VD. Therefore, the possible decrease of FH in dehardening conditions remains unknown. If we adopt the concept of steady-state in the control of FH by temperature, we may assume that FH was close to the steady-state corresponding to 5 °C in the dehardening conditions, with some variation among the cultivars (Repo and Pelkonen, 1986; Repo et al., 1990). In ‘Conference’ and ‘Clara Frijs’, FH was less in D2 than in D1 suggesting that the steady-state concept does not fully hold for these cultivars, and therefore would have required monitoring of the FH dynamics between D1, R1 and D2. In addition, FH may have reached in D1 the level where only partially reversible physiological processes were already commenced (Weiser, 1970). Several studies have demonstrated that plants are capable to reharden during dehardening depending on exposure time, frequency and magnitude (Gusta and Fowler, 1976; Repo, 1991). Even though not clearly observed here, dehardening of woody perennials exposed to warm temperature is more rapid than rehardening in cold temperatures (Chang and Reed, 2000) because the capacity to reharden is gradually lost with dehardening

(Leinonen et al., 1997). As an example, apple (*Malus domestica*) bark showed a loss of 15 °C in FH during one day in deacclimation conditions in the field but required three days to reverse (Howell and Weiser, 1970). On the other hand, hardening of peach (*Prunus persica* [L.]) bark and xylem tissues in autumn was significantly slower than the dehardening in spring (Arora et al., 1992).

4.3. Dormancy vs. susceptibility to change in FH

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' that 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 suggests that in 'Pepi' the chilling requirement was not fulfilled at the time of sampling. In temperate and boreal woody species, changes in FH occur at the same time as the dormancy status changes but their linkage changes during dormancy and with a rest break (Colombo, 1990). 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 as a critical component of tree fitness (Vitra et al., 2017). Because warm spells will promote irreversible deacclimation especially in buds in mid-winter, damage to buds would be of more concern than shoot damage in fluctuating temperature conditions (Arora and Taulavuori, 2016).

5. Conclusion

We aimed to determine if pear cultivars differ in the depth of dormancy and in their susceptibility to reharden after dehardening has commenced in winter. We could not find a clear relationship between the dormancy status and the extent of dehardening and rehardening in these cultivars. However, we found that 'Conference' was the least hardy cultivar in all conditions and it had the most rapid bud development in forcing conditions. The hardiest cultivar was typically 'Pepi' and it had the lowest proportion of budbreak in the forcing conditions. Following a short warm period, some rehardening was found in shoots but not in 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.

CRediT authorship contribution statement

Wu Dongxia: Methodology, Formal analysis, Visualization, Writing - original draft. **Palonen Pauliina:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **Lettojärvi Iiris:** Investigation, Data curation. **Finni Sanna:** Funding acquisition, Project administration, Formal analysis, Writing - review & editing. **Haikonen Tuuli:** Resources, Investigation, Writing - review & editing. **Luoranen Jaana:** Conceptualization, Methodology, Writing - review & editing. **Repo Tapani:** Supervision, Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2020.109638.

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