DOCTOR OF PHILOSOPHY

Studying the frost tolerance of Sitka spruce (Picea sitchensis [Bong.] Carr.)

Atucha Zamkova, Anastasia-Ainhoa

Award date:
2021

Awarding institution:
Bangor University

DOCTOR OF PHILOSOPHY

Studying the frost tolerance of Sitka spruce (Picea sitchensis [Bong.] Carr.)

Atucha Zamkova, Anastasia-Ainhoa

Award date:
2021

PRIFYSGOL BANGOR UNIVERSITY

STUDYING THE FROST TOLERANCE OF SITKA SPRUCE (*Picea sitchensis* [Bong.] Carr.)

A thesis submitted to Bangor University

by

Anastasia-Ainhoa Atucha Zamkova

In candidature for the degree of

Philosophiae Doctor

School of Natural Sciences,

Bangor University,

Thoday Building,

Deiniol Road,

LL57 2DF

Bangor (Gwynedd)

June 2021

Declaration

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

Anastasia-Ainhoa Atucha Zamkova,
In Bangor, on the 24/06/2021

Summary

Frosts can cause damage to the profitability of Sitka spruce (*Picea sitchensis* [Bong.] Carr.) tree plantations, as frost damage can cause dieback of the leader and loss of apical dominance resulting in a reduction of both wood quality and productivity. Damage to leaders can amount to a loss of productivity of up to seven years. Reducing frost damage could thus be an avenue for breeders to improve the resilience of Sitka spruce plantations.

Frost damage can occur in different forms, as plant tissue is affected by ice crystals and other, harmful effects caused by freezing temperatures. This damage can be counteracted by physiological and metabolic conditioning called frost tolerance. The mechanisms of frost tolerance in woody perennials are complex and controlled by many different genes influenced by abiotic and biotic factors. These adaptations are costly, and the plants' frost tolerance thus fluctuates throughout the year as temperatures decrease and increase throughout the season.

To understand the risk of frost damage to the forest industry, an investigation into the impact of climate change on the occurrence of frost damage in Sitka spruce was conducted. Modelling scenarios of frost tolerance in plantations throughout GB using temperatures modelled for different climate change scenarios indicated that Sitka spruce is not predicted to suffer an increased risk of early bud burst related frost damage. Conversely, climate change is predicted to improve the growing conditions and reduce the risk of frost damage in Sitka spruce throughout GB.

A systematic map of frost tolerance measuring techniques showed that measurement of frost tolerance involves controlling many factors, such as rate of freezing, thawing, and time of exposure, that affect the estimation of frost tolerance. Varying these factors can change the apparent frost tolerance, with measurements from the same tissue obtaining different estimates of frost tolerance. In order to improve the detection of frost tolerance, it is important to use the appropriate temperatures, with a wide enough range of temperatures to provide an accurate estimate of frost tolerance.

Field samples in the form of branch cuttings of Sitka spruce were collected from six forests in Scotland and one in Wales, from both frost damaged and undamaged trees. These cuttings were then rooted and grown in a controlled environment room, in addition to commercial Sitka spruce varieties. Once rooted, fresh needles grown from these trees were collected and their frost tolerance was tested by freezing treatments at +4 °C (control), -3 °C, -6 °C, -10 °C and -20 °C, and electrolyte leakage was determined by assessing changes in electroconductivity in pure water. No correlation between field observations of frost damage and electrolyte leakage was found.

Genotyping of DNA extracted from phenotyped samples was conducted by an external company, although the data has not yet been received due to the COVID-19 pandemic. A desk-based analysis was conducted to mitigate against the missing dataset. Four conifer studies revealing 165 genes associated with frost tolerance were identified. Of these, 84 possible candidate genes represented in Sitka spruce could be analysed to determine their function in relation to frost tolerance. Stress-related genes were found to be the most common category of genes identified by biological process ontology. Most genes were expressed in the cytoplasm and nucleus, and catalytic activity and protein binding were the main categories of molecular functions of the frost tolerance related genes. It was not possible to determine whether there were underlying genetic differences between the trees that conferred frost resilience among the 93 phenotyped samples. There was, however, evidence to suggest that Sitka spruce frost tolerance is in part genetic, as several genes related with frost tolerance in other conifers have been found to be present in Sitka spruce.

Acknowledgments

I would like to thank my supervisors, Dr Katherine Steele and Dr Andrew Smith at the School of Natural Sciences, Bangor University, for all their help and support. I would also like to thank Imam Sayyed and Chris Hardy at Maelor Forest Nurseries Ltd. for helping with the setup of the experiments. Professor John MacKay and Dr Hayley Thumas at the University of Oxford for their collaboration in genotyping my Sitka spruce samples. Gordon Dugan, John Lee, Andrew Noble, James Liley, Andrew MacQueen, Graham Chalk, and Simon Miller at TillHill Forestry, part of the BSW Timber Group Ltd., for helping me with the sampling collection. Dr Dave Buenavista, for his help in sampling and accompanying me on the trip. Llinos Hughes and Mark Hughes at Henfaes Research Centre, for their help with taking care of the plants I kept there. Helen Simpson, for helping me water the plants during my absence, and for the technical support provided. Sarah Chesworth, for her technical support. The team at the KESS office, for helping me deal with the funding paperwork, Dr Penny Dowdney and Brian Murcutt. Gillian Clayton for her help in navigating the university bureaucracy.

This PhD was funded by Knowledge Economy Skills Scholarships (KESS) II with Maelor Forest Nurseries Ltd. [Grant/Award Number: 80815]. KESS is a pan-Wales higher level skills initiative led by Bangor University on behalf of the HE sector in Wales. It is part funded by the Welsh Government's European Social Fund (ESF) convergence programme for West Wales and the Valleys.

Table of Contents

Index of figures

Index of tables

Abbreviations

ANOVA: Analysis of Variance

BPO: Biological Process Ontology

CAT: Catalase

CCO: Cellular Component Ontology

DTA: Differential Thermal Analysis

EIS: Electrical Impedance Spectroscopy

EL: Electrolyte Leakage

EPIC: Exon-primed Intron-crossing

EST: Expressed Sequence Tags

FC: Forestry Commission

FRDA: Forest Resource Development Agreement

GB: Great Britain

GCM: Global circulation models

GDD: Growing Degree Days

GO: Gene Ontology

GR: Glutathione reductase

GWAS: Genome-Wide Association Studies

HSP: Heat Shock Proteins

INA: Ice Nucleation Active

LD: Linkage Disequilibrium

LEA: Late Embryogenesis Abundant

LT_{50}: Lethal Time 50%

MAGIC: Multi-parent Advanced Generation Inter-Cross

MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry

MFO: Molecular Functional Ontology

POD: Peroxidase

PR: Pathogenesis Related

PSII: Photosystem II

QCI: Queen Charlotte Island (now called Haida Gwaii)

QTL: Quantitative Trait Locus

RCP: Representative Concentration Pathway

REL: Relative Electrolyte Leakage

RIL: Recombinant Inbred Line

ROS: Reactive Oxygen Species

SNP: Single Nucleotide Polymorphism

SOD: Superoxide Dismutase

SSR: Simple Sequence Repeats

VA: Visual Assessment

WGS: Whole Genome Sequencing

Chapter 1. Introduction

1. Overview

Frosts can cause severe damage to Sitka spruce (*Picea sitchensis* [Bong.] Carr.) plantations, as was shown in a 2015 frost that instigated this research. The damages were especially severe when they caused leader death, as side branches would have to compete to re-establish apical growth, in some cases causing two competing leaders to emerge. These damages can cause severe economic harm, eliminating the productivity of several years, and reduce timber quality. It has been estimated that in trees similar to Sitka spruce, such as Norway spruce (*Picea abies* (L.) H. Karst.), white spruce (*Picea glauca* (Moench) Voss), and black spruce (*Picea mariana* Mill.), a frost that affects the leader can slow the growth of a tree by as much as 7 years compared to an unaffected tree (Marquis et al., 2020). Sitka spruce is the main commercial softwood species in the UK, covering approximately 665,000 hectares and representing 25% of the total woodland area (Forestry Commission, 2019). The typical rotation length of Sitka spruce plantations in the UK is of 40 years (Herbert et al., 1999), and a reduction of 2-7 years of yield could cause a decrease of productivity of up to 5 to 17.5% for each individual tree, which in heavily affected areas could be a highly significant part of the forest.

Trees can tolerate frosts without damage by adaptations known as frost hardening, which are initiated by decreasing daylength and colder temperatures. Such adaptations, however, are costly, and are lost during dehardening, initiated by increased daylength and warmer temperatures. Climate change is expected to cause an increase in temperatures, and this increase could be beneficial by decreasing the total number of frosts, or not, by causing dehardening and making trees more vulnerable to frosts. Our company partner, Maelor Forest Nurseries, Ltd., and the Conifer Breeding Co-operative it is part of, was thus interested in identifying molecular markers that could be used for breeding Sitka spruce for frost tolerance. The unknown risks of climate change increased the need for this research, as forestry needs to take into account temperatures that occur in a longer time horizon than other industries.

2. Aims and objectives

- Review the mechanisms of frost damage and frost tolerance, as well as the internal and external factors that influence it (Chapter 2).
- Estimate the effects of climate change on Sitka spruce frost damage and examine the factors breeders need to take into account to avoid future frost damage (Chapter 3).
- Examine the frost tolerance measuring techniques currently used and their technical constraints (Chapter 4).

- Measure the frost tolerance of individual genotypes and identify populations that exhibited different patterns of frost tolerance (Chapter 5).
- Identify Sitka spruce genes that could be used for breeding for frost tolerance in the future (Chapter 6).

The project set out to test three hypotheses:

i) the differences in frost damage affecting Sitka spruce plantations that can be observed in the field are due to underlying genetic differences;
ii) these genetic differences can be found by measuring the frost tolerance of individuals and linking them to a SNP;
iii) climate change has an effect on the frost tolerance of Sitka spruce in the UK.

3. Thesis outline

The thesis is comprised of seven chapters, an introduction (Chapter 1), a review of the literature (Chapter 2), four investigative chapters (Chapters 3-6), and an overall discussion of the entire thesis that draws together the difference threads of the thesis (Chapter 7). The appendices included at the end of the thesis contain supplementary data used in Chapters 3 and 4. Each chapter is structured as a separate unit, with its own abstract, introduction, materials and methods, discussion and references.

Chapter 2: Frost tolerance of aboveground organs in gymnosperms

Provides a general review of frost damage and frost tolerance, the processes of acquiring and losing frost tolerance (*i.e.*, hardening and dehardening, respectively), and the internal and external factors that influence frost tolerance levels and development.

Aim:

- To examine the mechanisms of frost damage and frost tolerance, as well as the internal and external factors that influence it.

Chapter 3: Modelling the impact of climate change on the occurrence of frost damage in Sitka spruce (Picea sitchensis) in Great Britain

Predicts the degree of expected frost damage on Sitka spruce plantations in GB with climate change, by looking at predicted temperature trends as estimated by different models for different climate change scenarios and extrapolating the physiological and phenological status of Sitka spruce trees, giving an estimate of expected frost damage.

Aims:

- To approximate future trends of frost occurrence using three global and two regional climate change models.

- To utilise three indicators to determine how predicted future climate alters the occurrence and interaction of frosts with the physiological and phenological state of trees throughout the spring.
- To examine trends throughout GB to see whether there are regional differences in the expected changes to frost damage.
- To explore how the resolution of different climate models influences model prediction precision.

Chapter 4: Methods for measuring frost tolerance of gymnosperms: a systematic map

A systematic map that examines the techniques currently used to assess frost tolerance, and critically reviews the utility of these techniques as a proxy for determining the level of frost damage that a tree will experience in nature. I also examine the factors that should be accounted for in study design, in order to make frost tolerance measurements that are as accurate as possible and that reflect the results that would be expected in nature. This chapter was substantially and critically revised in light of my own experiences of estimating frost tolerance using the electrolyte leakage methodology. The chapter introduces many of the concepts and ideas that are useful to understand the problems identified in Chapter 5.

Aims:

- To document which frost tolerance measuring techniques are used and how they were used.
- To document the technical constraints faced when measuring frost tolerance.
- To note any reported correlations between different techniques in terms of results, by examining studies that use more than one method in further detail.

Chapter 5:

Describes the phenotyping of Sitka spruce for frost tolerance using electrolyte leakage of needles from 93 individual trees of known genotype that were collected at 6 sites across Scotland and Wales, following a wide-spread damaging frost event that occurred in 2015, and a selection of commercially available varieties of Sitka spruce seedlings and rooted stecklings supplied by my commercial partner Maelor Forest Nurseries Ltd.

Aims:

- Measure the frost tolerance of individual genotypes and identify populations that exhibited different patterns of frost tolerance.
- Check whether the results of the field observations correlate with the frost tolerance measured in the lab.

Chapter 6: Identifying potential frost tolerance genetic markers in Sitka spruce (Picea sitchensis) in silico

A desk-based study of the literature and analysis techniques used for genotyping, combined with a methodological description of the sample preparation for genotyping and an *in silico* analysis of publicly available databases using BLAST and BLASTN to explore the functional processes involved in the frost tolerance of Sitka spruce. It was intended that this chapter would comprise a genome-wide association study of genotyping data obtained with a SNP microarray. Unfortunately, due the COVID-19 pandemic the Infinium HTS iSelect analysis pipeline contracted out to a company in Canada was shut down, substantially delaying the provision of data. At the time of writing, I have been informed by my collaborators at the University of Oxford that the genotyping has been completed but I have not yet received the data to perform an analysis.

Aims:

- To generate SNP calls for the 93 phenotyped Sitka spruce samples which could subsequently be used for genome wide association studies.
- To identify which published frost-tolerant candidate genes/proteins have a homologue in Sitka spruce.
- To identify the predicted role of identified candidate genes (whether they are up or downregulated, and whether they play a positive or negative role in Sitka spruce frost tolerance).

***Chapter 7*: Synthesis**

I conclude the thesis by drawing from the content of each chapter and discussing my findings in relation to the overarching aims, hypotheses and chapter-specific objectives, giving recommendations for further research.

4. Contribution of author to each chapter

I have collected the data, analysed, and written all chapters included in this work, with a few exceptions mentioned below. The daily temperature data for Chapter 3 was obtained from a publicly available database, and the frost damage data used to verify the model was given by the TillHill Forestry Ltd. managers that collected it. My supervisors, Katherine Steele and Andy Smith, the co-authors for that paper, helped me with the design, analysis, and editing, but all the work was done by me. The SNP chip I used for genotyping the samples was designed for Professor John MacKay's group at the University of Oxford, who kindly let us share their genotyping platform. The chapters that were not written entirely by me state the individuals who contributed to the work in the chapter.

References

Forestry Commission, 2019. Chapter 1: woodland area and planting, in: *Forestry Statistics 2019*. pp. 1-37.

Herbert, R., Samuel, S., Patterson, G., 1999. Using local stock for planting native trees and shrubs. *For. Comm.* 1-8.

Marquis, B., Bergeron, Y., Simard, M., Tremblay, F., 2020. Growing-season frost is a better predictor of tree growth than mean annual temperature in boreal mixedwood forest plantations. *Glob. Chang. Biol.* **26**, 6537-6554.

Chapter 2. Frost tolerance of above ground organs in gymnosperms: a review.

Abstract

Frosts, resulting from minimum air temperatures below 0 °C, can cause damage to plant tissue through many mechanisms. The main two causes of frost damage are mechanical damage by ice crystal formation and by the denaturing effect of cold temperatures on proteins. Plant tissues are differentially affected by frost damage, as the structure of organs either facilitates frost damage or protects against it. Frost damage can be avoided by frost hardening, resulting in improved frost tolerance. There are many different mechanisms by which trees obtain frost tolerance, and gymnosperms differ from angiosperms in how they deal with frosts. Frost hardening is regulated by two main environmental factors, photoperiod and temperatures, and additional factors such as hydration and nutrition levels interact differently depending on the tree species. Many different genes are activated during hardening and dehardening, with many complex biological processes involved. Overall, it can be noted that frost tolerance is a very complex trait, regulated by complex environmental factors and a multitude of genes and gene products.

1. Introduction

Air frosts, for the purposes of this study, are defined as events that occur when daily minimum air temperatures drop below 0° C. Air frost events frequently lead to a decrease in air temperatures but not soil freezing. Soil takes longer to freeze and thaw than air due to its higher specific heat capacity. The mechanisms and causes of damage and resistance to the damage are different between roots and above ground plant tissues that are not protected by soil, as the latter are exposed to a wider range of temperatures. This review will only focus on the freezing tolerance of above ground tissues, which are exposed to air temperatures.

The frost tolerance level of the plant was defined as the warmest temperature that causes severe, unrecoverable harm. In many cases, this is estimated by a measurement called LT_{50}, which measures the temperature at which 50% of the tissue dies. In the context of this review, an increase in frost tolerance should be interpreted as a reduction of the frost tolerance temperature, *i.e.* an increase of the absolute value of the negative temperature.

Conifers are the largest group of gymnosperms, and they only dominate in dry, cold, or high-altitude habitats. Gymnosperms, unlike angiosperms, have not evolved xylem vessel elements, which have a bigger conduit diameter and a perforation plate at the end of the vessel (Figure 2.1). In angiosperms the evolution of vessel elements from tracheids increased hydraulic conductance, which increased the

volume of water reaching the crown during the growing season (Sperry et al., 1994). Conifers also tend to be evergreen, that is, they retain their leaves throughout the year, unlike angiosperms, which tend to be deciduous, and undergo senescence and leaf abscission in preparations for winter. Thus, evergreen trees have developed different mechanisms to deal with frosts.

Frosts have important economic effects on conifer plantations, as they inhibit the establishment of plantations. Cold air is heavier than warm air and sinks to the ground level, and this affects trees. This phenomenon also leads to frost hollows, low lying areas that trap cold air and tend to have more frequent frosts, with temperature differences as high as 14 K with the surrounding area (Trewin, 2005). In a study of Norway spruce (*Picea abies* (L.) H. Karst.), white spruce *(Picea glauca* (Moench) Voss), and black spruce (*Picea mariana* Mill.) lower parts of trees were more severely affected by frost (Marquis et al., 2020). Frost damage significantly affects trees below the 2 m height threshold deemed critical for frost tolerance (Cannell, 1985; Marquis et al., 2020). No effect of frost on tree growth was shown above this 2 m height threshold (Marquis et al., 2020). In trees shorter than 2 m in height, the effects of frosts on affected trees were economically significant. Affected trees needed 2 more years than non-affected trees to attain a height 50 cm above their height before the frost, and 7 more years to reach the 2 m height.

2. Frost damage in conifers

Frosts have a direct observable effect on conifer tissues. Frost damaged needles experience yellowing (chlorosis), and later browning, loss of turgor, and abscission (Figure 2.2). Damage to recently flushed buds can also be observed, with significant browning and necrosis in damaged tissues (Figure 2.3). Buds that have not gone through the phases of bud swelling and bud break can also be susceptible to damage (Figure 2.2). Damaged buds easily peel away from the stem, disintegrating and falling off when pulled (field observations by researcher). Stems can become damaged too, resulting in a loss of structure and a bent appearance that reduces wood quality (Figure 2.2).

At the lowest level of frost damage, Scots pine (*Pinus sylvestris* L.) suffers from partial needle discoloration and tip-burn (Reinikainen and Huttunen, 1989). This discoloration then extends to 50% of the needle, and the xylem in the stem and needle becomes brownish, while the phloem in the stem is lightened. Buds experience a change in colour but are still alive. At more severe stages of frost damage, needles experience more than 50% discoloration, and buds get considerably browned. Xylem tissue both in the stem and needles goes through browning as xylem cells disintegrate, while the phloem becomes dry. In the last stage of frost damage, the entirety of the needle experiences discoloration, and needles are shed. Buds are completely brown, and stem tissues also.

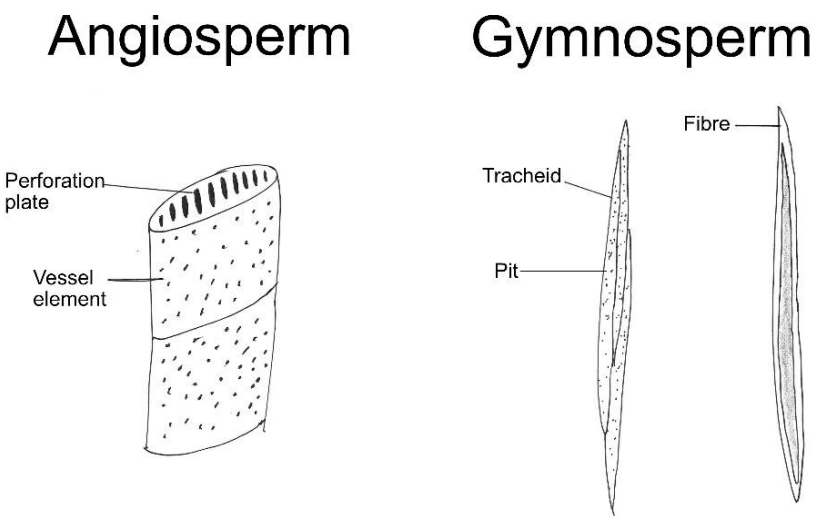

Figure 2.1. Representation of xylem cells in angiosperms and gymnosperms: vessel element (left) and tracheid (right).

Figure 2.2. Figure of frost damaged Sitka spruce (*Picea sitchensis* [Bong.] Carr.) tree. Black arrow points at branch with shed needles. White arrow points at branches bent by frost.

Figure 2.3. Frost damage to a bud after bud burst in Sitka spruce (*Picea sitchensis* [Bong.] Carr.).

2.1. Xylem conduit structure in conifers

Vessel elements, or trachea, the cell type of angiosperm xylem, are tubular vessels with end openings called perforation plates, which tend to have a bigger diameter than tracheids (Figure 2.1). Tracheids, the cell type of gymnosperm xylem, lack a perforation plate, tend to have a much smaller diameter, and carry water through pits, openings in the secondary cell wall to help adjacent cells exchange fluids. The bigger diameter of trachea, as well as the bigger water flow through the perforation plate allow for bigger water transport to the crown.

The greater ability of angiosperms to transport water with vessel elements, the trachea, is negatively correlated with resistance to cavitation by freezing and thawing, or freeze-thaw embolism (Davis et al., 1999; Feild and Brodribb, 2001; Sperry et al., 1994; Sperry and Sullivan, 1992). A non-vessel forming angiosperm such as *Tasmannia lanceolata* (Poir.) A.C.Sm., is as immune as gymnosperms from the same region to freeze-thaw cavitation, even as vessel forming plants suffer from significant freeze-thaw embolism (Feild and Brodribb, 2001).

Xylem conduits of conifers have a smaller mean diameter size compared to angiosperm trees, including diffuse porous (*e.g.*, maple and birch) and ring-porous (*e.g.*, oak and ash) tree species (Davis et al., 1999; Feild and Brodribb, 2001; Sperry and Sullivan, 1992). This is why gymnosperms tend to experience loss of conductivity only at very low xylem pressure, as they tend to have tracheid diameters that are much smaller than the diameters of xylem vessels in angiosperms (Davis et al., 1999).

Diffuse porous trees form vessel elements of the same diameter throughout the growing season, whereas ring-porous tree species tend to form the larger vessels early in the season. The mean conduit diameter, from smallest to largest, is that of conifers, shrubs, diffuse porous and ring-porous trees (Davis et al., 1999; Feild and Brodribb, 2001; Sperry and Sullivan, 1992). Conifer species lose much less xylem conductivity in comparison to angiosperm species, with 30% loss for conifers compared to more than 80% for angiosperms (Sperry et al., 1994).

Most of the conifers studied (Davis et al., 1999; Feild and Brodribb, 2001; Sperry and Sullivan, 1992) seem to have a tracheary element diameter lesser than 15 µm, which has been estimated to be the threshold mean diameter size for freeze-thaw embolism sensitivity in trees in Tasmania (Feild and Brodribb, 2001). For northern hemisphere conditions, the estimated threshold is higher, at 30 µm mean tracheary element diameter (Davis et al., 1999). This cut-off value is quite abrupt, as vessels with a higher diameter are sensitive to freeze thaw cavitation even at xylem tensions as high as -0.5 MPa (Davis et al., 1999). The difference between northern and southern hemisphere thresholds may be due to the very fast thawing rate used in the experiment, at 8 K h^{-1}, by Feild and Brodribb (2001). However, this thawing rate actually occurs on occasion in Tasmania (Feild and Brodribb, 2001) , so its use was justified. Tracheid diameter is correlated to susceptibility to freeze-thaw embolism (Mayr et al., 2003).

Diffuse porous trees prepare for growth in spring by refilling embolized vessels through positive root pressures, which happen when the soil warms, and by growing new vessels through the creation of earlywood in ring porous species (Sperry et al., 1994). In conifers, refilling of embolized tracheary elements happens through unknown mechanisms, one of the speculated mechanisms being the use of surface tension to dissolve air (Mayr et al., 2006; Sperry et al., 1994). The regrowth of vessels in ring porous trees means they start growing two weeks later than diffuse porous species, and a single late frost can completely destroy the year's growth (Sperry et al., 1994; Sperry and Sullivan, 1992).

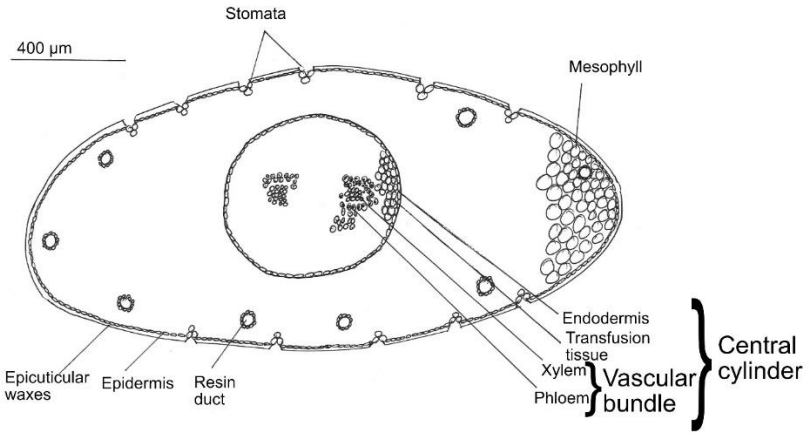

Figure 2.4. Schematic drawing of transverse cross section of conifer needle.

2.2. Effects of freezing on conifer tissue

Microscopy studies describing cellular frost damage in Sitka spruce were not found but studies in other species indicate that freezing affects conifer tissues both by the growth of ice crystals intra- or extra-cellularly and by other physical changes to the structure of the tissue. Freezing shrinks needles, as the air spaces are reduced when water moves into those spaces to form ice, as shown in a study in Monterey pine (*Pinus radiata* D. Don) (Roden et al., 2009).

Light microscopy examination of frost damage in Scots pine after freezing temperature exposure and recovery shows that severe frosts led to plasmolysis and disintegration of mesophyll cells in needles (Ryyppö et al., 1997). These plasmolysed cells did not recover even as the surrounding tissue recovered. Severity of frost damage occurred in a continuum that spanned from the non-lethal cellular process disruption to the complete disintegration of cells. The initial stage was a reduction in size of starch granules, followed by tannin precipitation in the central vacuole. At higher levels of damage, cells rupture, cell walls lyse, and large starch grains accumulate outside cells.

Another light microscopy study in Scots pine also shows the mesophyll cells underwent disintegration and plasmolysis at the relatively warm freezing temperature of -4.5 °C (Holopainen and Holopainen, 1988). Smaller scale observations were made with electron microscopy. In the first stage of frost damage, the endoplasmic reticulum appeared dissolved, and ribosomes precipitated in the chloroplast. In the later stages, all ribosome structure disappeared, the fibrous structure of the cell wall was lost, becoming granulated throughout as the tissue got more damaged. In subsequent phases, the tonoplast ruptured, and all the substances in the cytoplasm got mixed. In the final phase of injury, cell walls disintegrated, and protoplasts collapsed.

2.2.1. Ice Damage

Ice can form intra- or extra-cellularly, and the degree of harm will depend on the type of ice formation. Extracellular freezing leads to dehydration, as there is less water in the cell, which leads to the shrinking of the cell wall (Fujikawa et al., 1999; Kasuga et al., 2007). Extracellular ice formation without the accompanying dehydration did not lead to frost damage in dehardened needles of *Pinus canariensis* C.Sm. ex DC. (Luis et al., 2007). Intracellular ice formation leads to the deformation and at more severe stages, rupture of cells (Roden et al., 2009).

Light microscopy and electron microscopy can only detect what happens after thawing, while cryo-fixation preserves the distribution of water, ice, and gas spaces (reviewed by Roden et al., 2009). A cryo-scanning electron microscopy study in cryo-fixated unfrozen, frozen and thawed Monterey pine needles showed that within the vascular bundle of the needle (Figure 2.4), there was not as much damage as in the mesophyll (Roden et al., 2009) . Phloem cells were damaged by ice during freezing, particularly in tissue that had not been hardened, with no particular pattern in xylem cells. The degree

of harm from intracellular ice formation depends on the tissue and its degree of hardening. When ice formed in mesophyll spaces, it pressed against mesophyll cell walls and caused the dehydration of mesophyll cells. Upon thawing, mesophyll cells from tolerant tissue regained their previous volume and shape, and the space between them filled with air, while unacclimated cells did not recover to their previous size and the intercellular spaces were still filled with liquid. Transfusion tracheids, which with transfusion parenchyma form transfusion tissue (Figure 2.4), were usually compressed in unfrozen tissue, and expanded during freezing, becoming reservoirs of ice (Roden et al., 2009). Tracheids from frost hardened tissue shrank back to their original size after thawing, while non-hardened tracheids remained expanded and contained greater solute concentrations in the tracheid lumen. Transfusion parenchyma dehydrated, although they returned to their original size and structure upon thawing.

2.3. Chloroplast damage

Freezing irreversibly damages chloroplast membranes, especially if water percentage in the cell is high (Gillies and Binder, 1996). In the study of Feild and Brodribb (2001) on the effects of freezing on chlorophyll a fluorescence in Tasmanian trees, there was no correlation between resistance to freeze thaw embolism and photosystem II (PSII) degradation resistance.

Photoinhibition is a reversible decrease in chlorophyll fluorescence due to the inactivation of PSII (Gillies and Binder, 1996). When photosynthesis decreases due to stomata closure and decreased plant activity, PS II cannot discharge the energy absorbed from light, and PSII is degraded. This means that frosts occurring during intense light lead to more damage to chlorophyll and cell membranes (Gillies and Binder, 1996) than frosts occurring at lower light intensity. Freezing of hardened plants does not decrease chlorophyll fluorescence levels, but dehardened plants' chlorophyll fluorescence levels decrease below 6.2 °C, and permanently below -10 °C (Gillies and Binder, 1996).

3. Mechanisms of frost tolerance in conifers

Damage by frost can be decreased by a mechanism called frost hardening, a series of metabolic and physiological adaptations that allow the plant to withstand colder temperatures. Frost tolerance is costly, and there is a trade-off between growth and survival as a study in white spruce showed (Sebastian-Azcona et al., 2018). Local varieties tend to adapt, exhibiting the optimal trade-off for local conditions between growth and good survival rates.

There is a negative correlation between annual growth and frost tolerance, as measured by ice nucleation temperatures in beech (*Nothofagus pumilio* [Poepp. & Endl.] Krasser) (Molina-Montenegro et al., 2012). A negative correlation between tolerance and height was also found in loblolly pine (*Pinus taeda* L.) (Zapata-Valenzuela et al., 2015). Frost hardiness was correlated with survival, but not tree height, in white spruce (Sebastian-Azcona et al., 2018)

3.1. Structural changes for protection against frosts

As plant tissue adapts to frost, changes occur both at the cellular and organ level, with various barriers that prevent the spread of ice, avoid the rupture of membranes, or the degradation of proteins by ice. These changes also occur in tissues as they mature, with needles older than a year being much more frost tolerant than new needles (Luis et al., 2007; Neuner and Beikircher, 2010).

Growing tissue is less frost tolerant than tissue that has finished growing. Second flushing, an outburst of growth in shoots experienced in later summer after warm temperatures, leads to a decrease in frost hardiness (Anekonda et al., 1998). But as growing slows, hardiness increases, and longer flush length is correlated with frost tolerance (Zapata-Valenzuela et al., 2015).

There are differences in frost hardiness not only between older and newer growth but also differences depending on the type of tissue. Leaders determine the direction of growth and are very important for the tree to establish apical dominance. Frost tolerance of leader and lateral branch damage is correlated, with leaders having higher frost tolerance in Douglas fir (*Pseudotsuga menziesii* (Mirb). Franco) (Anekonda et al., 1998). The shoot tip where the terminal bud is located was significantly more resistant than the lower parts of the shoot, especially before hardening, in all branches (Colombo et al., 1995). Hardening increased the uniformity in the level of hardiness in a study in black spruce. There are also differences at the tissue level in frost hardiness.

Many structural changes at the organ level can help prevent frost damage. In an intraspecific study in Scots pine, it was found that more frost tolerant varieties had higher leaf mass per area, leaf density, a longer lifespan, they were shorter, had thicker epidermal cells and cell walls, wider resin ducts, and more collapse-resistant tracheids than needles from less resistant provenances (Jankowski et al., 2017).

The structure of buds is very important in protecting the primordium tissue inside them, as buds are the most sensitive organ, followed by needles and stem (Aitken and Adams, 1996; Beuker et al., 1998). Bud scales play an important role in preventing intracellular formation, as well as the lack of vascular tissue (Neuner and Beikircher, 2010). Once the shoot primordium, which until the onset of growth remains an undifferentiated mass of cells differentiates into the central cylinder or pith and the rest of the vascular tissue start growing, the spread of ice through the stem into the needles is facilitated (Figure 2.5).

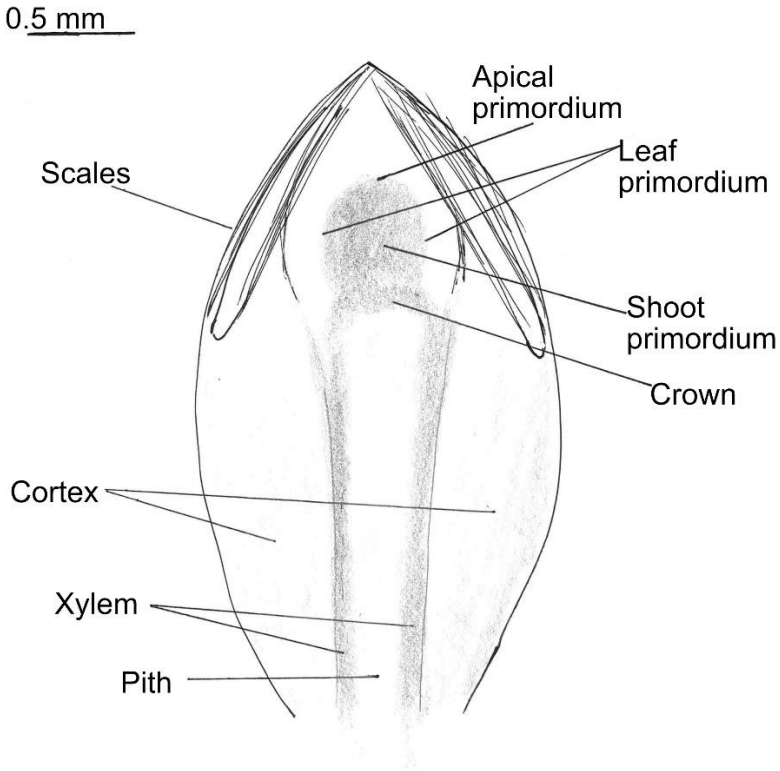

Figure 2.5. Schematic drawing of longitudinal section of a conifer bud.

Buds serve to protect primordia tissue. While slow freezing in intact Japanese larch (*Larix kaempferi* [Lamb.] Carr.) buds led to supercooling (a physical phenomenon in which water does not freeze at temperatures below 0 °C by reducing ice nucleation) and a slight shrinkage of cells, in isolated primordia it led to intracellular freezing and cell deformation (Endoh et al., 2014). In whole buds, ice forms in the empty space of pith beneath the crown tissue and in the basal area of scales (Figure 2.5). Isolated primordium tissue lacks such protection, so ice forms inside the cells.

Structural changes also occur at the cell level during hardening. Chloroplasts move from being lens-shaped and located near the cell walls to more irregular shapes and grouping together, as observed under an electron microscope in a study in Norway spruce (Jonsson et al., 2001). Chloroplasts also seemed to form dense bodies during hardening in Monterey pine endodermis and transfusion parenchyma (Roden et al., 2009).

While the structural changes increase frost tolerance, the changes that occur in tissues may lead to temporary troughs in frost tolerance. The mesophyll cell wall thickening process marked the most sensitive period for the needle: as cell walls became thicker, they regained their frost hardiness (Neuner and Beikircher, 2010).

3.2. Avoiding oxidative stress

Cold temperatures can harm plants through oxidation, as the level of reactive oxygen species (ROS) increases. In Norway spruce, dehardening was accompanied by an increase in ROS such as O_2^- radicals (Pukacki and Kamińska-Rozek, 2013). Antioxidants and the activity of enzymes which play a role in the decomposition of ROS serve to protect cells from oxidative damage.

3.2.1. Antioxidants

Low molecular weight ROS scavengers (such as glutathione, ascorbic acid and flavonoids) as well as amino acids (such as glutamic acid, aspartic acid and proline) prevent protein oxidation and are significantly correlated with hardiness (Pukacki and Kamińska-Rozek 2013; Repo 1992). Although the levels of these antioxidants decrease during dehardening, this does not seem to increase ROS levels (Pukacki and Kamińska-Rozek, 2013). Total levels of amino acids do seem to increase, especially the amino acids that are the by-products of the Krebs cycle (Repo 1992). The lack of change in ROS levels could be due to the fact that ROS are mainly produced by chlorophyll, and dehardening precedes the increase in photosynthesis (Doulis et al., 1993).

Ascorbic acid, glutathione, proline, and carotenoids increased during hardening in *Sabina* spp. (Chen et al., 2012). Ascorbic acid and flavonoids decreased during dehardening in Norway spruce (Pukacki and Kamińska-Rozek, 2013).

Glutathione is an antioxidant associated with increased levels of frost hardiness in Norway spruce (Pukacki and Kamińska-Rozek, 2013), where it has been observed that glutathione levels decreased by around 50% from the hardening to the dehardening period (Doulis et al., 1993; Pukacki and Kamińska-Rozek, 2013).

3.2.2. Enzymes

Oxidation of glutathione requires the enzyme glutathione reductase. Glutathione reductase (GR) activity levels are higher in more hardened trees of red spruce (*Picea rubens* Sarg.), with a 70% decrease in GR activity during the spring period, suggesting that glutathione antioxidant properties have a role in achieving cold tolerance (Doulis et al., 1993). GR is also more active in previous years' needles (Doulis et al., 1993), which have been shown to be more resistant than fresh needles (Gillies and Binder, 1996). Inversely, total glutathione levels are higher in fresh needles, as glutathione content decreases during dehardening (Doulis et al., 1993). It also appears that while glutathione levels may be controlled genetically, a comparison between different provenances of red spruce shows they have different levels of glutathione. Glutathione reductase levels do not change depending on the provenance, but the GR isozyme does change during hardening.

Another enzyme, superoxide dismutase (SOD), is negatively associated with hardiness (Doulis et al., 1993; Pukacki and Kamińska-Rozek, 2013). This enzyme functions in combination with GR, and the SOD/GR ratio needs to be kept low, so that toxic ROS intermediates do not accumulate (Doulis et al., 1993), but a very low SOD/GR ratio is inferior to an adequate SOD/GR ratio.

The activity of the enzymes catalase, peroxidase, and ascorbate peroxidase was positively correlated with frost hardiness in *Sabina* spp. (Chen et al., 2012). Peroxidase activity is also positively correlated with frost hardiness in Norway spruce (Pukacki and Kamińska-Rozek, 2013). Catalase activity was also correlated with frost hardiness in Japanese red pine (*Pinus densiflora* Siebold & Zucc.) (Meng et al., 2015).

3.3. Osmolytes' influence on cold hardiness

Extracellular freezing leads to dehydration, as there is less water in the cell, which leads to the shrinking of the cell wall (Fujikawa et al., 1999; Kasuga et al., 2007). Dehydration is caused by the outflow of water in the cytoplasm, which flows out to equilibrate osmotic pressures. One way to avoid this is to increase the internal osmotic pressure, increasing the concentration of osmolytes inside the cell. Freezing-point depression, decreasing the temperature of ice formation, is also achieved through an increased solute concentration in the cell cytosol, thus protecting the plant from intracellular freezing.

3.3.1. Carbohydrates

Soluble sugars play an important role in increasing frost tolerance (Charra-Vaskou et al., 2012; Chen et al., 2012; Jiang et al., 1994; Meng et al., 2015; Ögren et al., 1997; Repo, 1992). Soluble sugars are synthesized from starch during hardening (Ögren, 1997).

However, cold storage and cold exposure of white spruce seedlings (Jiang et al., 1994) and cold exposure of Scots pine seedlings (Ögren, 1997) contribute to the consumption of sugars due to the reduced production and increased need of energy. Soluble sugars were metabolised in all tissues (needles, stems and roots) for energy (Jiang et al., 1994; Ögren, 1997). Metabolic consumption of carbohydrates occurred even at sub-freezing temperatures in all types of plants. In a study examining carbohydrate consumption in Scots pine, Ögren (1997) showed that respiration continued at temperatures as low as -8.5 °C, although the rates of respiration did decrease. In addition, as sugar concentrations decreased, so did cold hardiness (Ögren, 1997). To compensate for this, plants convert stored starches into sugar via starch hydrolysis, increasing and maintaining cytosol osmotic pressures even at very low temperatures (Repo 1992).

The increase in starch hydrolysis (Repo 1992) is concurrent with a change in the concentration of enzymes which regulate starch-sugar conversion. Sucrose-phosphate synthase is upregulated, while sugar consuming enzymes such as neutral invertase and soluble acid invertase are downregulated by

cold temperatures (Chen et al., 2012), although the concentrations of total proteins does not seem to change (Jiang et al., 1994). However, the latter study also showed that total non-structural carbohydrate content slightly increased from autumn to spring, and starch concentrations increased significantly from autumn to spring, which would indicate that processes other than hydrolysis were also going on.

Carbohydrate composition also changes across different seasons, with increased concentrations of sucrose, raffinose and monosaccharides associated with cold hardiness (Aronsson et al., 1976). The change in composition of sugars is not due to novel compounds, but due to increased concentration of known frost tolerance metabolites, as a study in Siberian spruce (*Picea obovata* Ledeb.) showed (Angelcheva et al., 2014). Among them, sucrose was the most abundant carbohydrate in black spruce (Repo 1992). Although there was a variation in total non-structural carbohydrates depending on provenance, there was no difference for stachyose, raffinose and starch. The highest levels of non-structural carbons were found in needles, followed by stems and roots (Cannell et al., 1990), which probably indicates that the organs that are more isolated from the cold by either bark or soil need fewer osmolytes. Raffinose was increase 46 x in Siberian spruce during frost hardening, while galactinol increased 25 x (Angelcheva et al., 2014). Sucrose seemed to decrease, while fructose, glucose, melibiose and raffinose increased in extreme frost tolerance development. Galactose was also found to be significantly correlated with frost tolerance in loblolly pine (Zhao et al., 2017).

3.3.2. Other osmolytes

Other osmolytes, including amino acids such as proline and tryptophan, also play a role in frost hardiness. Tryptophan was increased 14 x in Siberian spruce during frost tolerance development (Angelcheva et al., 2014), and was also significantly correlated to frost hardiness in black spruce (Odlum et al., 1993). Proline was correlated with frost hardiness in Scots pine (Meng et al., 2015; Repo, 1992), *Sabina* spp. (Chen et al., 2012) and Norway spruce (Pukacki and Kamińska-Rozek 2013; Repo 1992).

3.4. Genetics of frost hardiness

Frost tolerance is a very complex characteristic controlled by environmental and genetic factors that involves multiple genes (Takata et al., 2007). It has been demonstrated that frost tolerance is heritable and correlated with region of provenance. In Russian larches (*Larix* Mill.) trees of more northern provenances were more tolerant both in the autumn and spring, at the same longitude, with no correlation with altitude (Eysteinsson et al., 2009). Northern origin was also correlated with autumn frost tolerance in Scots pine (Andersson and Fedorkov, 2004) and Norway spruce (Konttinen et al., 2007). The degree of continentality was important for autumn frost tolerance of Scots pine, as at the same latitude of origin more inland varieties of Scots pine exhibited higher degrees of tolerance

(Andersson and Fedorkov, 2004). Trees from same provenance had the same levels of frost tolerance in white spruce (Sebastian-Azcona et al., 2018). These differences are not only within, but also between species. Species from areas with high heterogeneity in temperatures showed higher levels of frost hardiness (Kreyling et al., 2015).

Autumn, winter and spring frost tolerance are different traits under different genetic control. Autumn and winter cold hardiness are correlated very weakly in Douglas fir (Aitken and Adams, 1996). Autumn and spring frost hardiness were found to be anti-correlated in Douglas fir, and controlled by different loci (Jermstad et al., 2001; O'Neill et al., 2001). Autumn and spring cold tolerance were weakly correlated in whitebark pine (*Pinus albicaulis* Engelm.), with spring cold tolerance under weaker genetic control than other species (Bower and Aitken, 2006). Spring frost tolerance seems to be under strong genetic control in many conifers, or at least stronger than autumn frost tolerance (Aitken and Adams, 1997; O'Neill et al., 2001).

Tissue type frost hardiness correlate positively with each other, which means that breeding by selecting plants with frost tolerance in tissues will result in more tolerant organisms (O'Neill et al., 2001). However, needle damage may give an underestimate of the overall frost tolerance of the plant, as buds can regrow living tissue even after the loss of all needles (Luis et al., 2007)

3.5. Proteins related to frost tolerance in conifers

Many proteins and enzymes help make plant tissues more frost tolerant. Dry weight fraction and vegetative storage protein concentration was correlated with frost tolerance in white spruce (Binnie et al., 1994). In a reverse-transcription PCR quantification study in common cypress (*Cupressus sempervirens* L.) the upregulated genes were those that coded for chaperonins, serine/threonine protein kinases, exonucleases, dehydrins, and senescence associated proteins (Baldi et al., 2011). Downregulated proteins included heat shock proteins and chlorophyll binding proteins.

A pathogenesis related (PR) protein from western white pine (*Pinus monticola* Douglas ex D. Don) with the modified cauliflower mosaic virus 35S promoter (a strong promoter that leads to overexpression of genes) gave frost resistance to modified *Arabidopsis thaliana* (L.) Heynh. (Liu et al., 2013). Chitinolytic activity (a type of PR protein) seemed to increase in Norway spruce during hardening (Dalen et al., 2014).

Using cDNA from three Scots pine bud tissue cDNA libraries filtered for frost tolerance, 1100 genes were identified as activated by frost tolerance development (Joosen et al., 2006). The ones that were most strongly and positively associated with frost tolerance development were abiotic stress related proteins such as dehydrins (class SK4, SK2, and K2), genes homologous to a loblolly pine drought-stress-induced gene (LP3); biotic stress related proteins, including several pathogenesis-related proteins; development related proteins such as LEA proteins; transcription factors and photosynthesis

related proteins. Translation-related and protein degradation enzymes were negatively correlated with frost tolerance. These findings seem to be in accordance with the positive association between frost tolerance development and the protection of proteins by denaturation.

In another study of differential expression in Norway spruce (Stattin et al., 2012), the genes that could be grouped into functional categories were involved in metabolism (*e.g.* genes involved in the biosynthesis of sugars, amino acids or lignin), cell defence (*e.g.* Heat Shock Proteins (HSPs), antifreeze genes such as dehydrins and pathogenesis-related genes, signal transduction pathways (hormonal), transcription, and cellular transport. Antifreeze proteins were also identified in Norway spruce protein assays for frost tolerant mature needles (Dalen et al., 2014).

The genes associated with supercooling in xylem parenchyma cells in larch are signal transduction proteins, metabolic enzymes, late embryogenesis abundant (LEA), HSP, chromatin constructed proteins, protein synthases, membrane transporters, metal binding (Takata et al., 2007). These genes are downregulated during dehardening, and all except one are in higher concentrations in xylem parenchyma cells than in cortical cells (Takata et al., 2007).

3.6. Lipids

Lipids form the plasma membrane, which is damaged by frost, and to avoid this damage lipid composition changes during acclimation with an accumulation of long-chain mono- and polyunsaturated fatty acids (Angelcheva et al., 2014). Not all polyunsaturated fatty acids increase frost tolerance, though; in red pine (*Pinus resinosa* Aiton), oleic and linoleic acids (unsaturated) decrease, while palmitic (saturated) and linolenic acids (unsaturated) increase in proportion of the plasma membrane fraction (Martz et al., 2006). It should be noted that it is the composition that changes, as total lipids do not seem to change even as phospholipids decreased during dehardening in several conifer species (Kedrowski, 1980).

4. Hardening/dehardening processes in conifers

To prevent frost damage, the level of frost tolerant of a plant needs to be lower than the coldest temperature that occurs during that day. Thus, to avoid frost damage, the frost tolerance levels should always remain below seasonal minimum temperature. In autumn, that would mean hardening should precede the decrease of temperatures, while in spring, dehardening should follow the increase in temperatures.

As will be examined further in this review, hardening and dehardening are initiated and controlled by two main external factors, temperatures and photoperiod, with one or both factors prevailing in different species. Hardening and dehardening also accompany other physiological processes, such as bud set and bud burst.

4.1. Frost tolerance and phenology

Both gymnosperms and angiosperms cease active growth of tissues during the colder winter periods. Bud set is accompanied by hardening in black spruce (Colombo et al., 2003). The growing primordium tissues are the most sensitive ones to frosts, which is why buds need to be protected from frosts. Bud set occurs during autumn, and frost tolerance is concurrently developed. Bud scales and other structural changes occur to protect the bud while it is dormant over winter. During bud burst, buds lose the scales and needles begin to develop from primordium tissues. Bud burst requires a genetically determined period of chilling, followed by a period of warmth that is accumulated over time, known as Growing Degree Days (GDD) (Cannell et al., 1990).

In spring, the accumulation of thermal time (usually defined as the accumulated temperatures above 5 °C) in combination with winter chill day exposure leads to bud break, and these newly grown shoots have the highest cold sensitivity throughout the year (Cannell and Sheppard, 1982). Primordium tissues are the most sensitive, followed by needles and stem (Aitken and Adams, 1996; Beuker et al., 1998). Bud burst date is negatively correlated with injury from frost, which means an earlier bud burst date leads to more damage (Aitken and Adams, 1997; Fløistad and Granhus, 2010). Bud burst is preceded with a decrease in frost tolerance, which occurs in response to warmer temperatures (Repo 1992). Some of the hardiness remains, though, despite increased apical activity (Cannell et al., 1990). The importance of bud break timing is illustrated by the delay in leader bud break. Apical buds help establish vertical growth and thus need to be protected for longer than lateral buds. Leaders flush a couple weeks later than lateral buds (Fløistad and Granhus, 2010), because they require 25 more growing degree days (Cannell, 1985).

Northern provenance trees show earlier bud break (Aldrete et al., 2008; Barney et al., 2013), but also seem to have superior height (Barney et al., 2013), although there does not seem to be a correlation between bud break date and tree height. Coastal provenance trees also seem to have an earlier bud break (Aitken and Adams, 1997). The reasons why northern provenance trees, which grow in colder sites with higher risks of lethal frosts, flush earlier, might be because the growing season is shorter in more northern regions. It might also be because shorter days during hardening induce earlier bud break, as has been shown in experiments where seedlings exposed to a late summer short day treatment before hardening happened earlier, set bud earlier and had an earlier bud break (Fløistad and Granhus, 2010). Late summer short day treatments also increase the occurrence of second flushes, and affect height (Fløistad and Granhus, 2010). However, despite making plants break bud earlier, late summer short day treatments followed by temperature decreases reduce frost damage (Fløistad and Granhus, 2010) and apparently speed dehardening (Bigras and D'Aoust, 1992).

In Douglas fir, there was no correlation between bud burst date and late spring frost tolerance (Hawkins and Stoehr, 2009), although there was a correlation between height and bud burst date. Non-coastal provenance trees also show earlier bud burst than coastal ones in Douglas fir and Norway spruce (Malmqvist et al., 2017). In a comparison study of exotic firs, higher cold hardiness levels seemed to be associated with earlier bud burst (Jones and Cregg, 2006).

Bud break has several phases, during which frost tolerance fluctuates. Frost resistance decreases during bud swelling, breaking, and shoot expansion, plateauing in the latter part of the expansion (Neuner and Beikircher, 2010). Flushing tissue is less frost tolerant than buds or mature needles, with a decreased solute concentration and higher ice nucleation temperatures.

4.2. Hardening in autumn

Hardening seems to have four different phases: pre-acclimation, early acclimation, late acclimation, and full acclimation, detected in the frost tolerance development to extreme frosts in Siberian spruce, with metabolites accumulating as hardening progressed (Angelcheva et al., 2014). Both temperatures and photoperiod influence frost hardening in different conifer species.

Although photoperiod starts the initial process of early acclimation in autumn, cold temperatures seem to play the more important role for hardening (Arnott et al., 1993). In a study in Scots pine and Norway spruce, reducing daylength from 8 to 5 h had no effect on frost tolerance, and even decreased it at the same ambient temperature (Hernandez Velasco, 2020). Temperature was more important than photoperiod in this study. Similarly, frosts not exceeding the plants' frost tolerance were found to accelerate frost hardening more than photoperiod in Scots pine, although shorter daylength induced hardening (Beck et al., 2004). Higher frost frequency leads to lower frost tolerance temperatures, although the rate of hardening does not increase.

The relationship between ambient temperatures and hardening rate was shown to be curvilinear in Monterey pine (Greer et al., 2000). Above a certain mean ambient air temperature threshold, 9.5 °C in Monterey pine, the hardening rate becomes the dehardening rate.

In some cases, though, temperatures seem to play a reduced role in frost hardening. A study in white pine (*Pinus strobus* L.) showed no effect from warming on frost hardening (Chang et al., 2015). Photoperiod had more influence than temperature on the level of frost hardiness in Scots pine, at positive ambient temperatures (Aronsson, 1975; Odlum et al., 1993). Photoperiod has a lasting effect, as autumn photoperiod affects spring frost tolerance and dehardening (Bigras and D'Aoust, 1992; Fløistad and Granhus, 2010; Rostad et al., 2006). The rate of hardening was positively correlated with photoperiod (Leinonen et al., 1997). Photoperiod only seems to affect frost tolerance to relatively mild frosts, though (down to a minimum temperature of -7 °C) (Rostad et al., 2006). This effect is quite important in the spring because in the spring frost tolerance reaches its lowest level, with damage

exhibited at temperatures as warm as -3 °C in Sitka spruce (Cannell and Sheppard, 1982). Photoperiod has no effect on the frost resistance of tissues not exposed to light, *i.e.* roots (Bigras and D'Aoust, 1992). The frost tolerance of the above ground organs, the stems and the needles, is correlated to each other (Bigras and D'Aoust, 1992).

Introducing an artificially short photoperiod in nurseries in early summer does not seem to have a positive effect on frost tolerance, unlike late summer treatments, which have an effect more attuned to the plants' natural growth cycles (Rostad et al., 2006). Late summer short day treatments for Norway spruce seedlings increase the number of new shoots after a frost, height (by 1 cm only) and significantly decrease the nitrogen content of tissues (Rostad et al., 2006). Photoperiod also increases levels of disaccharides, such as sucrose, but has a lesser, although still significant, effect on levels of monosaccharides such as glucose or on sugar alcohols, although it does seem to increase pinitol (Aronsson et al., 1976; Rostad et al., 2006).

Longer nights improved frost hardening in Norway spruce, although after a certain night length (10 h, which was longer than the critical night length of the spruce varieties in their provenance regions), there was no further improvement in frost tolerance (Konttinen et al., 2007).

While photoperiod is important, light quality also matters for frost hardening. Low light intensity and high night temperatures lead to earlier hardening than high light intensity and low night temperatures during autumn in Norway spruce (Johnsen and Skrøppa, 2000). Bud set also began earlier, and the level of bud set was positively correlated with increased frost tolerance.

4.3. Changes in winter

After trees have gone through the hardening process in autumn, the level of hardiness doesn't remain unchanged through the winter.

Winter frost hardiness in Swiss pine (*Pinus cembra* L.) was affected by both warm and cold winter temperatures by dehardening and further hardening, respectively (Buchner et al., 2011). Both soil and air warming had a similar effect on dehardening.

4.4. Dehardening

Dehardening is also affected by both photoperiod and temperatures, although there are many differences across species. Dehardening occurred in response to photoperiod only in yellow pine (*Pinus banksiana* Lamb.) while in lodgepole pine and white spruce it required warmer temperatures (Kedrowski, 1980). Scots pine can also deharden in response to longer daylength (Leinonen et al., 1997). Even when daylength remains short, dehardening can be forced by an accumulation of warm days in white spruce, black spruce, jack pine (*Pinus banksiana* Lamb.), and lodgepole pine (Man et al., 2017).

Initiation of the physiological processes of dehardening as the ambient temperatures warm up can occur very rapidly (Beuker et al., 1998; Leinonen et al., 1997; Repo, 1992), the rate of dehardening fitting an exponential curve (Man et al., 2017). In Scots pine, Repo (1992) showed that dehardening occurred at a rate of 11 K week^{-1}, whereas hardening occurred at a rate and 6 K week^{-1} when mean air temperatures are between 4-6 °C. Other species may have a slower dehardening rate, with a 6 K week^{-1} dehardening rate for Norway spruce (Repo 1992).

The onset of plant growth following dehardening does not prevent rehardening and fluctuations in temperature can result in cycles of the hardening and de-hardening processes (Leinonen et al., 1997). Plants cycling through the different physiological states of frost hardiness have also been shown to have higher levels of frost hardiness than plants grown in consistent temperatures, as long as the average temperature is the same.

5. Environmental factors that affect frost tolerance of conifers

5.1. Nutrients and frost hardiness

Many nutrients, such as Cu, Mg, P, N affected the frost hardiness of Norway spruce (Jonsson et al., 2001). Luoranen et al. (2008) added pre-mixed fertilizer during the summer and spring containing N, P, K to two years-old seedlings of Norway spruce grown in the field in fertilized, limed sphagnum peat using at a rate of 14, 7 and 14 mg per seedlings of N, P, K, and a further total of 4, 3, and 5 mg of N, P, K with irrigation, and found that autumn frost hardening improved when nutrients were added in the summer, but had no effect on dehardening in the spring.

Nitrogen is a very important nutrient, and its levels determine whether the plant can synthesize amino acids, the building blocks of proteins. Nitrogen levels significantly affect growth, and thus plant height (Fløistad and Kohmann, 2004). Additional nitrogen, especially when supplied during the hardening period, increases the frost hardiness of pine species. This increase is not related to cellular carbohydrate content (Toca et al., 2018). Additional nitrogen provided outside the hardening season has a species-dependant effect (Toca et al., 2018).

Nitrogen effects on frost hardiness are nonlinear and seem to highly depend on species. Although adding nitrogen to very poor soils does improve frost hardiness of trees, higher levels of nitrogen show decreased frost tolerance in Scots pine (Aronsson, 1980). In fact, sub-optimal levels of nitrogen (below 2.0-2.5 % of tissue content) seem to be best for improving frost hardiness in Norway spruce (Fløistad and Kohmann, 2004).

In a study in western redcedar (*Thuja plicata* Donn ex D.Don) and Douglas fir, very low levels of N supplementation (20 mg L^{-1}) reduced frost hardiness compared to the medium level of supplementation (100 mg L^{-1}), while at higher levels of supplementation (250 mg L^{-1}), the effect on

frost hardiness was uneven (Hawkins et al., 1996). This may be because nitrogen concentrations optimal for growth lead to earlier bud break (Fløistad and Kohmann, 2004).

Calcium is another nutrient that affects frost tolerance. Calcium increases levels of stress related polyamine putrescine during hardening, which is maintained into late winter (Schaberg et al., 2011). Total chlorophyll levels and chlorophyll a concentrations, which decrease due to frosts, were increased in late winter (February) in Ca rich plants (Schaberg et al., 2011).

Other mineral nutrients, such as K, Zn and Cu had no effect on hardiness of Scots pine (Aronsson, 1980). Boron deficiency decreases Norway spruce frost tolerance (Räisänen et al., 2007). Increased K and P, applied together, was positively correlated to frost tolerance (Jönsson et al., 2004).

5.2. CO_2 and its effects on frost hardiness

CO_2 has an effect on phenology and frost tolerance. Increasing levels of CO_2 raise the thermal requirement for bud burst, delaying bud burst up to a week, while hastening bud set (Bigras and Bertrand, 2006; Guak et al., 1998; Murray et al., 1994). Delayed bud burst and earlier bud set are correlated with frost tolerance (Kandemir et al., 2010).

CO_2 has also been shown to decrease frost tolerance by increasing ice nucleation temperatures (Lutze et al., 1998). Elevated atmospheric CO_2 concentration had no effect on frost tolerance under long photoperiods (Chang et al., 2016). In contrast, shorter photoperiods in elevated CO_2 conditions can lead to slightly lower levels of frost tolerance compared to ambient CO_2. Increases in CO_2 did not seem to affect the levels of frost hardiness in Norway spruce, at the same ambient temperatures (Levkoev et al., 2018). In deciduous larch (*Larix decidua* Mill.), CO_2 increased the degree of frost damage, without any effect on phenology (Rixen et al., 2012). While CO_2 affects the level of hardening, elevated atmospheric CO_2 concentrations do not seem to have an effect on the rate of dehardening (Bigras and Bertrand, 2006; Repo et al., 1996). In any case, the effects of temperature on frost tolerance are much greater than the effects of CO_2 (Martin et al., 2010; Repo et al., 1996).

5.3. Climate change and cold hardiness

Increased temperatures may decrease maximum frost hardiness of Douglas fir by as much as 7 K, by delaying the onset of dehardening (Guak et al., 1998). Increased temperatures also mean that bud burst happens more asynchronously across a region (Guak et al., 1998). Climatic warming seems to influence early flushing species more than later ones, as they have a smaller thermal time requirement for bud burst (Murray et al., 1989).

Climate change will change the conditions under which frosts occur, with warmer winter and spring periods. This may have an impact on bud set, bud break, winter hardiness and spring hardiness (Guak et al., 1998; Murray et al., 1989). Warmer winters will decrease winter hardiness (Guak et al., 1998; Murray et al., 1989), but will not necessarily mean an earlier bud break, because there is an inverse

relationship between the thermal time needed for bud burst and the chill days required (Cannell and Sheppard, 1982; Murray et al., 1989). Only species with a low chill requirement will benefit from a longer growing season provided by global warming (Murray et al., 1989).

Beuker et al., (1998) suggested that autumn frost incidence decreases with climate change because plants are prepared by photoperiod to withstand lower temperatures. Bud set and frost hardening were highly correlated in Scots pine (Hurme et al., 1997). Delays in bud set due to warmer summers would thus affect hardening.

Another effect of warming would be that decreased snow cover could leave root tissue unprotected from frost damage (Ambroise et al., 2020). Soil warming can worsen plant dieback by melting the snow cover, as happens with blueberry shrubs (*Vaccinium myrtillus* L.) (Rixen et al., 2012). The damage to fine root tissue would also result in greater damage to stems and needles due to dehydration (Ambroise et al., 2020).

5.4. Water stress and frost tolerance

Water stress was shown to deplete foliar starch, increase soluble sugars and increase frost tolerance following the event, although rewatering cancelled the increase in soluble sugars in red spruce (Amundson et al., 1993). Slightly lower levels of hydration in needles improved frost hardiness in white spruce and yellow pine (Kedrowski, 1980). Long-term frost tolerance, however, is worsened with water stress, as carbohydrates get depleted faster due to the stress. There was no consistent relationship between water content and frost hardiness in Norway spruce (Konttinen et al., 2007). Frost resistance is positively correlated with osmotic potential at the turgor loss point and with the total volume of liquid in the tissue, and negatively correlated with the dry weight % in western red cedar, meaning more hydrated tissues are more frost resistant (Grossnickle, 1992). Higher level of water stress led to death, as the loss of water absorption capability due to root freezing when air temperatures are positive leads to desiccation and death in Norway spruce (Danusevičius et al., 1999). Drought increases the rate of embolism (Mayr et al., 2006). Decreases in water potential increase embolism, and these decreases can be caused by sun exposure. Adaptations to drought are also beneficial for frost tolerance, as drier areas had higher levels of frost tolerance (Kreyling et al., 2015). Repeated frost-thaw events increase the damage to xylem conductivity (Aronsson, 1980; Mayr et al., 2006). When a freeze-thaw was combined with water stress, there was more or similar damage as with water stress alone. However, freeze-thaw embolism during water stress is correlated with conduit diameter, but not the degree of water stress resistance (Stefan Mayr et al., 2003; Sperry and Sullivan, 1992). Water stress embolism is not linked to vessel size, but to membrane pit permeability (Sperry and Sullivan, 1992), suggesting different mechanisms occur in each stress type. Water stress resistance, unlike frost resistance, does not depend on the season or on the altitude of trees (Charra-

Vaskou et al., 2012). Prolonged water stress or water stress during the growing season actually reduce frost tolerance (Aronsson, 1980), suggesting different adaptation mechanisms to drought and frost. While drought tolerance does not vary depending on tree provenance, frost tolerance does (Charra-Vaskou et al., 2012).

References

Aitken, S.N., Adams, W.T., 1997. Spring cold hardiness under strong genetic control in Oregon populations of *Pseudotsuga menziesii* var. *menziesii*. *Can. J. For. Res.* **27**, 1773-1780.

Aitken, S.N., Adams, W.T., 1996. Genetics of fall and winter cold hardiness of coastal Douglas-fir in Oregon. *Can. J. For. Res.* **26**, 1828-1837.

Aldrete, A., Mexal, J.G., Burr, K.E., 2008. Seedling cold hardiness, bud set, and bud break in nine provenances of *Pinus greggii* Engelm. *For. Ecol. Manage.* **255**, 3672-3676.

Ambroise, V., Legay, S., Guerriero, G., Hausman, J.F., Cuypers, A., Sergeant, K., 2020. The roots of plant frost hardiness and tolerance. *Plant Cell Physiol.* **61**, 3-20.

Amundson, R.G., Kohut, R.J., Laurence, J.A., Fellows, S., Colavito, L.J., 1993. Moderate water stress alters carbohydrate content and cold tolerance of red spruce foliage. *Environ. Exp. Bot.* **33**, 383-390.

Andersson, B., Fedorkov, A., 2004. Longitudinal differences in Scots pine frost hardiness. *Silvae Genet.* **53**, 76-80.

Anekonda, T.S., Adams, W.T., Aitken, S.N., 1998. Influence of second flushing on genetic assessment of cold hardiness in coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco). *For. Ecol. Manage.* **111**, 119-126.

Angelcheva, L., Mishra, Y., Antti, H., Kjellsen, T.D., Funk, C., Strimbeck, R.G., Schröder, W.P., 2014. Metabolomic analysis of extreme freezing tolerance in Siberian spruce (*Picea obovata*). *New Phytol.* **204**, 545-555.

Arnott, J.T., Grossnickle, S.C., Puttonen, P., Mitchell, A.K., Folk, R.S., 1993. Influence of nursery culture on growth, cold hardiness, and drought resistance of yellow cypress. *Can. J. For. Res.* **23**, 2537-2547.

Aronsson, A., 1980. Frost hardiness in Scots pine (*Pinus silvestris* L.) II. Hardiness during winter and spring in young trees of different mineral nutrient status. *Stud. For. Suec.* **155**, 1-27.

Aronsson, A., 1975. Influence of photo- and thermoperiod on the initial stages of frost hardening and dehardening of phytotron-grown seedlings of Scots Pine (*Pinus silvestris*) and Norway Spruce (*Picea abies* (L.) Karst.). *Stud. For. Suec.* **128**, 1-20.

Aronsson, A., Ingestad, T., Loof, L.G., 1976. Carbohydrate metabolism and frost hardiness in pine and spruce seedlings grown at different photoperiods and thermoperiods. *Physiol. Plant.* **36**, 127-132.

Baldi, P., Pedron, L., Hietala, A.M., La Porta, N., 2011. Cold tolerance in cypress (*Cupressus*

sempervirens L.): A physiological and molecular study. *Tree Genet. Genomes* **7**, 79-90.

Barney, D.L., Bauer, M., Jensen, J., 2013. Survival, frost susceptibility, growth, and disease resistance of corkbark and subalpine fir grown for landscape and Christmas trees. *Horttechnology* **23**, 194-200.

Beck, E.H., Heim, R., Hansen, J., 2004. Plant resistance to cold stress: Mechanisms and environmental signals triggering frost hardening and dehardening. *J. Biosci.* **29**, 449-459.

Beuker, E., Valtonen, E., Repo, T., 1998. Seasonal variation in the frost hardiness of Scots pine and Norway spruce in old provenance experiments in Finland. *For. Ecol. Manage.* **107**, 87-98.

Bigras, F.J., Bertrand, A., 2006. Responses of *Picea mariana* to elevated CO_2 concentration during growth, cold hardening and dehardening: phenology, cold tolerance, photosynthesis and growth. *Tree Physiol.* **26**, 875-888.

Bigras, F.J., D'Aoust, A.L., 1992. Hardening and dehardening of shoots and roots of containerized black spruce and white spruce seedlings under short and long days. *Can. J. For. Res.* **22**, 388-396.

Binnie, S.C., Grossnickle, S.C., Roberts, D.R., 1994. Fall acclimation patterns of interior spruce seedlings and their relationship to changes in vegetative storage proteins. *Tree Physiol.* **14**, 1107-1120.

Bower, A.D., Aitken, S.N., 2006. Geographic and seasonal variation in cold hardiness of whitebark pine. *Can. J. For. Res. Can. Rech. For.* **36**, 1842-1850.

Buchner, O., Neuner, G., Ball, M., 2011. Winter frost resistance of *Pinus cembra* measured in situ at the alpine timberline as affected by temperature conditions. *Tree Physiol.* **31**, 1217-1227.

Cannell, M.G.R., 1985. Analysis of risks of frost damage to forest trees in Britain., in: Tigerstedt, P.M.A., Puttonen, P., Koski, V. (Eds.), . Department of Plant Breeding, University of Helsinki, Helsinki, Finland, pp. 153-166.

Cannell, M.G.R., Sheppard, L.J., 1982. Seasonal changes in the frost hardiness of provenances of *Picea sitchensis* in Scotland. *Forestry* **55**, 137-153.

Cannell, M.G.R., Tabbush, P.M., Deans, J.D., Hollingsworth, M.K., Sheppard, L.J., Philipson, J.J., Murray, M.B., 1990. Sitka spruce and Douglas fir seedlings in the nursery and in cold storage - root growth potential, carbohydrate content, dormancy, frost hardiness and mitotic index. *Forestry* **63**, 9-27.

Chang, C.Y., Fréchette, E., Unda, F., Mansfield, S.D., Ensminger, I., 2016. Elevated temperature and CO_2 stimulate late-season photosynthesis but impair cold hardening in pine. *Plant Physiol.* **172**, 802-818.

Chang, C.Y., Unda, F., Zubilewich, A., Mansfield, S.D., Ensminger, I., 2015. Sensitivity of cold acclimation to elevated autumn temperature in field-grown *Pinus strobus* seedlings. *Front. Plant Sci.* **6**, 1-19.

Charra-Vaskou, K., Charrier, G., Wortemann, R., Beikircher, B., Cochard, H., Ameglio, T., Mayr, S., 2012. Drought and frost resistance of trees: a comparison of four species at different sites and altitudes. *Ann.*

For. Sci. **69**, 325-333.

Chen, T., Huijuan, P., Youfu, Z., Qian, Q., Pei, H., Zhang, Y., Qian, Q., 2012. Seasonal changes in non-structural carbohydrates and sucrose metabolism enzymes in two *Sabina* species. *Acta Physiol. Plant.* **34**, 173-180.

Chen, Y.P., Zhang, J., Yang, Z.J., Yu, F., Li, Y.Q., 2012. Relationship between nitric oxide accumulation, anti-oxidative system and freezing tolerance in the leaves of Sabina during cold adaptation. *JAPS, J. Anim. Plant Sci.* **22**, 1133-1141.

Colombo, S.J., Glerum, C., Webb, D.P., 2003. Daylength, temperature and fertilization effects on desiccation resistance, cold hardiness and root growth potential of *Picea mariana* seedlings. *Ann. For. Sci.* **60**, 307-317.

Colombo, S.J., Zhao, S., Blumwald, E., 1995. Frost hardiness gradients in shoots and roots of *Picea mariana* seedlings. *Scand. J. For. Res.* **10**, 32-36.

Dalen, L.S., Johnsen, Ø., Lönneborg, A., Yaish, M.W., 2014. Freezing tolerance in Norway spruce, the potential role of pathogenesis-related proteins. *Acta Physiol. Plant.* **37**, 1-9.

Danusevičius, D., Jonsson, A., Eriksson, G., 1999. Variation among open-pollinated families of *Picea abies* (L.) Karst. in response to simulated frost desiccation. *Silvae Genet.* **48**, 158-167.

Davis, S.D., Sperry, J.S., Hacke, U., 1999. The relationship between xylem conduit diameter and cavitation caused by freezing. *Am. J. Bot.* **86**, 1367-1372.

Doulis, A.G., Hausladen, A., Mondy, B., Alscher, R.G.C., Chevone, B.I., Hess, J.L., Weiser, R.L., 1993. Antioxidant response and winter hardiness in red spruce (*Picea rubens* Sarg.). *New Phytol.* **123**, 365-374.

Endoh, K., Kuwabara, C., Arakawa, K., Fujikawa, S., 2014. Consideration of the reasons why dormant buds of trees have evolved extraorgan freezing as an adaptation for winter survival. *Environ. Exp. Bot.* **106**, 52-59.

Eysteinsson, T.T., Karlman, L., Fries, A., Martinsson, O., Skulason, B., 2009. Variation in spring and autumn frost tolerance among provenances of Russian larches (*Larix* Mill.). *Scand. J. For. Res.* **24**, 100-110.

Feild, T.S., Brodribb, T., 2001. Stem water transport and freeze-thaw xylem embolism in conifers and angiosperms in a Tasmanian treeline heath. *Oecologia* **127**, 314-320.

Fløistad, I.S., Granhus, A., 2010. Bud break and spring frost hardiness in *Picea abies* seedlings in response to photoperiod and temperature treatments. *Can. J. For. Res.* **40**, 968-976.

Fløistad, I.S., Kohmann, K., 2004. Influence of nutrient supply on spring frost hardiness and time of bud break in Norway spruce (*Picea abies* (L.) Karst.) seedlings. *New For.* **27**, 1-11.

Fujikawa, S., Kuroda, K., Jitsuyama, Y., Sano, Y., Ohtani, J., 1999. Freezing behavior of xylem ray

parenchyma cells in softwood species with differences in the organization of cell walls. *Protoplasma* **206**, 31-40.

Gillies, S.L., Binder, W.D., 1996. The effect of sub-zero temperatures in the light and dark on cold-hardened, dehardened and newly hushed white spruce (*Picea glauca* [Moench.] Voss) seedlings. *New For.* **13**, 91-104.

Greer, D.H., Robinson, L.A., Hall, A.J., Klages, K., Donnison, H., 2000. Frost hardening of *Pinus radiata* seedlings: effects of temperature on relative growth rate, carbon balance and carbohydrate concentration. *Tree Physiol.* **20**, 107-114.

Grossnickle, S.C., 1992. Relationship between freezing tolerance and shoot water relations of western red cedar. *Tree Physiol.* **11**, 229-240.

Guak, S., Olsyzk, D.M., Fuchigami, L.H., Tingey, D.T., 1998. Effects of elevated CO(2) and temperature on cold hardiness and spring bud burst and growth in Douglas-fir (*Pseudotsuga menziesii*). *Tree Physiol.* **18**, 671-679.

Hawkins, B.J., Henry, G., Whittington, J., 1996. Frost hardiness of *Thuja plicata* and *Pseudotsuga menziesii* seedlings when nutrient supply varies with season. *Can. J. For. Res.* **26**, 1509-1513.

Hawkins, B.J., Stoehr, M., 2009. Growth, phenology, and cold hardiness of 32 Douglas-fir full-sib families. *Can. J. For. Res. Can. Rech. For.* **39**, 1821-1834.

Hernandez Velasco, M., 2020. Treatments for induction of cold hardiness in *Picea abies* (L.) Karst. and *Pinus sylvestris* L. seedlings pre-cultivated under light-emitting diodes-impact of photoperiod and temperature including energy consumption and seedling quality after cold storage. *Scand. J. For. Res.* **35**, 46-58.

Holopainen, J.K., Holopainen, T., 1988. Cellular responses of Scots pine (*Pinus sylvestris* L.) seedlings to simulated summer frost. *Eur. J. For. Pathol.* **18**, 207-216.

Hurme, P., Repo, T., Savolainen, O., Paakkonen, T., 1997. Climatic adaptation of bud set and frost hardiness in Scots pine (*Pinus sylvestris*). *Can. J. For. Res.* **27**, 716-723.

Jankowski, A., Wyka, T.P., Żytkowiak, R., Nihlgård, B., Reich, P.B., Oleksyn, J., 2017. Cold adaptation drives variability in needle structure and anatomy in *Pinus sylvestris* L. along a 1,900 km temperate-boreal transect. *Funct. Ecol.* **31**, 2212-2223.

Jermstad, K.D., Bassoni, D.L., Wheeler, N.C., Anekonda, T.S., Aitken, S.N., Adams, W.T., Neale, D.B., 2001. Mapping of quantitative trait loci controlling adaptive traits in coastal Douglas-fir. II. Spring and fall cold-hardiness. *Theor. Appl. Genet.* **102**, 1152-1158.

Jiang, Y., Zwiazek, J.J., MacDonald, S.E., 1994. Effects of prolonged cold storage on carbohydrate and protein content and field performance of white spruce bareroot seedlings. *Can. J. For. Res.* **24**, 1369-1375.

Johnsen, Ø., Skrøppa, T., 2000. Provenances and families show different patterns of relationship between bud set and frost hardiness in *Picea abies*. *Can. J. For. Res.* **30**, 1858-1866.

Jones, G.E., Cregg, B.M., 2006. Budbreak and winter injury in exotic firs. HortScience 41, 143-148.

Jönsson, A.M., Ingerslev, M., Raulund-Rasmussen, K., 2004. Frost sensitivity and nutrient status in a fertilized Norway spruce stand in Denmark. *For. Ecol. Manage.* **201**, 199-209.

Jonsson, A.M., Kivimaenpaa, M., Stjernquist, I., Sutinen, S., 2001. Frost hardiness in bark and needles of Norway spruce in southern Sweden. *Trees - Struct. Funct.* **15**, 171-176.

Joosen, R.V.L., Lammers, M., Balk, P.A., Bronnum, P., Konings, M.C.J.M.J.M., Perks, M., Stattin, E., van Wordragen, M.F., van der Geest, A.H.M., 2006. Correlating gene expression to physiological parameters and environmental conditions during cold acclimation of *Pinus sylvestris*, identification of molecular markers using cDNA microarrays. *Tree Physiol.* **26**, 1297-1313.

Kandemir, G.E., Kaya, Z., Temel, F., Önde, S., 2010. Genetic variation in cold hardiness and phenology between and within Turkish red pine (*Pinus brutia* Ten.) populations: Implications for seed transfer. *Silvae Genet.* **59**, 49-57.

Kasuga, J., Takata, N., Yamane, K., Kuroda, K., Arakawa, K., Fujikawa, S., 2007. Larch (*Larix kaempferi*) xylem parenchyma cells respond to subfreezing temperature by deep supercooling. *CryoLetters* **28**, 77-81.

Kedrowski, R.A., 1980. Changes in cold hardiness of introduced and native interior Alaskan evergreens in relation to water and lipid content during spring dehardening. *Physiol. Plant.* **48**, 438-442.

Konttinen, K., Luoranen, J., Rikala, R., 2007. Growth and frost hardening of *Picea abies* seedlings after various night length treatments. *Balt. For.* **13**, 140-148.

Kreyling, J., Schmid, S., Aas, G., 2015. Cold tolerance of tree species is related to the climate of their native ranges. *J. Biogeogr.* **42**, 156-166.

Leinonen, I., Repo, T., Hanninen, H., 1997. Changing environmental effects on frost hardiness of Scots pine during dehardening. *Ann. Bot.* **79**, 133-137.

Levkoev, E., Mehtätalo, L., Luostarinen, K., Pulkkinen, P., Zhigunov, A., Peltola, H., 2018. Development of height growth and frost hardiness for one-year-old Norway spruce seedlings in greenhouse conditions in response to elevated temperature and atmospheric CO_2 concentration. *Silva Fenn.* **52**, 1-15.

Liu, J.J., Ekramoddoullah, A.K.M.M., Hawkins, B., Shah, S., 2013. Overexpression of a western white pine PR10 protein enhances cold tolerance in transgenic *Arabidopsis*. *Plant Cell. Tissue Organ Cult.* **114**, 217-223.

Luis, V.C., Taschler, D., Hacker, J.J., Jiménez, M.S., Wieser, G., Neuner, G., 2007. Ice nucleation and frost resistance of *Pinus canariensis* seedlings bearing needles in three different developmental states.

Ann. For. Sci. **64**, 177-182.

Luoranen, J., Lahti, M., Rikala, R., 2008. Frost hardiness of nutrient-loaded two-year-old *Picea abies* seedlings in autumn and at the end of freezer storage. *New For.* **35**, 207-220.

Lutze, J.L., Roden, J.S., Holly, C.J., Wolfe, J., Egerton, J.J.G., Ball, M.C., 1998. Elevated atmospheric CO_2 promotes frost damage in evergreen tree seedlings. *Plant, Cell Environ.* **21**, 631-635.

Malmqvist, C., Wallin, E., Lindström, A., Säll, H., 2017. Differences in bud burst timing and bud freezing tolerance among interior and coastal seed sources of Douglas fir. *Trees Struct. Funct.* **31**, 1987-1998.

Man, R., Lu, P., Dang, Q.-L., 2017. Cold hardiness of white spruce, black spruce, jack pine, and lodgepole pine needles during dehardening. *Can. J. For. Res.* **47**, 1116-1122.

Marquis, B., Bergeron, Y., Simard, M., Tremblay, F., 2020. Growing-season frost is a better predictor of tree growth than mean annual temperature in boreal mixedwood forest plantations. *Glob. Chang. Biol.* **26**, 6537-6554.

Martin, M., Gavazov, K., Körner, C., Hättenschwiler, S., Rixen, C., Korner, C., Hattenschwiller, S., Rixen, C., 2010. Reduced early growing season freezing resistance in alpine treeline plants under elevated atmospheric CO_2. *Glob. Chang. Biol.* **16**, 1057-1070.

Martz, F., Sutinen, M.-L., Kiviniemi, S., Palta, J.P., 2006. Changes in freezing tolerance, plasma membrane H+-ATPase activity and fatty acid composition in *Pinus resinosa* needles during cold acclimation and de-acclimation. *Tree Physiol.* **26**, 783-790.

Mayr, S, Gruber, A., Bauer, H., 2003. Repeated freeze-thaw cycles induce embolism in drought stressed conifers (Norway spruce, stone pine). *Planta* **217**, 436-441.

Mayr, S., Hacke, U., Schmid, P., Schwienbacher, F., Gruber, A., 2006. Frost drought in conifers at the alpine timberline: xylem dysfunction and adaptations. *Ecology* **87**, 3175-3185.

Meng, P., Bai, X., Li, H., Song, X., Zhang, X., 2015. Cold hardiness estimation of *Pinus densiflora* var. *zhangwuensis* based on changes in ionic leakage, chlorophyll fluorescence and other physiological activities under cold stress. *J. For. Res.* **26**, 641-649.

Molina-Montenegro, M.A., Gallardo-Cerda, J., Flores, T.S.M., Atala, C., 2012. The trade-off between cold resistance and growth determines the *Nothofagus pumilio* treeline. *Plant Ecol.* **213**, 133-142.

Murray, M.B., Cannell, M.G.R., Smith, R.I., 1989. Date of budburst of fifteen tree species in Britain following climatic warming. *Br. Ecol. Soc.* **26**, 693-700.

Murray, M.B., Smith, R.I., Leith, I.D., Fowler, D., Lee, H.S.J., Friend, A.D., Jarvis, P.G., 1994. Effects of elevated CO2, nutrition and climatic warming on bud phenology in Sitka spruce (*Picea sitchensis*) and their impact on the risk of frost damage. *Tree Physiol.* **14**, 691-706.

Neuner, G., Beikircher, B., 2010. Critically reduced frost resistance of *Picea abies* during sprouting could be linked to cytological changes. *Protoplasma* **243**, 145-152.

O'Neill, G.A., Adams, W.T., Aitken, S.N., 2001. Quantitative genetics of spring and fall cold hardiness in seedlings from two Oregon populations of coastal Douglas-fir. *For. Ecol. Manage.* **149**, 305-318.

Odlum, K.D., Blake, T.J., Kim, Y.T., Glerum, C., 1993. Influence of photoperiod and temperature on frost hardiness and free amino acid concentrations in black spruce seedlings. *Tree Physiol.* **13**, 275-282.

Ögren, E., 1997. Relationship between temperature, respiratory loss of sugar and premature dehardening in dormant Scots pine seedlings. *Tree Physiol.* **17**, 47-51.

Ögren, E., Nilsson, T., Sundblad, L.G., 1997. Relationship between respiratory depletion of sugars and loss of cold hardiness in coniferous seedlings over-wintering at raised temperature: Indications of different sensitivities of spruce and pine. *Plant, Cell Environ.* **20**, 247-253.

Pukacki, P.M., Kamińska-Rozek, E., 2013. Reactive species, antioxidants and cold tolerance during deacclimation of Picea abies populations. Acta Physiol. Plant. 35, 129-138.

Räisänen, M., Repo, T., Lehto, T., 2007. Cold acclimation was partially impaired in boron deficient Norway spruce seedlings. *Plant Soil* **292**, 271-282.

Reinikainen, J., Huttunen, S., 1989. The level of injury and needle ultrastructure of acid rain-irrigated pine and spruce seedlings after low temperature treatment. *New Phytol.* **112**, 29-39.

Repo, T., 1992. Seasonal changes of frost hardiness in *Picea abies* and *Pinus sylvestris* in Finland. *Can. J. For. Res.* **22**, 1949-1957.

Repo, T., Hanninen, H., Kellomaki, S., 1996. The effects of long-term elevation of air temperature and CO_2 on the frost hardiness of Scots pine. *Plant, Cell Environ.* **19**, 209-216.

Rixen, C., Dawes, M.A., Wipf, S., Hagedorn, F., 2012. Evidence of enhanced freezing damage in treeline plants during six years of CO_2 enrichment and soil warming. *Oikos* **121**, 1532-1543.

Roden, J.S., Canny, M.J., Huang, C.X., Ball, M.C., 2009. Frost tolerance and ice formation in *Pinus radiata* needles: ice management by the endodermis and transfusion tissues. *Funct. Plant Biol.* **36**, 180-189.

Rostad, H., Granhus, A., Fløistad, I.S., Morgenlie, S., 2006. Early summer frost hardiness in *Picea abies* seedlings in response to photoperiod treatment. *Can. J. For. Res.* **36**, 2966-2973.

Ryyppö, A., Sutinen, S., Mäenpää, M., Vapaavuori, E., Repo, T., 1997. Frost damage and recovery of Scots pine seedlings at the end of the growing season. *Can. J. For. Res.* **27**, 1376-1382.

Schaberg, P.G., Minocha, R., Long, S., Halman, J.M., Hawley, G.J., Eagar, C., 2011. Calcium addition at the Hubbard Brook Experimental Forest increases the capacity for stress tolerance and carbon capture in red spruce (*Picea rubens*) trees during the cold season. *Trees* **25**, 1053-1061.

Sebastian-Azcona, J., Hacke, U.G., Hamann, A., 2018. Adaptations of white spruce to climate: strong intraspecific differences in cold hardiness linked to survival. *Ecol. Evol.* **8**, 1758-1768.

Sperry, J.S., Nichols, K.L., Sullivan, J.E.M., Eastlack, S.E., 1994. Xylem embolism in ring-porous, diffuse-porous, and coniferous trees of Northern Utah and Interior Alaska. *Ecology* **75**, 1736-1752.

Sperry, J.S., Sullivan, J.E.M., 1992. Xylem embolism in response to freeze-thaw cycles and water stress in ring-porous, diffuse porous, and conifer species. *Plant Physiol.* **100**, 605-613.

Stattin, E., Verhoef, N., Balk, P., van Wordragen, M., Lindström, A., 2012. Development of a molecular test to determine the vitality status of Norway spruce (*Picea abies*) seedlings during frozen storage. *New For.* **43**, 665-678.

Takata, N., Kasuga, J., Takezawa, D., Arakawa, K., Fujikawa, S., 2007. Gene expression associated with increased supercooling capability in xylem parenchyma cells of larch (*Larix kaempferi*). *J. Exp. Bot.* **58**, 3731-3742.

Toca, A., Oliet, J.A., Villar-Salvador, P., Maroto, J., Jacobs, D.F., 2018. Species ecology determines the role of nitrogen nutrition in the frost tolerance of pine seedlings. *Tree Physiol.* **38**, 96-108.

Trewin, B.C., 2005. A notable frost hollow at Coonabarabran, New South Wales. *Aust. Meteorol. Mag.* **54**, 15-21.

Zapata-Valenzuela, J.A., Ogut, F., Kegley, A., Cumbie, P., Isik, F., Li, B.L., McKeand, S.E., 2015. Seedling evaluation of Atlantic Coastal and Piedmont sources of *Pinus taeda* L. and their hybrids for cold hardiness. *For. Sci.* **61**, 169-175.

Zhao, X., Hui, B., Hu, L., Cheng, Q., Via, B.K., Nadel, R., Starkey, T., Enebak, S., 2017. Potential of near infrared spectroscopy to monitor variations in soluble sugars in loblolly pine seedlings after cold acclimation. *Agric. For. Meteorol.* **232**, 536-542.

Chapter 3: Modelling the impact of climate change on the occurrence of frost damage in Sitka spruce (*Picea sitchensis* [Bong.] Carr.) in Great Britain

A. A. Atucha-Zamkova, K. A. Steele, A. R. Smith

Published in the journal Forestry on the 17th May 2021. https://doi.org/10.1093/forestry/cpab020

Author contributions: AA, KS and AS conceived and planned the study. AA obtained the data used for verification from the forest managers at Tilhill Forestry Ltd. AA conducted all statistical analyses and led the writing of this chapter. KS and AS provided critical feedback on the writing and analysis process. This chapter is based on a published paper and was vastly improved thanks to anonymous feedback provided by peer review.

Abstract

Climate change is predicted to increase temperature and seasonal temperature variance in Great Britain (GB). Sitka spruce (*Picea sitchensis* (Bong.) Carr.) is the most important tree species used in commercial plantations throughout Europe and GB. Frosts that occur outside the winter dormancy period can negatively affect trees, since they happen after dehardening. Damage can be especially severe at bud burst, before emerging needles mature and form protective barriers. Here, we modelled the impact of climate change on frost sensitivity in Sitka spruce with temperature data from four climate models that use three emissions scenarios to a total of five datasets. The UKCP09 climate model HadRm3 uses emission scenario SRESA1B for the years 2020-2099. The global and downscaled versions of the UKCP18 HadGem3 model use the emissions scenario RCP 8.5. The global model CMCC-CM uses the RCP 4.5 and RCP 8.5 emissions scenarios. The predictions based on these models were compared with results from gridded historical data for the period 1960-2015. Three indicators that assessed the frost sensitivity of Sitka spruce were explored: the total number of frosts between the onset of dehardening and the end of summer, which use three different temperature thresholds (Index $1_{0\,°C}$, $1_{-3\,°C}$, $1_{-5\,°C}$); the total number of frosts after bud burst (Index 2); the number of days with minimum temperatures below the resistance level (backlashes) during the hardening-dehardening period (Sep-Aug) (Index 3). The indices were validated with historical data for frost damage across GB, and Index $1_{-3\,°C}$, Index $1_{-5\,°C}$ and Index 3 were shown to be significantly correlated. The frequency of all frosts and backlashes is expected to decrease with climate change, especially under higher emissions scenarios. Post bud burst frosts have been historically very rare in GB and remain so with climate change. Downscaled regional climate models detect geographic variability within GB and improve prediction of overall trends in frost damage in comparison to global climate change models for GB.

1. Introduction

1.1. Sitka spruce in Great Britain

Sitka spruce (*Picea sitchensis* [Bong.] Carr) is the most important commercial conifer species in Great Britain (GB). In the UK, Sitka spruce plantations cover approximately 665,000 hectares and represent 25% of the total woodland area (Forestry Commission, 2019). Observations of Sitka spruce in its native North America have indicated that genotypes from southern origins, such as Washington, experience higher levels of spring frost damage than northern origins such as QCI (Haida Gwaii/Queen Charlotte Island) (Forestry Commission, 1957). As QCI origin was suitable to local conditions in the UK, it was deemed unnecessary to identify trees of greater frost resistance from more northern origins, such as Alaska (Forestry Commission, 1957) and QCI was selected as the provenance of Sitka spruce for importation to the UK. Subsequent timber quality improvements were made by the breeding programmes of British tree nurseries (Lee, 1999).

1.2. Geographic regions of Great Britain

The climate of the island of GB is classified as temperate oceanic (Peel et al., 2007) with temperature and precipitation regimes that differ across geographic regions. To account for these differences, GB has been divided into four climatically similar regions of provenance that divide GB north to south and east to west (Figure 3.S1), as defined in the Forest Reproductive Material Regulations in 1977 for a range of native species (Herbert et al., 1999). It is important to note that the regions of provenance are defined for native species at current and historical conditions, which may change with climate change. Seed zones are sub-divisions of the regions of provenance, divided according to geoclimatic variation and natural boundaries.

1.3. Freezing damage

In plants, subfreezing temperatures can cause the destruction of cell walls and membranes due to ice formation (Holopainen and Holopainen, 1988), and protein denaturation. As perennial species, trees have evolved mechanisms to survive periods of cold temperatures during the winter season, such as protection of meristematic tissue by the formation of buds (Kuprian et al., 2014; Sakai, 1978) or cold hardening of sensitive tissues.

Cold hardening is triggered by shortening photoperiods and augmented by temperatures below 5 °C, environmental factors that also lead to the termination of bud development (Cannell et al., 1990). A study in white spruce *(Picea glauca* (Moench) Voss) found that a reduction in photoperiod is sufficient to start the hardening process, whereas cold temperatures alone do not induce hardening (Hamilton et al., 2016).

Cold hardiness is metabolically costly and there is little growth during the frost hardy period. This is why as soon as temperatures begin to rise in spring, trees lose these adaptations and deharden quickly, so that the threshold temperature at which frost damage could occur increases by up to 2 K day^{-1} (Neilson et al., 1972; Repo, 1992). During the process of dehardening the frost hardiness of bud tissue decreases to become the most sensitive tissue in trees, more so than needles to frost (Aitken and Adams, 1997; Anekonda et al., 2000). Buds also lose their scales during the period before bud burst, which confer protection during bud burst (Alfaro et al., 2000; Cannell and Sheppard, 1982; Cannell et al., 1990; Man et al., 2016).

Frost hardiness can be quantified using the median lethal time, LT_{50}, that is defined as the temperature at which 50% of trees subjected to a temperature die (Zhang and Willison, 1987). Spring frost damage is commonly reported after bud burst and during the growing stages, whereas damage to trees by frost in winter or before bud burst, is rare (Man et al., 2016), because the hardening process confers resistance to temperatures below the lowest temperature that typically occurs during this period (Sakai and Larcher, 1987).

1.4. Bud burst in Sitka spruce

Temperature is well-known to be the main abiotic driver of bud burst (Aitken & Hannerz, 2001; Cannell & Smith, 1983). Bud burst requires a genetically determined period of chilling, followed by a period of warmth that is accumulated over time, known as Growing Degree Days (GDD) (Cannell et al., 1990). Warmer temperatures seem to advance bud burst dates, although that advance is limited by an inverse relationship between the thermal time needed for bud burst and the chill days requirement (Bigler and Bugmann, 2018; Cannell and Sheppard, 1982; Fu et al., 2015; Morin et al., 2009, 2010; Murray et al., 1989). Higher temperatures seem to decrease chilling more than they increase thermal time, which reduces the effect of warming on bud burst hastening, resulting in a nonlinear effect (Bigler and Bugmann, 2018; Fu et al., 2015; Morin et al., 2009, 2010). Early models of changes in phenology were based on daily mean temperatures, but recent studies have found that models that account for maximum temperatures, T_{max}, and minimum temperatures, T_{min}, can improve accuracy (Fu et al., 2016) through the contrasting effects of chilling and warming. Recently, minimum temperatures were shown to contribute toward the chilling requirement for bud burst, whilst maximum temperatures provide the warming to advance bud burst (Meng et al., 2019; Meng et al., 2020). Understanding the contrasting effect of accumulated heating and chilling on bud burst has refined model accuracy.

The recent work of Peaucelle et al., (2019a) showed that GDD and chilling explain 30% of spatial variance of leaf unfolding, while the inclusion of water and light availability (intensity and photoperiod) combined explained another 10% of spatial variance. Indeed, a study that examined the phenology of

birch (*Betula pendula* Roth.), beech (*Fagus sylvatica* L.) and oak (*Quercus robur* L.) trees, found that maximum temperatures had a greater effect on birch and beech than on oak trees (Fu et al., 2016). Thus, species-specific phenological models need to be developed to account for this added complexity. In general, species with a low chilling requirement will have a longer bud burst to bud set period mediated by global warming (Ma et al., 2019; Morin et al., 2009; Murray et al., 1989). However, within species plasticity results in some populations possessing both chilling sensitive and insensitive characteristics (Morin et al., 2009). Plants that are adapted to cold climates generally have a longer chilling requirement, otherwise any short period of warmth in winter could prematurely deharden tissues and result in tissue death. Global warming is likely to result in a slight increase in the length of the bud burst-bud set period, but could also increase the cold hardiness threshold temperature in spring, thus increasing the likelihood of frosts with temperatures below the threshold (Richardson et al., 2018). However, this reduced frost tolerance may be compensated by a reduction in the total number of frosts (Pohl et al., 2019), and by an increased period between bud burst and damaging frosts (Bigler and Bugmann, 2018).

According to Cannell and Smith (1983), 92 % of the variation in bud burst dates in Sitka spruce trees is explained by the number of GDD needed for bud burst when exposed to a certain number of chill days (equation 1). As the number of days to bud burst decreases, so does cold hardiness, reaching the highest threshold temperature around the time of bud burst (Aitken and Hannerz, 2001). In the work of Cannell and Smith (1983) Scottish forests comprised of QCI provenance Sitka spruce were used to calculate the bud burst date, and the GDD model produced is still considered very accurate for current climate conditions (Cannell et al., 1985), and more complex models are frequently found to be less accurate (Olsson and Jönsson, 2015).

1.5. Climate change effects on frost tolerance

In an early study, Cannel and Smith (1983) reported that spring frosts that are potentially damaging to Sitka spruce have a return time of 3-5 years in upland regions. As trees are most sensitive to frosts during the first 3-4 years after planting, an increase in the return time of frosts could allow some young plantations to completely avoid frost damage. If frosts that cause severe damage to young plantations only happen every 7-8 years, some tree plantations could go for their entire establishment period without damages caused by frost.

Climate change will affect the conditions under which frosts occur through the increased likelihood of warmer winter, autumn and spring periods, which may impact bud set, bud burst, winter hardiness and spring hardiness (Guak et al., 1998; Murray et al., 1989). Although warmer temperatures hasten metabolic processes, the annual total duration of primary production may be unchanged, due to earlier seed maturation and senescence (Reyes-Fox et al., 2014). Thus, trees tend to start bud

formation earlier in autumn (Repo, 1992; Richardson et al., 2018), and bud burst starts later in spring, due to the reduction in chilling during the winter (Bigler and Bugmann, 2018; Morin et al., 2009, 2010; Murray et al., 1994).

1.6. Estimates of frost occurrence and impacts on vegetation

Estimates of the frequency and harmfulness of spring frosts can be achieved in two ways: (i) by observing the frequency of total frosts; or (ii) by linking frost occurrence to the expected phenological or physiological state of plant tissues and estimating the harmfulness of the frost based on that state. Modelling frost damage of coniferous tree species requires models that account for the damage to mature needles that have not been shed, as well as the tenderness of newly emerging tissues. Models suited for annual crops that simply time emergence to specific climatic events would not be suitable because of differences in the underlying biology. Subsequently, linking the expected frost damage to the phenological state, *e.g.* bud burst or leaf unfolding, seems to be the most common method of estimating frost damage for trees (Jeong et al., 2018; Jönsson et al., 2004; Ma et al., 2019; Meier et al., 2018; Morin and Chuine, 2014; Vitasse and Rebetez, 2018; Zohner et al., 2020).

Global circulation models (GCM) can estimate the occurrence of frost events under predicted climates. Thus, modelled climate data have often been used as the first approximation of predicted frost damage (Jönsson et al., 2004; Woldendorp et al., 2008). Recently, Jeong et al. (2018) have increased GCM complexity by the inclusion of additional microclimatic factors (*e.g.* humidity or snow cover) in an attempt to refine predictions. However, Ambroise et al. (2020) exemplified the complexity of including such factors in models without considering the associated plant interactions by showing that decreased snow cover could expose fine root tissue to damaging low temperatures that may result in stem and needle dehydration due to loss of hydraulic function.

Models that are also parameterised with the physiological state of the plant can take into account the interactions between frosts and physiologically mediated frost hardiness to improve accuracy (Jönsson et al., 2004; Kellomäki et al., 1995; Rochette et al., 2004; Woldendorp et al., 2008).

1.7. Aims and Objectives

The overarching aim of this study was to estimate the occurrence of frosts and potential frost damage to Sitka spruce trees under future climate change scenarios. Our specific objectives were to:

(i) approximate future trends of frost occurrence using three global and two regional climate change models;

(ii) utilise three indicators to determine how predicted future climate alters the occurrence and interaction of frosts with the physiological and phenological state of trees throughout the spring;

(iii) examine trends throughout GB to see whether there are regional differences in the expected changes to frost damage;

(iv) explore how the resolution of different climate models influences model prediction precision.

2. Methods

2.1. Climate Change indices

Index $1_{0°C}$, $1_{-3°C}$, $1_{-5°C}$, (Figure 3.1A) measures the number of days with daily minimum temperatures below a threshold of 0 °C, -3 °C and -5 °C following a five-day consecutive period of daily mean temperatures above 5 °C after the 1st of January (late winter to early spring), which is assumed to be the trigger that starts the dehardening process, to the end of August (Jönsson et al., 2004). The purpose of the index is to provide an estimate of the total number of frosts that occur between late winter and late summer, as a first approximation to the effects of climate change. The different temperature thresholds were selected for different reasons. First, that 0 °C is the freezing point of water and is a natural threshold to use. Second, -3 °C was chosen as the threshold where trees that had undergone supercooling to achieve baseline frost hardiness would likely be damaged. And third, -5 °C was chosen to determine how frequently temperatures below the natural frost hardiness level occurred. After validating Index 1, the threshold that was correlated with historical frost damage was used for Index 2.

Bud burst dates were calculated using equation 1, which estimates the thermal time, measured in GDD with a base temperature of 5 °C, that plants require for bud burst. For bud burst in Sitka spruce the chill days were defined in this study as days with mean temperatures below 5 °C that occurred between the 1st of November and bud burst. The model uses a floor value of 85 for the chill days, setting it at that number even when actual chill days are below that, and a maximum level of 180 (Cannell et al., 1985). The thermal time is defined as GDD(T_{base} > 5 °C) between 1st February and bud burst and is dependent on the chilling experienced by the trees.

$$GDD(T_{base} > 5\,°C) = a + b \times e^{(c \times chill\ days \leq 5°C)} \quad (1)$$

It was found that for Sitka spruce populations of QCI provenance (the most common provenance used in commercial forestry in GB), average values of the coefficients were a = 67.4, b = 4401.8, c = -0.042 (Cannell et al., 1985). To account for the variation that could be found among improved QCI origin trees coefficients at both extremes were taken, from a maximum of a = 100, b = 7500, to a minimum of a = 50, b = 2500.

Chill days are calculated first between the 1st November and the 31st May. An approximate bud burst date is then calculated from this value which is used to recalculate the value for the number of chill days that more accurately approaches reality, since chill days that occur after bud burst should not be

counted toward the chill days total. After the GDD needed for bud burst are calculated using equation 1, GDD accumulated from the 1st January is calculated, and the date at which the calculated value is achieved is considered the bud burst date.

Index 2 (Figure 3.1B) measures the frequency of daily minimum temperatures below -3 °C after bud burst (spring to summer period), calculated by equation 1, using the mean values of the coefficients (Cannell et al., 1985). Index 2_{max} does the same for the maximum value of the coefficients (late bud burst populations), while Index 2_{min} uses the minimum value of the coefficients (early bud burst date populations). These indices were used to evaluate whether frosts occurring after bud burst are a significant factor in expected historical and predicted future frost damage, for populations with differing sensitivities to chilling and warming.

Index 3 measures the number of days with a daily minimum temperature below the hardiness level during the hardening-dehardening period. The hardening-dehardening period is defined as the period between the 1st of September and the 31st of August of the next year. These dates were chosen because the autumn equinox, the 22nd September, was considered the starting point of the hardening process in autumn. The daily hardiness threshold was a function of daily mean temperatures, the rate of hardening, and the rate of dehardening, limiting the highest hardiness temperature to -3 °C (Cannell and Sheppard, 1982), and the lowest hardiness temperature to -40 °C (Cannell et al., 1990). This index gives a more detailed approximation of the expected levels of damage with climate change, depending on the effects of both the warming temperatures and the occurrence of frosts.

The rate of hardening and dehardening used for Index 3 were described by a sigmoidal function of the daily mean temperatures (equation 2). Where *d* defines the lower asymptote, or the minimum rate of hardening/dehardening, and *a* is the distance between the upper and lower asymptote, which when combined with *d* describes the maximum rate of hardening/dehardening, and *b* and *c* define the shape of the curve, with *c* defining the centre of the sigmoidal curve when $c = T_{mean}$. Parameter *b* defines whether the sigmoid slope is decreasing or increasing, depending on whether it is positive or negative.

$$Rate\ of\ (de)hardening = \frac{a}{1 + e^{b \times (Tmean - c)}} + d \qquad (2)$$

Since a decreasing photoperiod affects hardening rates even at warm temperatures, for the days between the autumn equinox and winter solstice, or the winter dormancy period, a minimum value of 0.1 K day^{-1} was established for hardening. For the rest of the year, the minimum hardening rate was set to 0 K day^{-1}. The maximum rate of hardening, 1.15 K day^{-1}, was estimated to be reached at -10 °C (Cannell et al., 1990), while the minimum rate of hardening was reached at 5 °C. The coefficients for the curve were calculated by simulating a dataset describing a symmetrical sigmoidal curve with the parameters contained in Table 3.1. For the hardening rate during the winter dormancy period, values

of a = 1.05, b = 0.96, c = 2.5, and d = 0.1, were estimated for equation 2, whereas for the rest of the year, the values were a = 1, b = 0.96, c = 2.5, and d = 0.

The maximum rate of dehardening in Sitka spruce was not found in the available literature, so a value of 1.25 K day^{-1} was used as a proxy, taken from the average of published dehardening rates (Repo, 1992) for Norway spruce (*Picea abies*; 6 K $week^{-1}$), and Scots pine (*Pinus Sylvestris*; 11 K $week^{-1}$). The minimum rate of dehardening, 0 K day^{-1}, was assumed to be reached at 5 °C, whereas the maximum rate was reached at 15 °C. For the dehardening rate, values of a = 5, b = -1.69, °C = 10.07, and d = 0, were estimated for coefficients of the sigmoid curve.

All the indices were calculated for all years using R 3.6.3 (R Foundation for Statistical Computing and R Core Team, 2019) with the RStudio IDE 1.3.959 (RStudio Team, 2019). The ncdf4 1.17 R package (Pierce, 2019) was used to open and manipulate the minimum and mean temperature ncdf format datafiles. The raster 3.1-5 (Hijmans et al., 2020) and rgdal 1.4-8 (Bivand et al., 2020) R packages were used to manipulate the mean and minimum daily temperature data, extracted from the ncdf datafile and organized into a uniform grid of GB. The dplyr 1.0 R package (Wickham et al., 2020a) was used to calculate the indices for each grid coordinate. The PMCMRplus 1.4.4 R package (Pohlert, 2020) was used for the Bonferroni-Dunn post-hoc test after the Kruskal-Wallis H test.

Table 3.1. Parameters used to calculate the cold hardiness threshold for Index 3.

	Hardening		**Dehardening**	
	Max	**Min**	**Max**	**Min**
Rate (K day^{-1})	0	1.15	1.25	0
Temperature at which rate was reached	5 °C	-10 °C	15 °C	5 °C

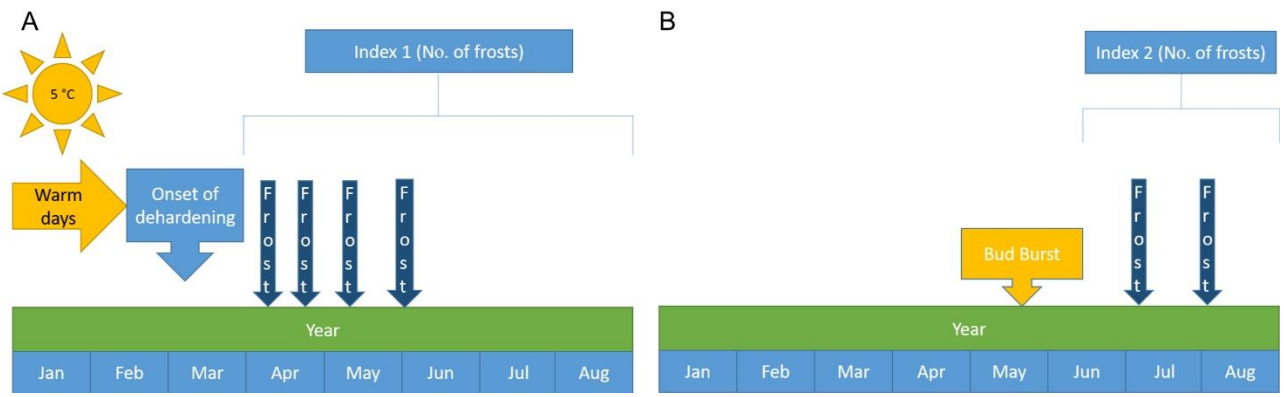

Figure 3.1. Illustration of the indices. A) Index 1 measures the number of frosts between the onset of dehardening and the end of August. B) Index 2 measures the number of frosts after bud burst until the end of August.

The indices did not follow a normal distribution. The distribution of each index is the sum of different non-correlated non-normal distributions in different zones, thus the overlap of different distributions across different zones was not normal and could not be transformed. The non-parametric Kruskal-Wallis H test was used to compare the indices by different factors, with a post-hoc Bonferroni-Dunn test used for pairwise comparisons.

2.2. Datasets

A dataset of daily mean and minimum air temperatures above ground obtained by interpolating meteorological station data onto a uniform 5 × 5 km grid at a daily timescale for the period 1961-2016 was used (Met Office et al., 2017) to calculate frost damage indices.

For the climate change predictions for 2020-2100, the UKCP09 Met Office HadRM3-PPE UK run for the HadRM3 model (Hadley Centre for Climate Prediction and Research, 2014) was used. The UKCP09 dataset used here uses a 25 × 25 km grid of GB (Hadley Centre for Climate Prediction and Research, 2014). The HadRM3 model uses the historical and medium (SRESA1B) emissions scenario. The HadRM3Q14 (afixl) variant (one of eleven variants of the MOHC Regional Climate Model) was used with a climate sensitivity of 4.88 K.

A model that uses a higher emissions scenario, the UKCP18, was also selected. The UKCP18 Regional Projections uses a 12 × 12 km grid over the UK for 1980-2080 for the dynamically downscaled HadGEM3 model (Met Office Hadley Centre, 2018b). The HadGEM3 model uses the Representative Concentration Pathways (RCP) 8.5, which estimates high future greenhouse gas concentrations. The dataset for model run 1 was used, for a shorter time period, 2020-2080, because the data for 2080-2100 was not released.

To compare the predictions made by downscaled models to global ones, the UKCP18 Global Climate Model Projections for the HadGEM3 model (Met Office Hadley Centre, 2018a) were also used. The dataset used was variant 1 of the Global Climate Model Projections for the HadGEM3 model (Met Office Hadley Centre, 2018a), for the years 2020-2100, which uses a 60 × 60 km grid, in order to observe the local implications of downscaling.

To see the effect of a different global model with the same emissions scenario, CMIP5 project's CMCC-CM model run for RCP 8.5 (Centro Euro-Mediterraneo per I Cambiamenti Climatici, 2017) was used. The CMCC-CM model run for RCP 4.5 (Centro Euro-Mediterraneo per I Cambiamenti Climatici, 2012) was also used to see the difference between different emissions scenarios that use the same global

model. The datasets for the r1i1p1 variants for both CMIP5 project's CMCC-CM model runs were used. Both use a 0.75 × 0.75 degree grid, roughly equivalent to an 83 × 83 km grid on the UK scale.

2.3. Spatial differences

The gridded data for each year was grouped into provenance regions in order to establish differences between biologically relevant geographical areas across GB (Forestry Commission, 2012). The matrix was then transformed into a data frame with the addition of coordinates for each grid point. The sp 1.4-2 R package (Pebesma et al., 2019) was used to extract the polygon coordinates for each provenance region from the GB Forestry Commission (FC) provenance region shapefile, overlay the data frame and group all points by zone. A Kruskal-Wallis H test was conducted with indices as dependants and the zones as factors.

The level of spatial autocorrelation was measured by calculating Moran's I, with a Markov chain of 10000. The shapefiles 0.7 (Stabler, 2013) and sp 1.4-2 (Pebesma et al., 2019) R packages were used to load shapefiles into the R environment. The CARBayes 5.2 (Lee, 2019) and sp 1.4-2 R packages were used to combine the dataset with the .shp file containing the polygon information of each area, and the .dbf file which links the polygons in the .shp file to the data with the same unique identifier in the dataset. The spdep 1.1-3 (Bivand et al., 2019) R package was used to calculate the binary neighbourhood matrix and Moran's I.

2.4. Figures

Figures of the values of the indices for all seed zones were prepared in RStudio 3.6.3 (RStudio Team, 2019). The sp 1.4-2 (Pebesma et al., 2019) R package was used to extract the shape of the polygons out of the FC provenance region shapefile, and the mean values for each index were joined with the coordinates extracted. The maptools 1.0-1 (Bivand et al., 2019) and ggplot2 3.3.1 (Wickham et al., 2020b) R packages were used to draw the maps, while the RColorBrewer 1.1-2 (Neuwirth, 2014) package was used to select the colours used for the maps.

2.5. Validation

Data on the level of tissue damage to 41 commercial forests across GB by frosts in the spring of 2015 was obtained from Tilhill forestry. Individual trees were scored on a scale of 0-100 points scale by evaluating both the presence of leader damage (the most harmful frost damage for commercial forests, thus assigned 50 points if present) and the level of observed frost damage among lateral branches. The final score was a sum of the overall percentage of damage and the presence/absence of leader damage (Table 3.S1).

In order to calculate the coefficient of correlation between the indices and the observed values of frost damage, a correlation matrix was calculated using the Hmisc 4.4-1 R package (Harrell, 2020), giving the value of the Pearson's r correlation coefficient and the p-value for the correlation.

3. Results

3.1. Validation

The percentage value of frost damage was correlated with the number of frosts below -3 °C, Index $1_{-3°C}$ (Pearson's r = 0.47, p-value < 0.01), and with the number of frosts below -5 °C ,Index $1_{-5°C}$ (Pearson's r = 0.34, p-value < 0.05), for the forests evaluated. A correlation was also found between the percentage of damage and the number of backlashes, Index 3 (Pearson's r = 0.23, p-value < 0.05).
No correlation was found between the percentage of damage and the number of frosts below 0 °C, (Index $1_{0°C}$), or frosts occurring after bud burst (Index 2).

3.2. Overall changes with climate change

The number of frosts between the onset of dehardening and late summer for all three reference temperatures of Index 1 (0 °C, -3 °C, and -5 °C), was significantly reduced with climate change in all climate change scenarios ($p < 0.001$), when compared with the average for 1960-2015, with the exception of the CMCC RCP 4.5 model for both the -3 °C and -5 °C threshold temperatures (Figure 3.2). This follows a general trend during the historical period, with reductions in the occurrence of frosts between 1960-2015 (Table 3.S2).
For the CMCC RCP 4.5 predicted scenario, the frequency of frosts <-3 °C and <-5 °C increased, and the change was significantly different from the historical average for the -5 °C threshold temperature ($p < 0.001$). All predicted climate change scenarios were significantly different between each other ($p < 0.001$). The scenarios with higher CO_2 levels showed a higher decrease in the incidence of frosts. However, there were differences between different models that used the same emissions scenarios; the CMCC model showed a lower decrease of frost incidence than the UKCP18 model (Figure 3.2). There was also a difference in the scope of the modelled area; the global HadGEM3 model showed a higher decrease than the downscaled version.
The number of late season frosts that occur after bud burst (Index 2) decreased significantly in all scenarios, for both average, early, and late bud burst dates (Index 2, 2_{min}, 2_{max}), when compared with the average for 1960-2015. There was, however, an exception for the UKCP09 model, based on the SRESA1B emissions scenario, which, for early bud burst dates, showed an increase in the number for frosts after bud burst (Figure 3.S2).

There were no significant differences in Index 2_{min} between all climate change models used. For Index 2, only UKCP09 and UKCP18 downscaled outputs remained significantly different ($p < 0.001$). For Index 2_{max}, the UKCP09 model differed significantly from the rest ($p < 0.001$), while the other four showed no significant differences.

The number of frosts after bud burst was very low, both historically and with changes due to climate change. In fact, the average number of post bud burst frosts < -3 °C per year, Index 2, was < 0.05, for all provenance regions and periods (Table 3.S3). The average return time for a post bud burst frost was found to extend to the entire length of the period evaluated (55 years for the historical period, 80 years for the 2020-2100 period).

Bud burst dates were significantly hastened ($p < 0.001$) in all climate models except the CMCC RCP 4.5 model, which showed a delay in bud burst by 3 days (Table 3.S4). The UKCP18 model hastened bud burst dates by more than 50 days for both the global and downscaled model resolution. UKCP09 also hastened the bud burst date by 17 days, two weeks more than the CMCC RCP 8.5. All differences between models were significant ($p < 0.001$). This follows a similar trend between 1960-2015, with a hastening of the bud burst date between 1960-1979 to 1980-1999 and 1980-1999 to 2000-2015 (Table 3.S5).

The number of chill days ($T_{mean} < 5$ °C) during winter decreased significantly with all climate change scenarios ($p < 0.001$), except for the CMCC RCP 4.5 model, by a range of 1-15 days (Table 3.S4). The CMCC model showed the smallest decrease, and the higher CO_2 emissions scenario showed a higher effect on chill days (Table 3.S4). All differences between different climate change models were significant ($p < 0.001$). Chill days were also decreased in the 20-year periods between 1960-2015 (Table 3.S5).

The number of backlashes (Index 3) significantly decreased with all climate change scenarios ($p < 0.001$), except for the UKCP09 model, where backlashes increased by 70% (Figure 3.3). All the models that used higher CO_2 emissions scenarios showed a decrease in backlashes, with the largest decrease in higher CO_2 emission scenarios (Figure 3.3). The CMCC model estimated a greater decrease in backlashes than the UKCP18 model, and the downscaled version of UKCP18 showed a smaller change in backlashes than the global model (Figure 3.3). All differences between climate change model results were significant ($p < 0.001$). This follows the historical trend, where the number of backlashes decreases between 1960-1979 to 1980-1999, although the decrease slows between 1980-1999 and 2000-2015 (Table 3.S6).

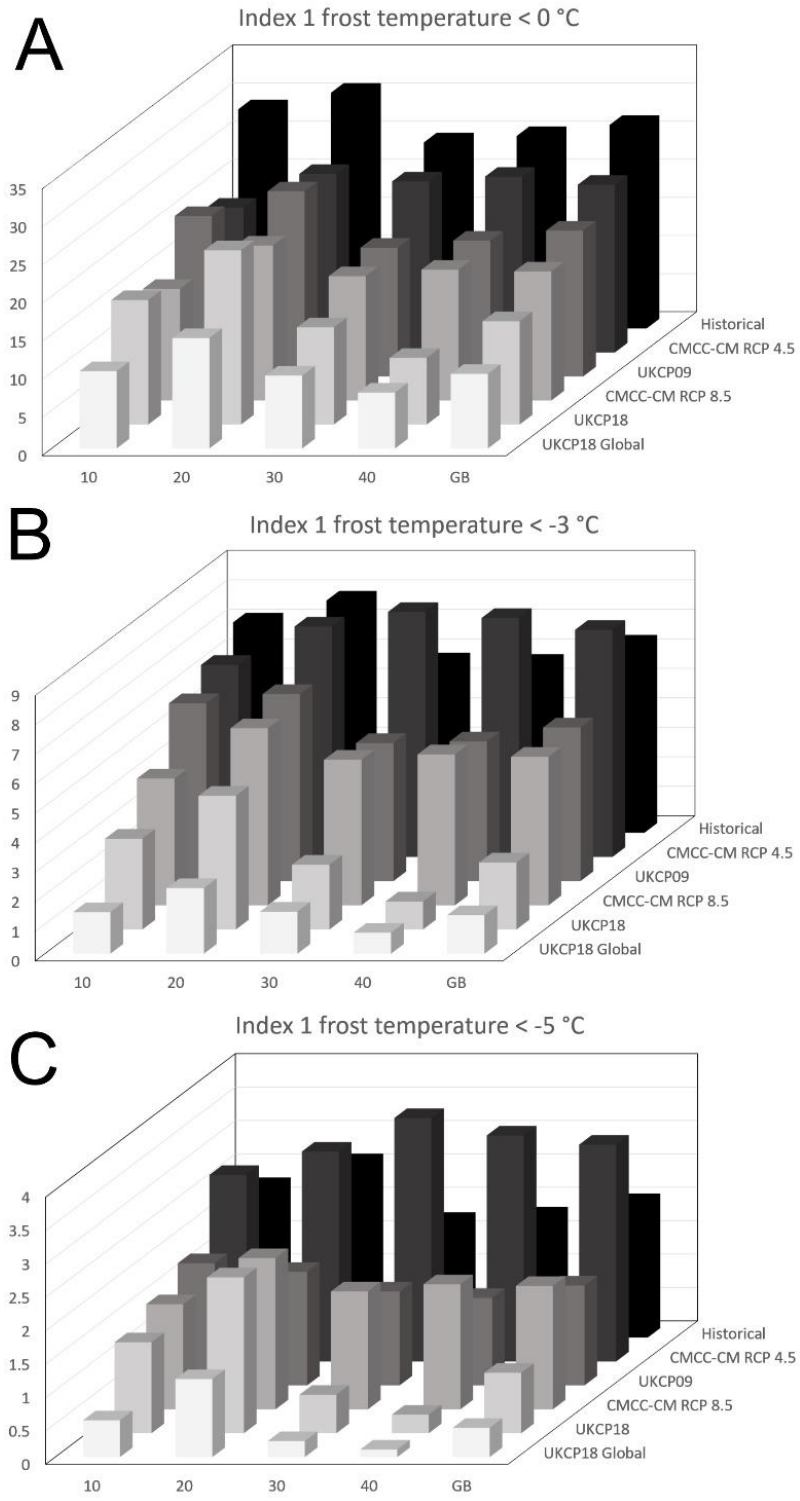

Figure 3.2. The average number of frosts < 0, -3 and -5 °C between the onset of dehardening and late summer (Index 1) for the historical (1960-2015) and the predictions for 2020-2100 made by five climate change datasets (CMCC model's data for RCP4.5, the UKCP09 datasets for model HadRM3, the CMCC model's data for RCP 8.5, the UKCP18 dataset for both the downscaled and global HadGEM3 model). Values given for all four provenance regions of GB (10, 20, 30, 40), and the average for the entire GB. Note that Y axes in all three figures have different scales.

3.3. Geographical differences within Great Britain.

All values showed a significantly ($p < 0.01$) high level of spatial autocorrelation for the historical (1960-2015) dataset (Table 3.S7). All indices, except Index 2_{min}, were significantly spatially autocorrelated for UKCP09, as were all indices, except for Index 2_{max} for UKCP18. For the global model of UKCP18, fewer indices were significantly spatially autocorrelated, while for the CMCC global model, both the RCP 4.5 and RCP 8.5 had fewer significantly spatially autocorrelated indices and the autocorrelation was weaker.

The climatic gradient of temperatures did not change with climate change, with the coldest provenance region being the northeast (zone 20), followed by the northwest (10), southwest (30), and southeast (40) of GB (Table 3.S4).

However, geographical differences in the number of frosts below -3 °C (Index $1_{-3°C}$) between the onset of dehardening and late summer between provenance regions generally reduced with climate change, especially with the higher CO_2 emissions scenario (Figure 3.4). Geographic differences were more visible in the downscaled model when compared to the global UKCP18. Geographic differences in the incidence of frosts below -5 °C (Index $1_{-5°C}$) were non-existent historically and were predicted to remain so with climate change.

The number of frosts < -3 °C (Index $1_{-3°C}$) was reduced more in the southern regions, where the historical average number of frosts was lower, than in the northern regions (Figure 3.4). There are also longitudinal differences in the rate of change. The northeast showed a higher rate of decrease of frost incidence than the northwest, whereas in the south of GB the southwest (provenance region 40, Figure 3.S2) showed a higher rate of decrease than the southeast (provenance region 30, Figure 3.S2).

The provenance regions were significantly different from each other in the number of frosts < -3 °C (Index $1_{-3°C}$) they experienced, and the differences remained significant ($p < 0.001$) with climate change. The CMCC global model showed fewer pairs of provenance regions remaining significantly different ($p < 0.001$) from each other, with no differences between provenance regions 20, 30 and 40 for the RCP 8.5, and no differences between provenance regions 30-40 for the RCP 4.5.

For the historic period 1960-2015 for GB the geographical variation in the number of frosts after bud burst, whether early, average or late (Index 2_{min}, 2, and 2_{max}), was low but significant ($p < 0.001$). Whereas for all climate change models, no significant geographical differences in the number of frosts after bud burst were observable (Figure 3.S2).

The geographical variation in the number of backlashes, Index 3, was found to increase with climate change, at least for the downscaled models (Figure 3.5). Geographic differences were much less observable with the global models.

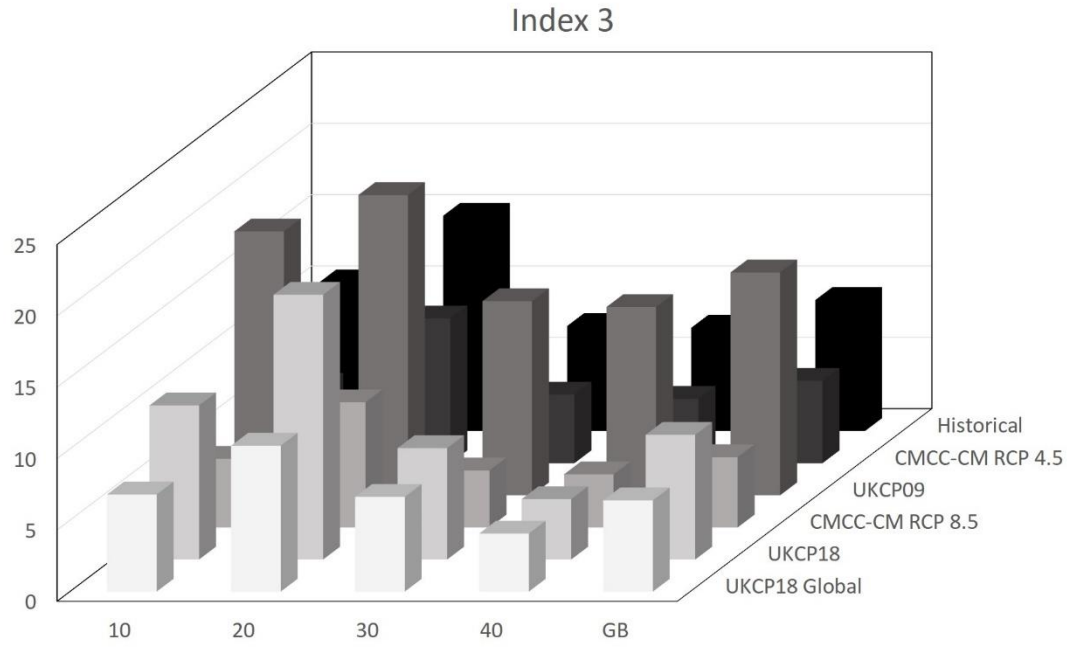

Figure 3.3. Average number of backlashes, days during which the frost tolerance of a tree is predicted to be higher than the minimum temperatures achieved that day (Index 3). Values given for the historical (1960-2015) and the predictions for 2020-2100 made by five climate change models (CMCC model's data for RCP4.5, the UKCP09 datasets for model HadRM3, the CMCC model's data for RCP 8.5, the UKCP18 dataset for both the downscaled and global HadGEM3 model). Values given for all four provenance regions of GB (10, 20, 30, 40), and the average for the entire GB.

The same pattern of change was observed for Index 1 and Index 3, with differences in the rate of change across longitudinal and latitudinal clines (Figures 2 and 3).

The hastening of bud burst occurred more markedly in the south than in the north, although differences in the rate of bud burst change between east-west were not apparent. Geographical differences in the date of bud burst remained significantly different ($p < 0.001$) both on the longitudinal and latitudinal cline (Table 3.S5).

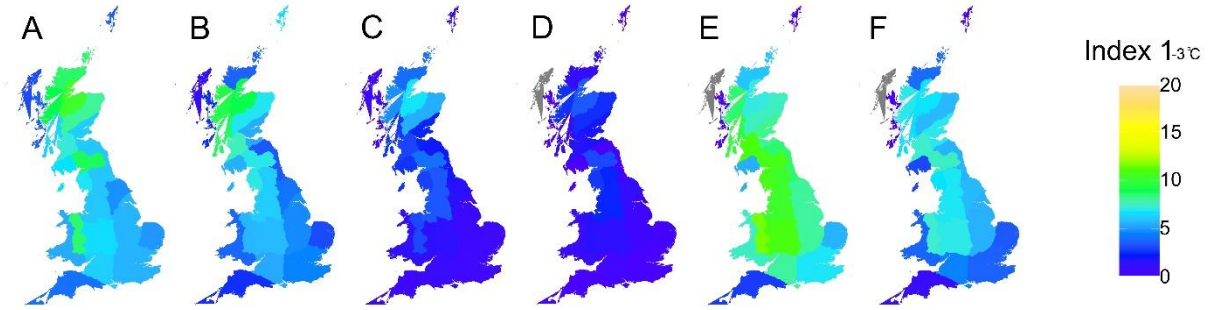

Figure 3.4. Frequency frosts < -3 °C in GB. Panes show different predictions based on different datasets. A) historical period, 1960-2012, B) UKCP09, C) UKCP18, D) UKCP18 Global model, E) CMCC-CM RCP 4.5, F) CMCC-CM RCP 8.5. Contains Forestry Commission information licensed under the Open Government License v3.0.

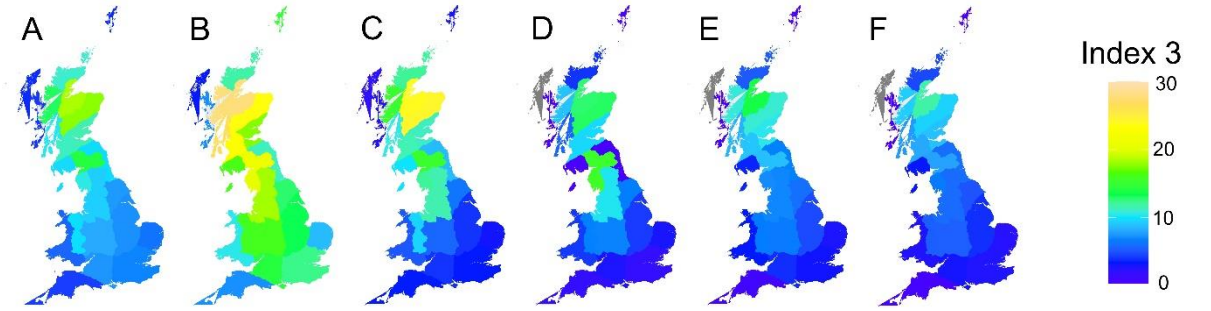

Figure 3.5. Number of backlashes (days with minimum temperatures below the level of frost hardiness) in GB. Panes show predictions based on the different datasets. A) historical period, 1960-2012, B) UKCP09, C) UKCP18, D) UKCP18 Global model, E) CMCC-CM RCP 4.5, F) CMCC-CM RCP 8.5. Contains Forestry Commission information licensed under the Open Government License v3.0.

4. Discussion

4.1. Validation of indices

Frost damage observed by commercial foresters in the field was most often correlated with a threshold temperature of -3 °C. This suggests that the natural hardiness of Sitka spruce trees to daily minimum temperatures between 0 °C and -3 °C is sufficient to prevent damage, with the bifurcation threshold where damage occurs being close to -3 °C. The weak correlation between frost damage and the number of frosts below -5 °C in British commercial forests, despite the increased severity of the frosts, can be explained by the rarity of these events, as GB has a mild, temperate climate. Thus, it can be expected that frosts cause severe damage more frequently due to unusual timing than due to their severity.

The correlation of tissue damage with the number of times minimum temperatures drop below the natural frost hardiness of a tree (*i.e.*, backlashes; Index 3) suggests that Index 3 can be used to predict expected levels of damage due to frosts. Whilst we have shown that Index 3 has utility in frost prediction, it should be noted that the data used for validation was based on frost damage observations made in a single year and that correlation of frost damage observation with indices does not necessarily indicate causation. For example, a single severe frost event could have caused all the observed damage, although this is unlikely, because forest managers were unable to ascribe the damages to a single event. This suggests that an additional measure of frost severity could be useful in making predictions.

4.2. Effects of climate change

Climate change, with warmer winter and spring periods, will change the conditions under which frosts occur in GB. Our modelling shows that predicted climate change will result in a decrease in the number of backlashes (Index 3) during the entire year thereby decreasing the chance of damage to Sitka spruce (Figure 3.3). Less frost damage is likely despite the delay of the onset of hardening in autumn, which could decrease maximum winter frost hardiness thresholds by as much as 7 K (Guak et al., 1998).

The total number of frosts (Index $1_{-3^{\circ}C}$) between the onset of dehardening and late summer is also predicted to decrease with climate change. However, the number of frosts matters less than their timing. Our data showed that climate change-driven increases in spring daily mean temperatures (average temperatures above 5° C) will advance dehardening and bud burst, resulting in conifers that are at their lowest level of frost hardiness earlier in the year (Cannell and Sheppard, 1982; Cannell and Smith, 1983; Ma et al., 2019; Morin et al., 2009, 2010). Frosts that occur before trees have dehardened do not affect trees severely, as harm occurs when air temperatures are below the frost hardiness level of the tree tissues. Therefore, the timing of the frosts is very important, and frosts occurring after a tree has dehardened or during bud burst are expected to be much more harmful (although rarer) than those occurring when a tree is hardened.

Despite the overall significant decrease in chill days during the winter with climate change, the simultaneous significant increase in thermal time, as measured by GDD (Table 3.S4), is expected to lead to earlier bud burst dates for Sitka spruce across GB, as calculated by equation 1 (Table 3.S4). Bud burst is expected to occur 2 to 57 days earlier, according to the downscaled climate models. It should be noted that the error of bud burst dates is about 12 days on the national scale (Peaucelle et al., 2019b).

Cannel and Smith's (1983) bud burst model is based exclusively on GDD and ignores the difference between the effects of daily maximum and minimum temperatures. Despite this omission the GDD based model seems to accurately describe bud burst dates for the 1960-2015 historical period used in

this study (Olsson and Jönsson, 2015). However, our study found that accounting for the different effects of maximum and minimum temperatures would lead to a model too specific to a site to be useful at larger resolutions, where generic models tend to work best (Peaucelle et al., 2019b). Thus, the generic model works best for the scale that is being used, especially considering it is calibrated to the most common local origin of Sitka spruce (QCI).

Historically, the number of post bud burst frosts precited using the Cannell and Smith (1983) GDD model is low throughout the entire 1960-2015 period, with extremely long return times between post bud burst frosts (Table 3.S3). Our GDD model is specific to Sitka spruce varieties introduced to GB. It is not locally calibrated to each seed zone as: (i) Sitka spruce is an introduced species; (ii) most commercial Sitka spruce varieties are of the same origin; and (iii) local adaptations have not occurred yet due to the limited time scale. However, due to the extremely low value for Index 2 of post-bud burst frosts (an average occurrence of 0.05 post bud burst frosts a year), it is expected that changes in phenology would not affect the amount of frost damage in commercial forests in GB (Table 3.S3). Even considering early bud burst varieties of Sitka spruce (Index 2_{min}), the number of post bud burst frosts remains extremely low both historically and with climate change.

Thus, despite the inaccuracies that result from the lack of calibration of the phenological models to local adaptations, the size of the effect means that, for Sitka spruce in GB, climate change is unlikely to change the number of post bud burst frosts by orders of magnitude to a level where it has consequences for commercial plantations. Occurrences of post bud burst frosts (Index 2) with climate change were significantly ($p < 0.001$) different to the number of historical frosts, but, at the UK scale, the extremely low frequency of frosts means that they are unlikely to cause noteworthy damage to tree form and productivity. Thus, whilst the damages inflicted on Sitka spruce plantations by post bud burst frosts may be significant when they do occur, the rarity of modelled return times (55-80 years ; Table 3.S3) suggests that post bud burst frosts are unlikely to be a factor that should be considered in relation to commercial forestry. Although the rotation length of a Sitka spruce forest in GB is typically 35-45 years, frosts are most damaging in the first 3-4 years of the establishment of a young plantation, after which damage is unlikely to affect tree form and timber quality. Due to the rarity of such frosts the length of the return time calculated in this study is limited to the length of the period studied and is likely to be even longer than predicted.

Phenology-based predictions of frost damage with climate change are very common with many authors considering the frequency of frosts after bud burst for indigenous tree species (Jönsson et al., 2004; Ma et al., 2019; Morin and Chuine, 2014) or for all vegetation (Rigby and Porporato, 2008). It should be noted that post-bud burst damage only affects some tree species (Ma et al., 2019), and our study shows Sitka spruce is not affected. Since phenology is such a common concern for breeders

when it comes to frost damage, it is useful to highlight that efforts to alter Sitka spruce phenology are unlikely to reduce frost damage in plantations throughout GB.

Locations where late spring frosts are more common, such as North America, are typically populated by tree species that are late-leafing, so these should suffer from less frost damage, while Europe and Asia have native species that quickly react to warm temperatures, and these will thus in the future become more vulnerable (Zohner et al., 2020). This suggests that Sitka spruce, which is of North American origin, is likely to benefit from climatic change when compared to native species in GB in terms of frost tolerance.

Bud burst date is negatively correlated with injury from frost, which means an earlier bud burst date leads to more damage (Aitken and Adams, 1997; Fløistad and Granhus, 2010). However, the number of frosts that occur after bud burst (Index 2) is significantly reduced with climate change (Table 3.S2). This means that, despite bud burst happening earlier, the levels of harm from frosts do not increase as trees are most sensitive in the period preceding and following bud burst when the number of frosts happening during this most critical period is expected to decrease.

These anticipated reductions in frost harm will depend on whether the warming caused by climate change is uniform. A warmer climate during the winter and spring without a reduction of frosts would increase the chances of potential frost damage experienced by trees. Despite this, the onset of plant growth following dehardening does not prevent rehardening, and fluctuations in temperature can result in cycles of hardening and dehardening (Leinonen et al., 1997). Plants cycling through the different physiological states of frost hardiness have also been shown to have higher levels of frost hardiness than plants grown in consistent temperatures, as long as the average temperature is the same (Leinonen et al., 1997).

There is an overall decrease in the occurrence of Index 3, which calculates the number of days with minimum temperatures below the frost hardiness level achieved during daily hardening-dehardening (Figure 3.3, Table 3.S3), following the historical trend (Table 3.S6). However, it should be noted that this reduction is based on the absence of sudden local fluctuations that cannot be predicted by big - scale climate models.

An observational study in north-eastern Ontario showed that warming in that area was not as uniform as was predicted by previous modelling studies. There was an increase in extreme events, like a sudden -8 °C frost which was preceded and followed by daily maximum temperatures up to 30 °C (Man et al., 2009). Therefore, we cannot rule out wider fluctuations in temperatures without the general reduction of frosts in some areas in GB where overall increases in temperature lead to an increase in sensitivity, which would mean that in those areas, the levels of harm could increase.

Changes that accompany warming, such as an increase in dry seasons (Gazol et al., 2019; Vitasse et al., 2019), may also counteract the overall beneficial effects of warming. Thus, drought years will harm the growth of trees due to the reduction of xylem conductivity (Vitasse et al., 2019). Frosts can also induce xylem embolism (Gazol et al., 2019), in addition to harming needles.

4.3. Geographical differences in Great Britain

All these climate improvements are happening to a greater degree in the north of GB relative to the south, which would facilitate an increased productivity of plantations in the north, through lower risk of frost during establishment of young plantations and increased productivity and yield due to warmer growing periods. Decreases in subfreezing temperatures are expected to lead to increases in yield of only 10-15% for Sitka spruce in GB (Waring, 2000). This is because the main limitations to growth in Sitka spruce plantation in GB seems to be solar radiation and soil fertility more than the length of the bud burst to bud set period. In fact, even winter growth is limited by solar radiation rather than temperatures for Sitka spruce in GB (Waring, 2000).

However, these decreases in the predicted number of harmful frosts depend on the reliability of climate models. Some studies show that models over-estimate mean temperatures, while better predicting increases in maximum temperatures (Pohl et al., 2019). There are also issues in prediction certainty because model predictions are based on the data from close meteorological stations, but local microclimatic conditions (*e.g.* frost hollows) may create conditions for frost damage. Thus, estimated temperatures are a proxy for real temperatures in certain locations, which are unknown (Keenan et al., 2020). This means that even if an area is predicted to have decreased frost events, local conditions may contribute to higher than expected levels of harm from frosts (Man et al., 2009).

Autocorrelation measures the level to which the value of a variable depends on its neighbouring values. If the autocorrelation is significant, the spatial distribution of high values will be spatially clustered with other high values, contrary to what would occur if the spatial processes were random. This means that the underlying geography significantly correlates with the values obtained. Thus, different regions of GB will have different spatial trends based on their geography. Global models that are not downscaled have much lower levels of spatial autocorrelation. This would indicate that low resolution global models are unable to account for underlying geography of smaller regions, thus ignoring important regional differences.

The UKCP18 Regional Projections model used in this study uses RCP 8.5, and the UKCP09 Met Office HadRM3-PPE UK run for the HadRM3 model uses the SRESA1B scenario. Improvements to the modelling accuracy of this study could be achieved if datasets of T_{min} and T_{max} generated from local scale (grid size < 25 km) models with different RCPs were available to estimate future effects of climate change.

5. Conclusion

The overall effect of climate change on Sitka spruce in GB is predicted to result in earlier bud burst, a decrease in the occurrence of frosts, and a decrease in the occurrence of frost events during a vulnerable period for plant tissues, known as backlashes. The lengthening of the bud burst to bud set metabolically active period due to earlier bud burst and the decreased risk of harm from frosts, are likely to increase the productivity of tree plantations, improve the quality of wood products, and could reduce establishment and management costs incurred by the commercial forest industry.

Data availability

Research code available at: **https://data.mendeley.com/datasets/4vvdtr9597/2**

DOI: 10.17632/4vvdtr9597.2

References

Aitken, S. N., and Adams, W. T. 1997. Spring cold hardiness under strong genetic control in Oregon populations of *Pseudotsuga menziesii* var. *menziesii*. *Can. J. Forest Res.* **27**, 1773-1780.

Aitken, Sally N., and Hannerz, M. 2001. Genecology and Gene Resource Management Strategies for Conifer Cold Hardiness. *Conifer Cold Hardiness*, 1st edn., Springer Netherlands, pp. 23-53.

Alfaro, R. I., Lewis, K. G., King, J. N., El-Kassaby, Y. A., Brown, G., and Smith, L. D. 2000. Budburst phenology of sitka spruce and its relationship to white pine weevil attack. *Forest Ecol. Manag.*, **127**, 19-29.

Ambroise, V., Legay, S., Guerriero, G., Hausman, J. F., Cuypers, A., and Sergeant, K. 2020. The roots of plant frost hardiness and tolerance. *Plant Cell Physiol*., **61**, 3-20.

Anekonda, T.S., Adams, W.T., Aitken, S.N., Neale, D.B., Jermstad, K.D., Wheeler, N.C., 2000. Genetics of cold hardiness in a cloned full-sib family of coastal Douglas-fir. *Can. J. Forest Res*. **30**, 837-840.

Bigler, C., and Bugmann, H. 2018. Climate-induced shifts in leaf unfolding and frost risk of European trees and shrubs. *Sci. Rep-UK*, **8**, 1-10.

Bivand, R., Altman, M., Anselin, L., Assunção, R., Berke, O., Bernat, A., Blanchet, G., Blankmeyer, E., Carvalho, M., Christensen, B., Chun, Y., Dormann, C., Dray, S., Gómez-Rubio, V., Gubri, M., Halbersma, R., Krainski, E., Legendre, P., Lewin-Koh, N., Li, A., Li, H., Ma, J., Mallik, A., Millo, G., Mueller, W., Ono, H., Peres-Neto, P., Piras, G., Reder, M., Tiefelsdorf, M., Westerholt, R., Yu, D. 2019. spdep: Spatial Dependence: Weighting Schemes, Statistics. Retrieved 1st July 2020 from https://github.com/r-spatial/spdep/

Bivand, R., Keitt, T., Rowlingson, B., Pebesma, E., Sumner, M., Hijmans, R., Rouault, E., Warmerdam, F., Ooms, J., Rundel, C. 2020. rgdal: Bindings for the "Geospatial" Data Abstraction Library. Retrieved

1st July 2020 from http://rgdal.r-forge.r-project.org

Cannell, M. G. R., Murray, M. B., and Sheppard, L. J. 1985. Frost Avoidance by Selection for Late Budburst in *Picea sitchensis*. *J. Appl. Ecol.*, **22**, 931-941.

Cannell, M. G. R., and Sheppard, L. J. 1982. Seasonal Changes in the Frost Hardiness of Provenances of *Picea sitchensis* in Scotland. *Forestry*, **55**, 137-153.

Cannell, M. G. R., and Smith, R. I. 1983. Thermal time, Chill days and prediction of budburst in *Picea sitchensis*. *J. Appl. Ecol.*, **20**, 951-963.

Cannell, M. G. R., Tabbush, P. M., Deans, J. D., Hollingsworth, M. K., Sheppard, L. J., Philipson, J. J., and Murray, M. B. 1990. Sitka spruce and Douglas fir seedlings in the nursery and in cold storage - root growth potential, carbohydrate content, dormancy, frost hardiness and mitotic index. *Forestry*, **63**, 9-27.

Centro Euro-Mediterraneo per I Cambiamenti Climatici. 2012. cmip5.output1.CMCC.CMCC-CM.rcp45.day.atmos.day.r1i1p1.v20120518. Retrieved October 1, 2020, from https://esgf-node.llnl.gov/search/cmip5/

Centro Euro-Mediterraneo per I Cambiamenti Climatici. 2017. cmip5.output1.CMCC.CMCC-CM.rcp85.day.atmos.day.r1i1p1.v20170725. Retrieved October 1, 2020, from https://esgf-node.llnl.gov/search/cmip5/

Fløistad, I. S., and Granhus, A. 2010. Bud break and spring frost hardiness in *Picea abies* seedlings in response to photoperiod and temperature treatments. *Can. J. Forest Res.*, **40**, 968-976.

Forestry Commission. 1957. Exotic forest trees in Great Britain. *Forestry Commission Bulletin*, 30.

Forestry Commission. 2012. Forest Reproductive Materials Sites GB. Retrieved 1st July 2020 from https://data.gov.uk/dataset/ebC3fd45-27fc-499f-968e-ba4398f088fb/forest-reproductive-materials-sites-gb

Forestry Commission. 2019. Chapter 1: Woodland Area and Planting. *Forestry Statistics 2019* pp. 1-37.

Fu, Y. H., Liu, Y., De Boeck, H. J., Menzel, A., Nijs, I., Peaucelle, M., Peñuelas, J., Piao, S., Janssens, I. A. 2016. Three times greater weight of daytime than of night-time temperature on leaf unfolding phenology in temperate trees. *New Phytol.*, **212**, 590-597.

Fu, Y. H., Zhao, H., Piao, S., Peaucelle, M., Peng, S., Zhou, G., Ciais, P., Huang, M., Menzel, A., Peñuelas, J., Song, Y. Vitasse, Y., Zeng, Z., Janssens, I. A. 2015. Declining global warming effects on the phenology of spring leaf unfolding. *Nature*, **526**, 104-107.

Gazol, A., Camarero, J. J., Colangelo, M., de Luis, M., Martínez del Castillo, E., and Serra-Maluquer, X. 2019. Summer drought and spring frost, but not their interaction, constrain European beech and Silver fir growth in their southern distribution limits. *Agr. Forest Meteorol.*, **278**, 107695.

Guak, S., Olsyzk, D. M., Fuchigami, L. H., and Tingey, D. T. 1998. Effects of elevated CO_2 and

temperature on cold hardiness and spring bud burst and growth in Douglas-fir *Pseudotsuga menziesii*. *Tree Physiol.*, **18**, 671-679.

Hadley Centre for Climate Prediction and Research. 2014. UKCP09: Met Office HadRM3-PPE UK model runs. Retrieved 1st July, 2020, from http://catalogue.ceda.ac.uk/uuid/465ecd8a305ffb9df2bd8b54cada669f

Hamilton, J. A., El Kayal, W., Hart, A. T., Runcie, D. E., Arango-Velez, A., and Cooke, J. E. K. 2016. The joint influence of photoperiod and temperature during growth cessation and development of dormancy in white spruce *Picea glauca*. *Tree Physiol.*, **36**, 1432-1448.

Harrell, F. E. J. 2020. Hmisc: Harrell Miscellaneous. Retrieved 1st July 2020 from https://cran.r-project.org/package=Hmisc

Herbert, R., Samuel, S., and Patterson, G. 1999. Using Local Stock for Planting Native Trees and Shrubs. *Forestry Commission*, 8.

Hijmans, R. J., van Etten, J., Sumner, M., Cheng, J., Bevan, A., Bivand, R., Busetto, L., Canty, M., Forrest, D., Ghosh, A., Golicher, D., Gray, J., Greenberg, J.A., Hiemstra, P., Hingee, K., Institute for Mathematics Applied Geosciences, Karney, C., Mattiuzzi, M., Mosher, S., Nowosad, J., Pebesma, E., Perpinan Lamigueiro, O., Racine, E.B., Rowlingson, B., Shortridge, A., Venables, B., Wueest., R. 2020. raster: Geographic Data Analysis and Modeling. Retrieved 1st July 2020 from https://cran.r-project.org/package=raster

Holopainen, J. K., and Holopainen, T. 1988. Cellular responses of Scots pine *Pinus sylvestris* L. seedlings to simulated summer frost. *Eur. J. Forest Pathol.*, **18**, 207-216.

Jeong, Y., Chung, U., and Kim, K. H. 2018. Predicting future frost damage risk of kiwifruit in Korea under climate change using an integrated modelling approach. *Int. J. Climatol.*, **38**, 5354-5367.

Jönsson, A. M., Linderson, M.-L. L., Stjernquist, I., Schlyter, P., and Bärring, L. 2004. Climate change and the effect of temperature backlashes causing frost damage in *Picea abies*. *Global Planet. Change*, **44**, 195-207.

Keenan, T. F., Richardson, A. D., and Hufkens, K. 2020. On quantifying the apparent temperature sensitivity of plant phenology. *New Phytol.*, **225**, 1033-1040.

Kellomäki, S., Hänninen, H., and Kolström, M. 1995. Computations on Frost Damage to Scots Pine Under Climatic Warming in Boreal Conditions. *Ecol. Appl.*, **5**, 42-52.

Kuprian, E., Briceño, V. F., Wagner, J., and Neuner, G. 2014. Ice barriers promote supercooling and prevent frost injury in reproductive buds, flowers and fruits of alpine dwarf shrubs throughout the summer. *Environ. Exp. Bot.*, **106**, 4-12.

Lee, D. 2019. CARBayes: Spatial Generalised Linear Mixed Models for Areal Unit Data. Retrieved 1st July 2020 from http://github.com/duncanplee/CARBayes

Lee, S.J. 1999. Improving the timber quality of Sitka spruce through selection and breeding. *Forestry* **72**, 123-133.

Leinonen, I., Repo, T., and Hanninen, H. 1997. Changing Environmental Effects on Frost Hardiness of Scots Pine During Dehardening. *Ann. Bot.-London*, **79**, 133-137.

Ma, Q., Huang, J. G., Hänninen, H., and Berninger, F. 2019. Divergent trends in the risk of spring frost damage to trees in Europe with recent warming. *Glob. Change Biol.*, **25**, 351-360.

Man, R., Colombo, S., Lu, P., and Dang, Q.L. 2016. Effects of winter warming on cold hardiness and spring budbreak of four boreal conifers. *Botany*, **94**, 117-126.

Man, R., Kayahara, G. J., Dang, Q. L., and Rice, J. A. 2009. A case of severe frost damage prior to budbreak in young conifers in Northeastern Ontario: Consequence of climate change? *Forestry Chronicle*, **85**, 453-462.

Meier, M., Fuhrer, J., and Holzkämper, A. 2018. Changing risk of spring frost damage in grapevines due to climate change? A case study in the Swiss Rhone Valley. *Int. J. Biometeorol.*, **62**, 991-1002.

Meng, F., Zhang, L., Zhang, Z., Jiang, L., Wang, Y., Duan, J., Wang, Q., Li, B., Liu, P., Hong, H., Lv, W., Renzeng, W., Wang, Z., Luo, C., Dorji, T., Zhou, H., Du, M., Wang, S. 2019. Opposite effects of winter day and night temperature changes on early phenophases. *Ecology*, **100**, 1-8.

Meng, L., Zhou, Y., Li, X., Asrar, G. R., Mao, J., Wanamaker, A. D., and Wang, Y. 2020. Divergent responses of spring phenology to daytime and nighttime warming. *Agr. Forest Meteorol.*, **281**, 107832.

Met Office Hadley Centre. 2018a. UKCP18 Global Climate Model Projections for the entire globe. Retrieved 1st July 2020 from http://catalogue.ceda.ac.uk/uuid/f1a2fc3c120f400396a92f5de84d596a

Met Office Hadley Centre. 2018b. UKCP18 Regional Projections on a 12km grid over the UK for 1980-2080. Retrieved October 1, 2020, from https://catalogue.ceda.ac.uk/uuid/589211abeb844070a95d061c8cc7f604

Met Office, Hollis, D., and McCarthy, M. 2017. UKCP09: Met Office gridded and regional land surface climate observation datasets. Centre for Environmental Data Analysis. Retrieved 1st July 2020 from http://catalogue.ceda.ac.uk/uuid/87f43af9d02e42f483351d79b3d6162a

Morin, X., and Chuine, I. 2014. Will tree species experience increased frost damage due to climate change because of changes in leaf phenology? *Can. J. Forest Res.*, **44**, 1555-1565.

Morin, X., Lechowicz, M. J., Augspurger, C., O'Keefe, J., Viner, D., and Chuine, I. 2009. Leaf phenology in 22 North American tree species during the 21st century. *Glob. Change Biol.*, *15*4, 961-975.

Morin, X., Roy, J., Sonié, L., and Chuine, I. 2010. Changes in leaf phenology of three European oak species in response to experimental climate change. *New Phytol.*, **186**, 900-910.

Murray, M. B., Cannell, M. G. R., and Smith, R. I. 1989. Date of budburst of fifteen tree species in Britain following climatic warming. *British Ecological Society*, **26**, 693-700.

Murray, M. B., Smith, R. I., Leith, I. D., Fowler, D., Lee, H. S. J., Friend, A. D., and Jarvis, P. G. 1994. Effects of elevated CO_2, nutrition and climatic warming on bud phenology in Sitka spruce *Picea sitchensis* and their impact on the risk of frost damage. *Tree Physiol.*, **147**, 691-706.

Neilson, R. E., Ludlow, M. M., and Jarvis, P. G. 1972. Photosynthesis in Sitka Spruce *Picea sitchensis* Bong. Carr.. II. Response to temperature. *J. Appl. Ecol.*, **9**, 721-745.

Neuwirth, E. 2014. RColorBrewer: ColorBrewer Palettes. Retrieved 1st July 2020 from https://cran.r-project.org/package=RColorBrewer

Olsson, C., and Jönsson, A. M. 2015. Budburst model performance: The effect of the spatial resolution of temperature data sets. *Agr. Forest Meteorol.*, **200**, 302-312.

Peaucelle, M., Janssens, I. A., Stocker, B. D., Descals Ferrando, A., Fu, Y. H., Molowny-Horas, R., Ciais, P., Peñuelas, J. 2019a. Spatial variance of spring phenology in temperate deciduous forests is constrained by background climatic conditions. *Nat. Commun.*, **10**, 1-10.

Peaucelle, M., Ciais, P., Maignan, F., Nicolas, M., Cecchini, S., and Viovy, N. 2019b. Representing explicit budburst and senescence processes for evergreen conifers in global models. *Agr. Forest Meteorol.*, **266-267**, 97-108.

Pebesma, E., Bivand, R., Rowlingson, B., Gomez-Rubio, V., Hijmans, R., Sumner, M., MacQueen, D., Lemon, J., O'Brien, J., O'Rourke, J. sp: Classes and Methods for Spatial Data 2019. Retrieved 1st July 2020 from https://github.com/edzer/sp/

Peel, M. C., Finlayson, B. L., and McMahon, T. A. 2007. Updated world map of the Köppen-Geiger climate classification. *Hydrol. Earth Syst. Sc.*, **11**, 1633-1644.

Pierce, D. 2019. Interface to Unidata netCDF Version 4 or Earlier Format DataFiles. Retrieved 1st July 2020 from http://cirrus.ucsd.edu/~pierce/ncdf/

Pohl, B., Joly, D., Pergaud, J., Buoncristiani, J. F., Soare, P., and Berger, A. 2019. Huge decrease of frost frequency in the Mont-Blanc Massif under climate change. *Sci. Rep-UK*, **9**, 1-7. https://doi.org/10.1038/s41598-019-41398-5

Pohlert, T. 2020. Calculate Pairwise Multiple Comparisons of Mean Rank Sums Extended. Retrieved 1st July 2020 from https://cran.r-project.org/package=PMCMRplus

R Foundation for Statistical Computing, and R Core Team. 2019. R: A language and environment for statistical computing. Retrieved 1st July 2020 from http://www.r-project.org/index.html

Repo, T. 1992. Seasonal changes of frost hardiness in *Picea abies* and *Pinus sylvestris* in Finland. *Can. J. Forest Res.*, **22**, 1949-1957.

Reyes-Fox, M., Steltzer, H., Trlica, M. J., McMaster, G. S., Andales, A. A., Lecain, D. R., and Morgan, J. A. 2014. Elevated CO_2 further lengthens growing season under warming conditions. *Nature*, **510**, 259-262.

Richardson, A. D., Hufkens, K., Milliman, T., Aubrecht, D. M., Furze, M. E., Seyednasrollah, B., Krassovski, M.B., Latimer, J.M., Nettles, W.R., Heiderman, R.R., Warren, J.M., Hanson, P. J. 2018. Ecosystem warming extends vegetation activity but heightens vulnerability to cold temperatures. *Nature*, **560**, 368-371.

Rigby, J. R., and Porporato, A. 2008. Spring frost risk in a changing climate. *Geophys. Res. Lett.*, **35**, 1-5.

Rochette, P., Bélanger, G., Castonguay, Y., Bootsma, A., and Mongrain, D. 2004. Climate change and winter damage to fruit trees in eastern Canada. *Can. J. Plant Sci.*, **84**, 1113-1125.

RStudio Team. 2019. RStudio: Integrated Development for R. Retrieved 1st July 2020 from http://www.rstudio.com/

Sakai, A. 1978. Low temperature exotherms of winter buds of hardy conifers. *Plant Cell Physiol.*, **19**, 1439-1446.

Sakai, A., and Larcher, W. 1987. Mechanisms of Frost Survival. *Frost survival of plants*. Springer -Verlag, pp. 59-96.

Stabler, B. 2013. shapefiles: Read and Write ESRI Shapefiles. Retrieved 1st July 2020 from https://cran.r-project.org/package=shapefiles

Vitasse, Y., Bottero, A., Cailleret, M., Bigler, C., Fonti, P., Gessler, A., Lévesque, M., Rohner, B., Weber, P., Rigling, A., Wohlgemuth, T. 2019. Contrasting resistance and resilience to extreme drought and late spring frost in five major European tree species. *Glob. Change Biol.*, **25**, 3781-3792.

Vitasse, Y., and Rebetez, M. 2018. Unprecedented risk of spring frost damage in Switzerland and Germany in 2017. *Climatic Change*, **149**, 233-246.

Waring, R. H. 2000. A process model analysis of environmental limitations on the growth of Sitka spruce plantations in Great Britain. *Forestry*, **73**, 65-79.

Wickham, H., François, R., Henry, L., Müller, K., and Rstudio. 2020a. dplyr: A Grammar of Data Manipulation. Retrieved 1st July 2020 from http://dplyr.tidyverse.org

Wickham, Hadley, Chang, W., Henry, L., Pedersen, T. L., Takahashi, K., Wilke, C., Woo, K., Yutani, H., Dunnington, D., RStudio. 2020b. ggplot2: Create Elegant Data Visualisations Using the Grammar of Graphics. Retrieved 1st July 2020 from http://ggplot2.tidyverse.org

Woldendorp, G., Hill, M. J., Doran, R., and Ball, M. C. 2008. Frost in a future climate: Modelling interactive effects of warmer temperatures and rising atmospheric CO_2 on the incidence and severity of frost damage in a temperate evergreen *Eucalyptus pauciflora*. *Glob. Change Biol.*, **14**, 294-308.

Zhang, M. I. N., and Willison, J. H. M. 1987. An improved conductivity method for the measurement of frost hardiness. *Can. J. Botany*, **65**, 1774-1774.

Zohner, C. M., Mo, L., Renner, S. S., Svenning, J. C., Vitasse, Y., Benito, B. M., Ordonez, A., Baumgarten, F., Bastin, J. F., Sebald, V., Reich, P. B., Liang, J., Nabuurs, G. J., De-Migueln, S., Alberti, G.,Antón-Fernández, C.,Balazy, R., Brändli, U. B., Chen, H. Y. H., Chisholm, C., Cienciala, E., Dayanandan, S., Fayle, T. M., Frizzera, L., Gianelle, D., Jagodzinski, A. M., Jaroszewicz, B., Jucker, T., Kepfer-Rojas, S., Khan, M. L., Kim, H. S., Korjus, H., Johannsen, V. K., Laarmann, D., Langn, M., Zawila-Niedzwiecki, T., Niklaus, P. A., Paquette, A., Pretzsch, H., Saikia, P., Schall, P., Seben, V., Svoboda, M., Tikhonova, E., Viana, H., Zhang, C., Zhao, X., Crowther, T. W. 2020. Late-spring frost risk between 1959 and 2017 decreased in North America but increased in Europe and Asia. *P. Natl. Acad. Sci. USA*, **117**, 1-9.

Chapter 4: Methods for measuring frost tolerance of gymnosperms: a systematic map

Abstract

Frost tolerance is the temperature threshold below which plants start experiencing damage. Measuring frost tolerance involves various steps, each of which will vary depending on the objectives of the study. This systematic map takes an overall view of the literature that uses frost tolerance measuring techniques. Many different techniques were used for testing, although the gold standard remains the field observation study, which, due to its cost, is frequently substituted by other techniques. Closed enclosure freezing tests combine all non-field freezing tests, are done using various types of equipment. An examination of the literature indicates that several factors have to be controlled in order to measure frost tolerance similarly to what could be observed in a field study. Equipment that allows controlling the freezing rate, frost exposure time and thawing rate would obtain results closer to field studies. Other important factors in study design were the number of test temperatures used, the range of temperatures selected and the decrements between the temperatures, which should be selected based on expected frost tolerance of the tissue and species.

1. Introduction

Frost tolerance was the mechanism that protects plants from severe, unrecoverable harm caused by freezing temperatures. Damage can be caused by ice, leading to cell wall damage (Fujikawa et al., 1999; Kasuga et al., 2007), protein denaturation (Welling and Palva, 2006), and cell and chloroplast membrane damage (Gillies and Binder, 1996). Frosts can cause damage when combined with other factors, like photoinhibition, the phenomenon where freezing was combined with an abundance of light energy and photosystem II (PSII) cannot discharge the excess of energy (Gillies and Binder, 1996). This results in the degradation of PSII, causing damage to chlorophyll. Although rarer in conifers than in other species (Sperry et al., 1994), the combination of frosts with drought (or a frozen ground, which complicates the absorption of water by the roots), can lead to freeze-thaw embolism, thus increasing frost damage (Davis et al., 1999; Mayr et al., 2003; Sperry and Sullivan, 1992).

Gymnosperms, which tend to be evergreen, need to develop mechanisms to deal with frost damage differently from angiosperms, since they cannot use the strategy of shedding vulnerable tissue during cold times. Needles tend to have a lower frost tolerance than stems, as they were less protected from the cold (Aitken and Adams, 1996; Beuker et al., 1998).

Extensive reviews were available on the nature of frost hardiness of plants (Bannister and Lord, 2006; Hughes and Dunn, 1996), cereals (Frederiks et al., 2015), woody plants (Li et al., 2004; Neuner, 2014; Strimbeck et al., 2015), trees (Rodrigo, 2000; Welling and Palva, 2006) and the molecular mechanisms of frost hardiness (Hughes and Dunn, 1996; Li et al., 2004; Sheppard, 1994; Welling and Palva, 2006). A book has also been published on the frost hardiness of conifers (Bigras and Colombo, 2001), which includes descriptions of the ecology, hardening, interaction of frost hardiness with biotic factors, and even a description of methods used for measuring frost hardiness.

We have only found three compilations of techniques used to measure frost tolerance in gymnosperms, a Canadian Forest Resource Development Agreement (FRDA) report (Keates, 1990), a review (Warrington and Rook, 1980), and a book chapter (Bigras and Colombo, 2001). Keates (1990) focuses on sample selection, conditioning, freezing, testing, and statistical analysis, while Warrington and Rook (1980) focuses on the techniques used for freezing and testing. The book chapter by Bigras and Colombo (2001) focuses on the description of the measurement techniques and their advantages and disadvantages. Updating the current literature on frost tolerance techniques, comparing the techniques between each other, and analysing the technical details that make the techniques different from their idealized form could be useful for somebody designing a study measuring frost tolerance. The primary goal of this review was thus to provide the information necessary to design a study measuring frost tolerance. The objectives were:

(i) to document which techniques were used and how they were used;
(ii) to document the technical constraints faced when measuring frost tolerance;
(iii) to note any reported correlations between different techniques in terms of results, by examining studies that use more than one method in further detail.

The review also aims to answer the central research question, 'Which were the current state of the art techniques used to measure frost hardiness in gymnosperms?', as well as secondary questions: 'How robust were these techniques?' and 'How well do the techniques measure frost hardiness?'.

2. Methods

2.1. Search strategies

The peer reviewed literature searching was conducted using 'topic' for a basic search in Web of Science (Clarivate Analytics, USA), using the entire time span available, 'all years' (1970-2020), and 'keywords' for a basic search in the Cab Direct database (CAB International) on 20th November 2020, which includes articles between 1968-2020. The search used the terms outlined in Table 4.1. No additional attempt at retrieving grey literature was made, although some of the search results through Cab Direct were part of the grey literature. One study passed all three rounds of selection.

The search strategy was optimized during a scoping phase, which tried to find an appropriate balance between depth (number of papers found) and specificity (how well the papers found fit the search criteria). This was achieved through an explorative search (Table 4.S1). The search terms were given a broader range by using the asterisk wildcard, which enables matching a word with multiple beginnings or endings. For example, the '*freezing' term would find words such as freezing or subfreezing, and the 'test*' term will find words such as test, tests or testing. Search terms were concatenated using the Boolean operators 'AND' and 'OR'.

Papers were accessed through the Bangor University access portal and through green open access literature. No additional effort was made to find inaccessible articles published before the year 2010. Only English and Spanish language papers were included, other languages were discarded.

2.2. Article screening and inclusion criteria

Literature results were exported into Excel (Microsoft Corporation, USA), and duplicates deleted. Results were screened based on the inclusion/exclusion criteria listed in Table 4.2. Only original research papers that directly study the measurement of frost tolerance of above ground tissues in gymnosperms were included (Table 4.2). Three rounds of selection were conducted. In the first selection round search results were excluded based on the title, and more leeway was given with the criteria in Table 4.2 than in the second round. Ambiguity as to the species studied or the part of the plant studied was interpreted favourably, leading to inclusion. In the second round, where the articles were included based on the abstract, the criteria in Table 4.2 were adhered to strictly. All reviews and modelling studies were excluded, and the abstract had to mention a gymnosperm species and frost tolerance measurements. Papers that studied species other than gymnosperms were included as long as they included at least one gymnosperm species whose frost tolerance was studied. In the final selection round, selected papers that were available were excluded if they did not explain the technique used for measuring frost tolerance.

Table 4.1. Definition of the main components of the search and the search terms used.

	Definition	Search terms[1]
Population	All gymnosperms	All gymnosperm Latin species names: (*Cycas OR Dioon OR Bowenia OR Macrozamia OR Lepidozamia OR Encephalartos OR Stangeria OR Ceratozamia OR Microcycas OR Zamia OR Ginkgo OR Welwitschia OR Gnetum OR Ephedra OR Cedrus OR Pinus OR Cathaya OR Picea OR Pseudotsuga OR Larix OR Pseudolarix OR Tsuga OR Nothotsuga OR Keteleeria OR Abies OR Araucaria OR Wollemia OR Agathis OR Phyllocladus OR Lepidothamnus OR Prumnopitys OR Sundacarpus OR Halocarpus OR Parasitaxus OR Lagarostrobos OR Manoao OR Saxegothaea OR Microcachrys OR Pherosphaera OR Acmopyle OR Dacrycarpus OR Dacrydium OR Falcatifolium OR Retrophyllum OR Nageia OR Afrocarpus OR Podocarpus OR Sciadopitys OR Cunninghamia OR Taiwania OR Athrotaxis OR Metasequoia OR Sequoia OR Sequoiadendron OR Cryptomeria OR Glyptostrobus OR Taxodium OR Papuacedrus OR Austrocedrus OR Libocedrus OR Pilgerodendron OR Widdringtonia OR Diselma OR Fitzroya OR Callitris OR Actinostrobus OR Neocallitropsis OR Thujopsis OR Thuja OR Fokienia OR Chamaecyparis OR Callitropsis OR Cupressus OR Juniperus OR Xanthocyparis OR Calocedrus OR Tetraclinis OR Platycladus OR Microbiota OR Austrotaxus OR Pseudotaxus OR Taxus OR Cephalotaxus OR Amentotaxus OR Torreya)*. And ordinary names for the most common gymnosperms (*OR cedar OR celery-pine OR cypress OR fir OR juniper OR larch OR pine OR redwood OR spruce OR yew)*. The common name for the largest division among gymnosperms *(OR conifers)*, as well as the common name for conifer wood (*OR softwood)*.
Trait	Frost resistance	Synonyms for **frost** (frost OR *freezing OR subzero OR cold*), joined with synonyms for resistance (*toleran* OR hard* OR resistan**), joined by the AND Boolean operator.
Technique/Method	Techniques used to measure frost resistance	Synonyms for techniques and technologies (*test* OR technique* OR measure* OR treat* OR trait OR analys**)

1. Separate strings in brackets and joined by the AND Boolean operator.

2.3. Coding of the articles

Metadata from all included research papers was recorded in an Excel workbook, with columns including basic publication data available (year, title, publication, DOI, language).

Information was extracted from the papers on the basis of three main categories: source and conditions of original biological study material (species, growing conditions, tissue studied); the treatment given (how freezing treatment was conducted, how thawing was handled, the temperature treatments used, the length of the treatment and its accuracy); and the measurement technique used (Visual Assessment (VA), Electrolyte Leakage (EL), Differential Thermal Analysis (DTA), fluorometry, and others). For studies where more than one technique was used, information on the correlation between the results of the techniques was also noted. On the rare occasion when a field assessment was done in natural conditions, this was noted. Equipment used for freezing tests was classified into categories according to its functionality and technology employed.

The categories for coding were decided *a priori* based on experience and practice with frost phenotyping methods. Examples of the extracted data files can be viewed in Tables 4.3, 4.4 and 4.5 for each of the three categories (source material, treatment and measuring technique).

Table 4.2. Inclusion and exclusion criteria for entries to be included in the systematic map (decided *a priori*).

Inclusion criteria	Exclusion criteria
• Original research.	• Reviews, modelling studies, projections.
• Studies done on gymnosperms.	• No gymnosperm species studied.
• Directly measures frost tolerance.	• Uses indirect methods only to measure frost tolerance (*e.g.* DNA markers, amino acid levels, sugar levels, antioxidants, tree rings).
• Measures frost tolerance of above ground tissues.	• Does not measure frost tolerance of above ground tissue at all.

3. Results

3.1. Summary of the evidence

In total, 3,095 publications were found, of which 677 duplicates were deleted (Figure 4.1). After screening by title, 495 articles passed the inclusion criteria. Of these, 400 were included after examining the abstracts, and 88 were unavailable. Since all the unavailable articles were published before 2010, no additional effort was made to obtain them. Only English language articles were scored, plus a Spanish language article (other languages were excluded). Upon examination of the entire paper, 283 were selected (Table 4.S2).

3.2. Overview of the included articles and studies

Various publication types were included in this systematic map, specifically journal articles (n = 264, from 70 journals), conference proceedings (n = 10), notes in journals (n = 5), professional forester organization bulletins (n = 1), and research theses (n = 3) (Figure 4.1). The journals publishing the most articles were *Canadian Journal of Forest Research* (n = 49), *Scandinavian Journal of Forest Research* (n = 31), *Tree Physiology* (n = 24), *New Forests* (n = 12), *Forest Ecology and Management* (n = 11) and *Physiologia Plantarum* (n = 11). The rest of the journals were represented by < 10 articles; one journal was represented by nine articles, three journals were represented by seven articles, one by five, one by four, seven by three, thirteen by two, and the rest were included only once.

Most of the research on frost tolerance was published in the 1990-1999 decade, with subsequent decline in the posterior decades (Figure 4.2). The techniques described in this review were old, with little change in the methodology used in the papers published after 2000.

3.3. Key findings

3.3.1. Sample selection

Among gymnosperms studied for frost tolerance, Norway Spruce (*Picea abies* (L.) H. Karst.) was the most studied species (n = 56), Scots Pine (*Pinus sylvestris* L.) the second (n = 50) and Douglas fir (*Pseudotsuga menziesii* (Mirb). Franco) the third (n = 46) (Table 4.6). Overall, spruces were the most studied genus among the studies included in this review.

Studies that did not consider the frost tolerance of gymnosperms were excluded in the initial steps. For this review, in studies where both gymnosperms and angiosperms were studied, only the gymnosperm species were annotated.

Most studies focused on 1-4 species, with only ten studies researching the frost tolerance of more than five gymnosperm species. These studies were designed to measure the frost tolerance of either species that belonged to the same taxonomic group (Climent et al., 2009; Hodge et al., 2012; Jones and Cregg, 2006; Mabaso et al., 2017), or species that belonged to the same geographical area (Bannister and Lord, 2006; Sakai and Malla, 1981; Sakai and Wardle, 1978).

Studies used cut branches (n = 101, 35.6%), whole plants (n = 100, 35.3%), needles (n = 94, 33.2%) (Table 4.7), on their own or in combination (n = 41, 14.5%). The exact combinations can be seen in Table 4.7, with the sum adding up to 283. Needles were the organ most studied. Buds (n = 25, 8.8%) or stems (n = 15, 5.3%) were studied in only 40 cases.

Table 4.3. Example of ten coded research articles for source material.

Source material			Article
Gymnosperm species[1]	**Tissue studied**	**Growing conditions**	
Picea abies	Whole plant	Field	Buchner and Neuner, 2009
Picea glauca	Whole plant exposure, shoots assessed.	Tunnel	Carles et al., 2012
Picea rubens	Shoots	Field	Adams and Perkins, 1993
Picea sitchensis	Branches	Field in boxes	Nicoll et al., 1996
Pinus sylvestris	Needles	Greenhouse	Aho, 1994
Pinus sylvestris	Whole plant	Greenhouse	Andersson, 1992
Picea abies	Whole plant	Phytotron	Fløistad and Granhus, 2010
Pinus sylvestris	Needles	Field and growth chamber	Leinonen et al., 1997
Picea abies	Branches exposed, needles cut and assessed.	Field	Kathke and Bruelheide, 2011
Pinus sylvestris	Needles	Field	Repo et al., 2000

1. Only the gymnosperm species studied in the paper were mentioned; even if other plant species were studied, those were not listed.

Table 4.4. Example of ten coded research articles for freezing treatment.

Freezing treatment						Article
Freezing Technique	**Freezing rate**	**Thawing method**	**Length exposure**	**Temperature treatments used**	**Accuracy**	
Field exposure chambers that use deeply cooled ethanol	5 K h^{-1}	5 K h^{-1}, using the field exposure chambers.	6 h	Treatment temperatures of -30 °C, -40 °C and -50 °C.	Not given	Buchner and Neuner, 2009
Programmable freezer T20RS, Versa Tenn controller; temperatures first lowered to -2 °C and kept there for 40 h to ensure freezing, then lowered at a faster rate.	2 K h^{-1}	Kept at refrigerator at 4 °C until thawed.	1 h	A +4 °C control, and -4 °C, -8 °C, -12 °C and -20 °C treatment temperatures.	Not given	Carles et al., 2012
Formatemp ethanol bath.	10 K h^{-1}	Thawed at incubator at 5 °C.	Not given	Treatment temperatures from 0 °C to -25 °C at 5K intervals, then at 2.5K intervals to -65 °C.	Not given	Adams and Perkins, 1993
Natural temperatures.	Natural freezing rate.	Natural thawing rate.	Not given	Recorded temperatures of -3 °C to -5 °C.	Not given	Nicoll et al., 1996
Freezing chambers.	6 K h^{-1}		3 h	Treatment temperatures ranging from -1 °C to -33 °C.	Not given	Aho, 1994
Not mentioned.	5 K h^{-1}	Thawing rate of 5 K h^{-1} given without mentioning how it was achieved.	2 h	Treatment temperatures ranging from -10 °C and -15 °C.	Not given	Andersson, 1992
Not mentioned.	2 K h^{-1}	Thawing rate of 2 K h^{-1} given without mentioning how it was achieved.	4 h	Different temperature treatment according to photoperiod treatment.	Not given	Floistad and Granhus, 2010
Not mentioned.	5 K h^{-1}	Thawing rate of 2 K h^{-1} given without mentioning how it was achieved.	4 h	Not given	Not given	Leinonen et al., 1997
Climate chamber (Sanyo)	Cooled to first four temperatures at a 4 K h^{-1} freezing rate, last four at 6 K h^{-1}.	Thawed at 4 °C overnight	30 min	Negative control, +4 °C, positive control, -196 °C (liquid nitrogen). Treatment temperatures of 0 °C, -8 °C, -16 °C, -24 °C, -32 °C, -40 °C, -80 °C.	Not given	Kathke and Bruelheide, 2011
Air cooled chamber	5 K h^{-1}	Air cooled chambers at a rate of 5 K h^{-1}	4 h	Temperatures ranging down to -70 °C	Not given	Repo et al., 2000

Table 4.5. Example of ten coded research articles for source material.

Damage assessment		Field assessment	Article
Technique used[1]	**Correlation between methods[2]**		
VA; Tetrazolium assay	Not given		Buchner and Neuner, 2009
EL	Not given		Carles et al., 2012
VA; Fluorometry	Measurements by two techniques correlated with a Pearson's r = 0.81.		Adams and Perkins, 1993
EL; VA	Not given	Field assessment done by studying the effect of frosts in a forest planted outside.	Nicoll et al., 1996
VA; Impedance measurements	Not given		Aho, 1994
VA			Andersson, 1992
VA			Floistad and Granhus, 2010
VA			Leinonen et al., 1997
EL			Kathke and Bruelheide, 2011
EL; VA	There was a close correlation (r=0.97, p<0.001) between frost hardiness assessed by EL and VA.		

1. Abbreviations: VA, Visual Assesment; EL, Electrolyte Leakage.
2. Only given when more than two methods of assessment were given.

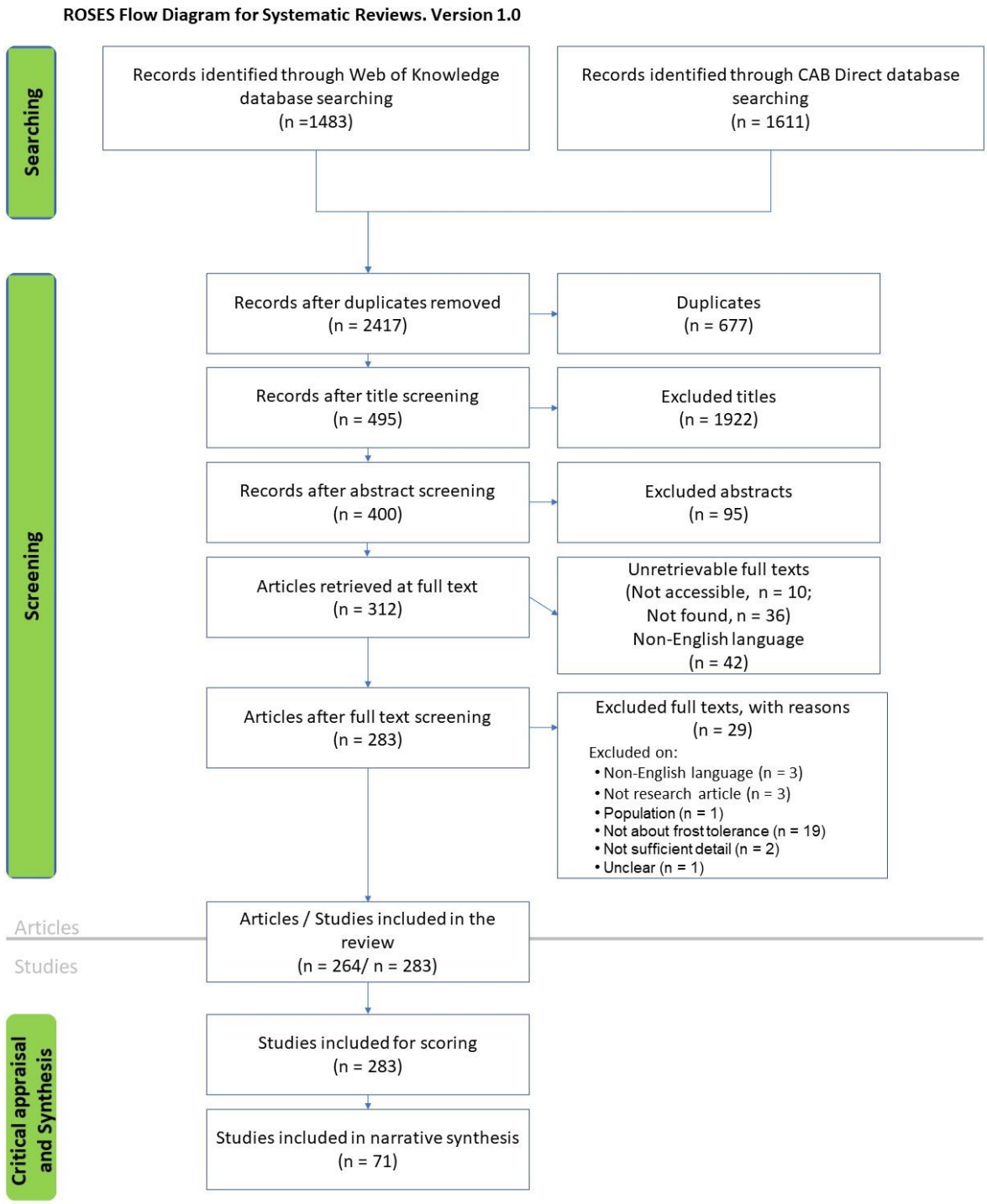

Figure 4.1. ROSES diagram outlining the search, screening and critical appraisal stages. Adapted from Haddaway et al., (2018).

Table 4.6. Gymnosperm species included in this review, and the number of studies which include them.

Species name	N	Species name	N	Species name	N
Picea abies	56	*Pinus maximinoi*	2	*Abies chensiensis*	1
Pinus sylvestris	50	*Pinus tecumannii*	2	*Abies grandis*	1
Pseudotsuga menziesii	46	*Podocarpus lawrenci*	2	*Abies koreana*	1
Picea glauca	26	*Podocarpus nivalis*	2	*Abies homolepis*	1
Picea rubens	22	*Abies procera*	2	*Tsuga dumosa*	1
Picea mariana	20	*Abies nephlorepsis*	2	*Tsuga sieboldii*	1
Pinus contorta	16	*Abies holophylla*	2	*Tsuga diversifolia*	1
Picea sitchensis	12	*Abies veitchii*	2	*Tsuga yunnanensis*	1
Picea engelmanni	9	*Abies nordmanniana*	2	*Larix sukaczewii*	1
Pinus banksiana	8	*Abies fraseri*	2	*Larix sibirica*	1
Chamaecyparis nootkatensis	8	*Abies sachaliensis*	2	*Larix gmelinii*	1
Pinus radiata	7	*Thuja occidentalis*	2	*Larix potanini*	1
Thuja plicata	7	*Larix leptolepis*	2	*Larix potanini*	1
Pinus halepensis	5	*Cupressocyparis leylandii*	2	*Larix occidentalis*	1
Pinus taeda	5	*Pseudotsuga sinensis*	1	*Larix cajanderi*	1
Pinus strobus	5	*Pinus albicaulis*	1	*Diselma archeri*	1
Pinus resinosa	5	*Pinus densiflora*	1	*Phyllocladus aspleniifolius*	1
Pinus nigra	4	*Pinus pseudostrobus*	1	*Cupressus sempervirens*	1
Pinus ponderosa	4	*Pinus monticola*	1	*Sabina przewalskii*	1
Pinus pinaster	4	*Pinus bungeana*	1	*Cedrus libani*	1
Abies alba	4	*Picea pungens*	1	*Cedrus deodara*	1
Larix decidua	4	*Picea smithiana*	1	*Keteleeria evelyniana*	1
Pinus cembra	3	*Picea brachytyla*	1	*Juniperus sinensis*	1
Pinus greggii	3	*Picea likiangensis*	1	*Juniperus recurva*	1
Pinus oocarpa	3	*Picea jezoensis*	1	*Agathis australis*	1
Pinus wallichiana	3	*Picea glehnii*	1	*Agathis vicennia*	1
Pinus elliotii	3	*Picea asperata*	1	*Dacrydium colensoi*	1
Pinus mugo	3	*Podocarpus macrophyllus*	1	*Dacrydium bidwillii*	1
Pinus caribaea	3	*Podocarpus oleifolius*	1	*Dacrydium cup res sinum*	1
Podocarpus totara	3	*Podocarpus ferrugineus*	1	*Dacrydium biforme*	1
Abies lasiocarpa	3	*Podocarpus hallii*	1	*Dacrydium laxifolium*	1
Abies balsamea	3	*Podocarpus salignus*	1	*Dacrydium colensoi*	1
Tsuga mertensiana	3	*Podocarpus latifolius*	1	*Libocedrus plumosa*	1
Tsuga heterophylla	3	*Podocarpus henkelii*	1	*Libocedrus bidwillii*	1
Larix laricina	3	*Abies amabilis*	1	*Araucaria cunninghamii*	1
Pinus brutia	2	*Abies spectabilis*	1	*Araucaria bidwillii*	1
Pinus canariensis	2	*Abies ernestii*	1	*Callitris oblonga*	1
Pinus pinea	2	*Abies delavayi*	1	*Athrotaxis selaginoides*	1
Pinus hartwegii	2	*Abies mariesii*	1	*Dacrycarpus dacrydioides*	1
Pinus patula	2	*Abies firma*	1	*Callitropsis nootkatensis*	1

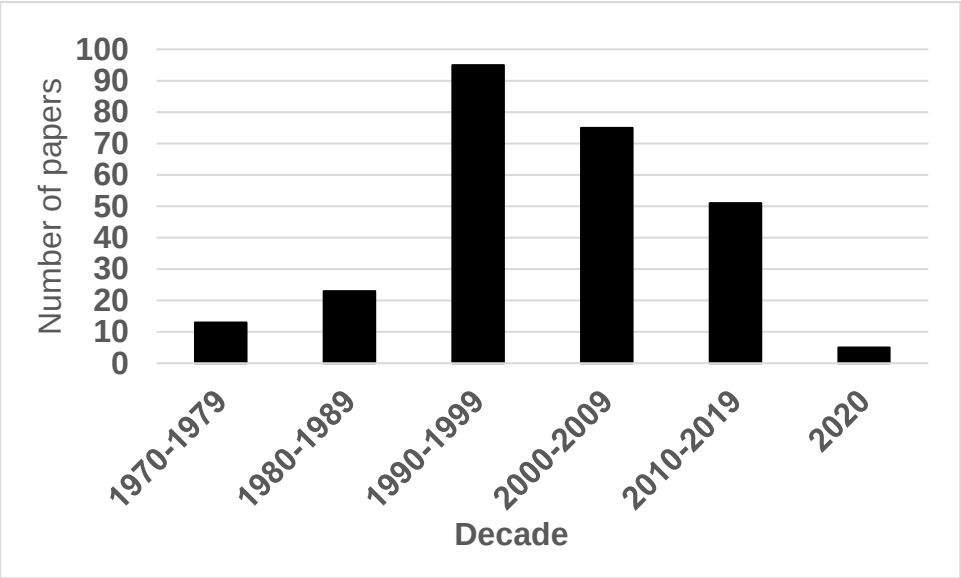

Figure 4.2. Distribution of selected papers by decade.

Table 4.7. Combinations used in the studies, by number of studies.

Tissue studied	Number of studies
Whole plant, branch cuttings, needles, and buds	1
Whole plant, branch cuttings, needles	1
Whole plant and branches	4
Whole plant, needles, buds	3
Whole plant, needles	5
Whole plant	86
Branch cuttings, needles, buds and stems	1
Branch cuttings, needles and buds	3
Branch cuttings and needles	6
Branch cuttings, buds and stems	1
Branch cuttings and buds	1
Branch cuttings	83
Needles, buds and stems	7
Needles and buds	5
Needles and stem	2
Needles	60
Buds and stems	1
Buds	2
Stems	3
Whole plant with cut roots	1
Not mentioned	7

3.3.2. Pre-conditioning

The growing conditions were classified according to the level of control exercised by the researcher, sometimes including categories with a wide range of variability. Thus, both pot-grown seedlings placed outside, irrigated and non-irrigated fields, and old-growth forests were scored into the 'field' category.

As can be seen in Table 4.8, field-grown samples were most common (n = 149), followed by greenhouse (n = 71) and growth chamber (n = 35) grown samples. Many studies tested the effect of different growing conditions, growing plants in different conditions for comparisons (Bigras and D'Aoust, 1993; Nielsen and Rasmussen, 2009; Nilsson and Eriksson, 1986). Other conditions, like open top chambers used in the field, to control the air around the plant (n = 9), indoor growth rooms that allowed for the complete filtration of air (n = 7), phytotrons (n = 6), cold storage, tunnels, and indoor rooms, were much rarer (Table 4.8).

Table 4.8. Combinations of growth conditions, by number of studies. This table shows the totality of studies.

Combination of growth conditions	Number of studies
Open top chambers and growth chamber	1
Greenhouse and cold storage	1
Greenhouse and growth chamber	2
Field and growth room	1
Field and cold room	1
Field and phytotron	1
Field and growth chambers	5
Field and greenhouse	6
Field, greenhouse and growth chambers	3
Field	132
Greenhouse	59
Open top chambers	8
Growth chamber	24
Tunnel	3
Growth room	6
Cold storage	2
Indoors	1
Phytotron	5
Not mentioned	21

3.3.3. Freeze testing

3.3.3.1. Freeze testing equipment

Field testing

Studies that performed field studies were noted, and in the cases where the results were compared with lab results, this information was used to verify that freeze testing correlated with the desired characteristic.

Of the sixteen studies that did direct field observations, eleven (Table 4.9) measured material frozen in the field, without any freezing experiments. Most of them did visual assessment (n = 9), while two

of them collected the field material for assessment by EL. Seven studies did both field testing and lab testing, only three of which measured correlation.

Some studies combined field observations with lab testing and measuring (Table 4.9), either checking for correlation between the two (n = 2) or not checking for correlation (n = 4).

In the four studies that did both field observations and lab assessments but did not check for the correlation, two did the observations separately, with the same plants but not providing any information that allowed to compare the results for the same plants (Coursolle et al., 1997; Jones and Cregg, 2006). Another was about freezing tolerance in indoor Christmas trees that were later grown outside (Gooch et al., 2009). The study by Hodge et al., (2012), while not explicitly measuring correlation between field and lab results, found that the ranking of species coincided in both methods.

In a study on different populations of *Pinus oocarpa* Schiede ex Schltdl., field observations, obtained by visual scoring, were correlated with laboratory based Electrolyte Leakage (EL) measurements (r^2 = 0.79; if control excluded, r^2 = 0.32) (de Waal et al., 2018). The authors noted the importance of using a large sample when performing artificial freezing tests, as correlation between field observations and lab based EL measurements was poor for the smaller groups (families vs provenances).

In a study in red spruce (*Picea rubens* Sarg.), field observations were done after establishing the level of frost hardiness of field-collected samples in the lab by EL, and field damage was strongly correlated (r^2 = 0.61) with the EL measurement (Vann et al., 1992).

Controlled enclosure testing

According to Warrington and Rook (1980), there were two types of controlled enclosure tests, that depended on the equipment they used: cold rooms and freezer cabinets (divided into laboratory units, field units and liquid nitrogen-based systems) and controlled environment rooms (divided into radiation frost chambers and advective frost enclosures).

The most frequently used type of equipment was the freezer, in non-programmable (n = 45) and programmable (n = 46) versions (Table 4.10). Many modifications were used with freezers to control the rate of freezing. Some freezers were modified (n = 2). In one case the modification was not stated (Hansen, 1992). Gillies and Binder (1996) used extensive modifications, replacing the lid with a glass window, in order to provide light inside the freezer. In other cases, while the freezer itself was not modified, additional equipment was used to control the rate of freezing, such as programmable controllers (n = 6), or cyclic timers (n = 1). In some cases, insulating material was used to slow the rate of freezing, such as insulated boxes (n = 1), a Styrofoam chest (n = 1), plywood boxes (n = 1), thermo flasks (n = 2) or aluminium foil (n = 1). Additional materials were used to provide a more spatially even temperature, such as aluminium shelves (n = 1). When freezing beyond the capacity of the freezers was needed, liquid nitrogen was used (n = 5).

Table 4.9. Studies that included field observations.

	Frost tolerance measurement					Organ tested			
	Total studies	**EL**	**Visual**	**Fluorometry**	**Whole plant**	**Branches**	**Needles**	**Buds**	**Measure correlation**
Field only	11	2	9	0	7	1	3	1	*
Field and lab testing	2	2	2	0	1	2	1	0	Yes
Field and lab testing	4	1	4	2	3	2	1	0	No

*Not applicable; correlation could only be measured when more than one technique was used in the same study.

Table 4.10. Studies according to the freezing equipment used.

Equipment type	Non-programmable	Modifications[1]	Programmable	Listed
Cold room	4		2	
Field chamber	2			
Freezer	45	6 programmable controllers; 5 liquid nitrogen; 2 thermoses; 2 modified; 1 insulated boxes; 1 aluminium shelf; 1 cyclic timer; 1 Styrofoam chest; 1 plywood boxes; 1 aluminium foil	46	
Freezing chamber	52	1 Conviron controlled; 1 programmable fan; 1 temperature controller; 1 external alcohol circulating system	25	
Liquid bath	27			
Not mentioned	63			
Other	7			2 Growth chambers; 1 Precision BOD incubator; 1 Portable freezing system; 1 Refrigeration unit; 1 Refrigerator

1. Modifications to non-programmable equipment that allowed to control freezing rates.

The studies that used freezing chambers (n = 52 non-programmable, n = 25 programmable) did not use advective frost enclosures nor radiation frost chambers (Table 4.10) but simply the mechanism of freezing air. Some of the non-programmable units had modifications that allowed for the control of the rate of freezing, such as a Conviron (n = 1), a programmable fan (n=1), a temperature controller (n = 1), or an external alcohol circulating system (n =1).

The third most frequently used technology (Table 4.10) were liquid baths (n = 27). Due to water's freezing temperature of 0 °C, other liquids were used to provide sub-zero temperatures. The most frequently used liquid in order of number of studies was ethanol (n = 11), methanol (n = 3) or an unspecified alcohol (n = 3). Separate cases of use of polyethylene glycol (n = 1), glycol (n = 1), ethylene glycol (n = 1), an ethanol:water solution (n = 1), and antifreeze solvent (n = 1) were noted.

Other technologies, such as field chambers (n = 2), cold rooms (non-programmable n = 4, programmable n = 2), or growth chambers, were much rarer, whereas some equipment was only used in one study (Table 4.10). They include a refrigerator (n = 1), a precision BOD incubator (n = 1), a portable freezing system (n = 1) and a refrigeration unit (n = 1).

Equipment used was also classified into programmable and non-programmable versions. Programmable versions allow for the control of the freezing and sometimes thawing rate. A substantial proportion of the equipment used, 25.7% (Table 4.10), was programmable, while 48.4% was non-programmable. Many studies, 22.2%, do not mention the type of equipment they use for freezing. The leftover 3.7% was made by the use of field freezing tests, which do not require any equipment.

3.3.3.2. Freeze testing regimes

Studies differed in their testing regimes that affect the measured frost tolerance by freezing rate, frost exposure time and thawing conditions. Other factors, such as the temperature range used, the numbers of temperatures, and the decrements between temperatures, affect the accuracy of measurements but not the measured frost tolerance.

Freezing rate

Freezing rates were measured or given in 75.2 % of studies, and the results presented below only apply to those. Defined here as the rate of temperature decrease per hour (in K h^{-1}), the scoring ignored some edge studies that were noted as follows.

Some studies (n = 17) first equilibrated the sample at -2 °C (Table 4.11), from room temperature to -2 °C, so the ice would form slowly, and a different freezing rate below -2 °C. The rate scored was the one below -2 °C.

In some studies (n = 8), in addition to freezing treatments using freezers and other equipment (Table 4.11), samples were immersed in liquid nitrogen, as a positive control for freezing damage. This meant

that the rate of freezing for the liquid nitrogen exposed sample would be of 196 K s^{-1}, with the temperature immediately jumping to -196 °C.

Some of the studies (n = 19) that used a broad range of temperatures sometimes used different freezing rates for different temperatures, using higher freezing rates for lower temperatures (Table 4.11). This was done in a stepwise manner, first decreasing the temperature to a certain threshold at a certain rate, and then increasing the freezing rate. An average of the freezing rates used was scored. Most studies used a freezing rate slower than or equal to -5 K h^{-1} (Figure 4.3), with a small proportion of studies (17.8 %) using freezing rates faster than -5 K h^{-1}. It should be noted that most studies had freezing rates faster than -4 K h^{-1} and up to -5 K h^{-1}, with 48.4 % of studies having freezing rates in that range (Figure 4.3).

Table 4.11. Studies by variations to the freezing rate.

Variation	Number of studies
Equilibrate at - 2 °C	17
Use liquid nitrogen (-196 °C)	8
Different rates	19

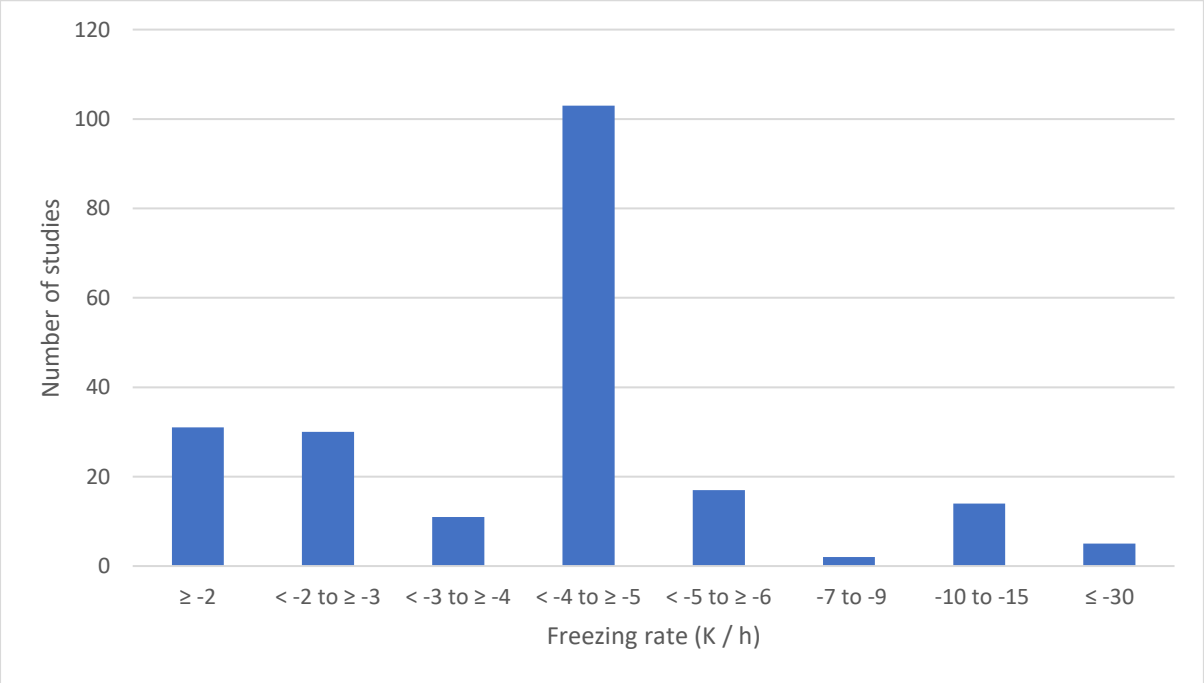

Figure 4.3. Number of studies binned according to the freezing rate used.

Frost exposure time

Frost exposure time was scored as the time the sample spent exposed to the desired air temperature. In some cases, it should be noted that larger samples, such as seedlings or large trees, will take a longer time to equilibrate with the air than smaller samples, but only the length of the air temperature exposure was noted, as the true value of the exposure was not available.

The most frequently used (20.1 %) exposure time was of 1 h (Figure 4.4), followed by the exposure time above 3 h and up to 4 h. Flash exposure, where samples were taken out when temperature in the freezer was reached, was the third most frequently used method (11.6%). It should be noted that a few studies use different exposure times for different organs. Overall, the majority (71.7 %) of studies use a frost exposure time up to 4 h.

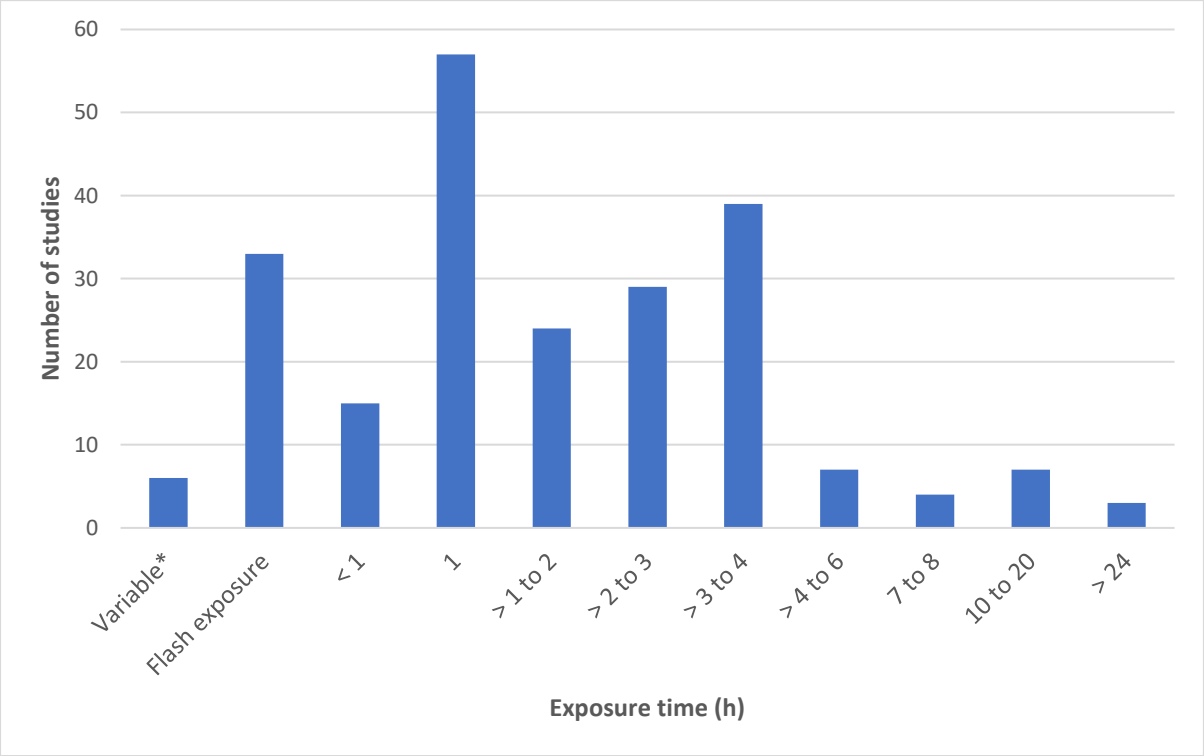

Figure 4.4. Number of studies binned according to the exposure time they used.

* Uses different exposure times in the study.

Thawing rate

Thawing rate was only measured in 39.9% of the studies, with the most common thawing rate being 5 K h^{-1}, followed by 7-10 K h^{-1}, with 2 K h^{-1} being the third most frequent (Figure 4.5).

It should be noted that 43.1 % of studies did not mention the thawing rate used, as it was difficult to control. Different techniques were used to slow the thawing rate even when precise control was unavailable. Some studies (n = 9) used a stepwise procedure, where frozen samples were placed at

temperatures until they equilibrated, at several temperature decrements (Table 4.12). This helped reduce the rate of thawing by reducing the temperature differential between the frozen sample and the surrounding temperature.

In order to avoid the extremely high temperature differential between the frozen sample and ambient temperature, in most cases (n = 107) the sample was placed in refrigerators or other such freezing devices at temperatures between 0 and 5 °C before it was exposed to the freezing temperature (Table 4.12).

In a minority of cases (n = 6) samples were left at warm ambient temperatures to warm (Table 4.12).

Table 4.12. Number of studies by thawing conditions, in cases where the thawing rate was not given.

Thawing conditions	Number of studies
Thawed at positive temperatures up to 5 °C	107
Thawed at temperatures above 5 °C	6
Stepwise	9

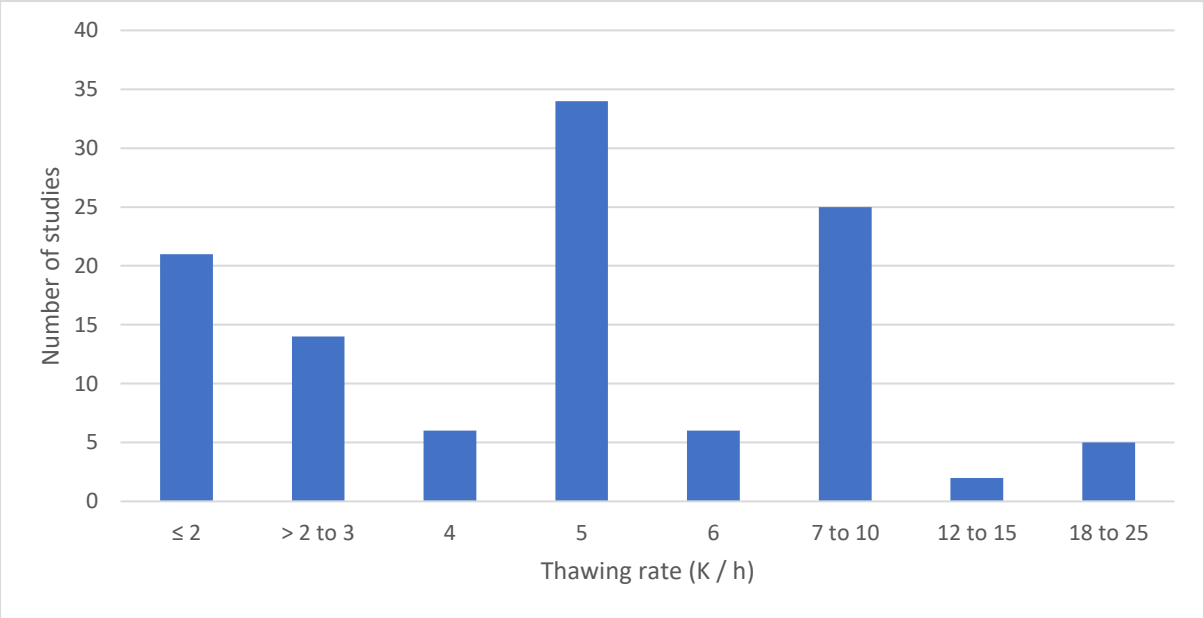

Figure 4.5. Number of studies binned according to thawing rate, among the studies for which the thawing rate is known.

Freezing temperature

Temperature range (the difference between the highest and lowest test temperature used in the study) was scored for 33.9 % of the studies. The most frequently used temperature range was of 10-19 K or below (Figure 4.6). Higher temperature ranges were much less frequent, but the ranges

extended quite widely, with the highest temperature range being 193 K (the difference between 0 °C and -193 °C, the temperature of liquid nitrogen).

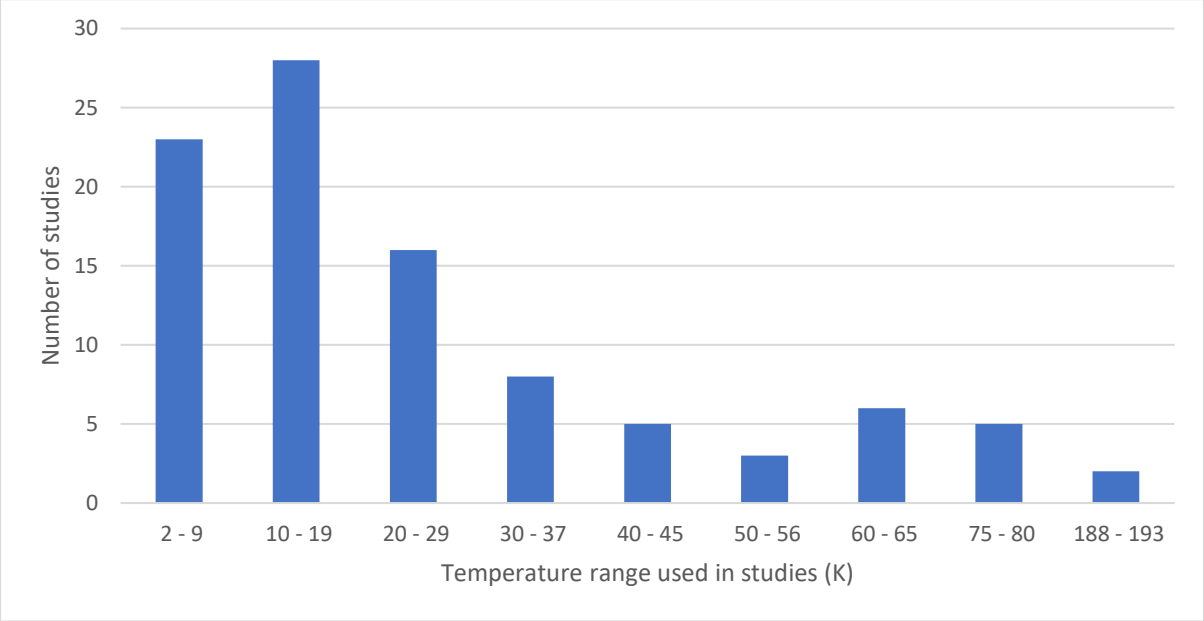

Figure 4.6. Number of studies binned by temperature range used (difference between the highest treatment temperature, excluding the control, and the lowest temperature).

The number of test temperatures was scored in 56.5% of studies. The most frequent type of study (n = 36, 22.5 % of those scored) only used one test temperature (Figure 4.7). The second and third most common set up involves the use of 3 and 4 test temperatures, respectively, making a combined 36.2 % of studies.

The majority of studies used temperature steps of 3 K or above, which fell outside the accuracy range (Table 4.14).

3.3.4. Measuring freezing damage

Visual Assessment (VA) was the most common method used for measuring frost damage, in 61.8% of studies (Table 4.15).

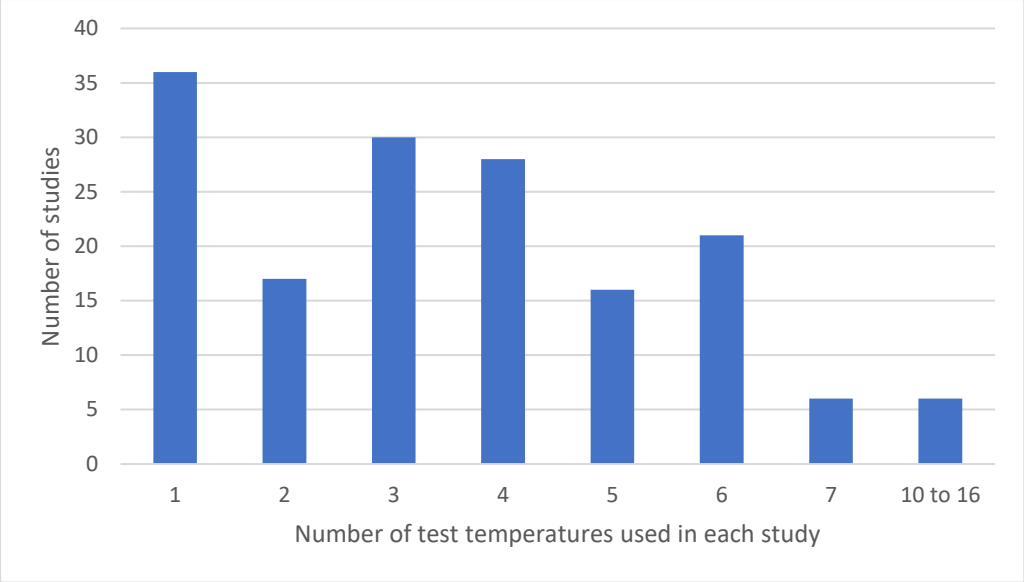

Figure 4.7. Number of studies according to the number of test temperatures used in each study.

Temperature decrements were defined as the smallest distance between two adjacent test temperatures used in a study. Temperature decrements of 1-2K were quite frequently used, in 20.6 % of studies for which temperature steps can be calculated (Table 4.13). This value fell within the range of accuracy of reached temperatures (the difference between temperatures programmed and actual temperatures achieved), which was between 0.1 - 2.0 K, for the studies where it was measured (Table 4.14).

Table 4.13. Number of studies according to temperature decrements (difference between two adjacent test temperatures) used in the studies. Table only includes those studies where the temperature decrements were given or could be calculated.

Temperature decrements	Number of studies
1 to 2 K	13
3 K	6
4 K	10
5 K	13
More than 5 K	10
Different steps depending on temperature*	11

*Use different temperature steps depending on the temperature, *e.g.* use a temperature step of 2.5 K between 0 °C and -20 °C, and temperature step of 10 K between -20 °C and lower.

Table 4.14. Number of studies by the accuracy of the achieved test temperatures (the difference between temperatures programmed and actual temperatures achieved), for the studies that give this value.

Accuracy (K)	Number of studies
0.1	3
0.2	2
0.3	1
0.5	5
0.7	1
1	5
1.5	3
2	2

The second most used technique (n = 119) was EL (Table 4.15), which consisted of placing a treated sample in pure water and measuring the change in conductivity. The third most used technique was fluorometry (n = 27). Differential Thermal Analysis, DTA, was the fourth most used technique (n = 12). The Electrical Impedance Spectroscopy (EIS) technique was also used in some cases (n = 10). Techniques such as the Tetrazolium Assay and the PM-ATPase activity measurement, were rare (Table 4.15), and were not used after 2004.

A total of 58 studies (Table 4.15) combined different measuring techniques, two of them the previously discussed studies that combined field and laboratory measurements that checked for the correlation between the two (de Waal et al., 2018; Vann et al., 1992). Of the other 56, only 48.2 % checked for correlation or agreement between the different techniques.

The most common comparison (n = 12) was between VA and EL, the second most used technique (Table 4.15), and they seemed to be well correlated. High degrees of correlation between the two were measured in studies in black spruce (*Picea mariana* (Mill.) Britton, Sterns & Poggenb.), r^2 = 0.67 to 0.81 (Odlum and Blake, 1996); white spruce (*Picea glauca* (Moench) Voss), r^2 = 0.95 (Gillies and Binder, 1996); in five different species, r^2 = 0.81 (L'Hirondelle et al., 2006); in European larch (*Larix decidua* Mill.) and mountain pine (*Pinus mugo* Turra), r^2 = 0.85 (Martin et al., 2010); red pine (*Pinus resinosa* Aiton) and Austrian pine (*Pinus nigra* J.F. Arnold), r^2 = 0.94 (Sutinen et al., 1992). In some cases, the correlation between EL and VA was statistically significant, such as in studies in maritime pine (*Pinus pinaster* Aiton), r^2 = 0.31 in spring, r^2 = 0.79 in autumn, $p < 0.05$ (Prada et al., 2014); only below -30 °C in Scots pine, r^2 = 0.94, $p < 0.0001$ (Repo et al., 1996); and in a study of multiple pine species, r^2= 0.64, $p < 0.0001$, (Mabaso et al., 2017). Some studies, while not measuring correlation, find agreement between the results of VA and EL, in Sitka spruce (*Picea sitchensis* [Bong.] Carr.)

(Jalkanen et al., 1998); in multiple conifer species (Man et al., 2017, 2016); and Douglas fir (*Pseudotsuga menziesii* (Mirb). Franco) (van den Driessche, 1976).

The second most common comparison (n = 10) was between VA and Fluorometry. Fluorometry was also highly correlated with VA, for red spruce, $r^2 = 0.81$ (Adams and Perkins, 1993); white spruce, $r^2 = 0.76$ (Binder and Fielder, 1996); in another study in white spruce, $r^2 = 0.96$ (Gillies and Binder, 1996); in five different species, $r^2 = 0.93$ (L'Hirondelle et al., 2006); and in Douglas fir, $r^2 = 0.81$ October, $r^2 = 0.93$ November (Rose and Haase, 2002). Some correlations between the results of VA and fluorometry were even statistically significant, like a study in multiple species ($r^2 = 0.85$, $p < 0.001$) (Bannister and Lord, 2006); Aleppo pine (*Pinus halepensis* Mill.), $r^2 = 0.97$, significance stated but p-value not provided (Fernandez et al., 2003); in exotic firs, $r^2 = 0.36$ for needles, $r^2 = 0.48$ for stems, and $r^2 = 0.21$ for buds, $p < 0.0001$ (Jones and Cregg, 2006); maritime pine, $r^2 = 0.19$ in spring, $r^2 = 0.61$ in autumn, $p < 0.05$ (Prada et al., 2014); and in Aleppo pine, $r^2 = 0.67$ at 200 h (p-value not mentioned, but significance stated) (Puértolas et al., 2005).

The third most common comparison (n = 3) was between EL and fluorometry. Wulff et al. (1994) found similar values when measuring frost tolerance by using the EL method or the fluorescence method in Sitka spruce. High degrees of correlation were also found in common cypress (*Cupressus sempervirens* L.), $r^2 = 0.45$ (Baldi et al., 2011). In some cases, this correlation was significant, such as in a study in maritime pine, $r^2 = 0.50$ in spring, $r^2 = 0.55$ in autumn, $p < 0.05$ (Prada et al., 2014).

VA and EIS measurements of frost hardiness in Scots pine were correlated, $r^2 = 0.95$ (Toivonen et al., 1991), but no correlation was found in another study of Scots pine (Repo et al., 1994). A study in Douglas fir, found agreement between the ranking achieved by VA and EIS (van den Driessche, 1973). EL and EIS were found to be correlated ($r^2 = 0.91$) in a study of *Pinus bungeana* Zucc. ex Endl.(Zhang et al., 2010).

In a three way comparison between EL, DTA and VA done in ponderosa pine (*Pinus ponderosa* Douglas ex C. Lawson), Douglas fir and Engelmann spruce (*Picea engelmannii* Parry ex Engelm.), it was seen that, while the measurements agreed, EL was more precise than DTA, with the VA, done following a whole-plant freezing test, was the least precise (Burr et al., 1990). In another three-way comparison, between EL, VA and fluorometry, an overall correlation of $r^2 = 0.85$ was found for Douglas fir, white spruce, Engelmann spruce, contorta pine (*Pinus contorta* Douglas ex Loudon), and western larch (*Larix occidentalis* Nutt.) (L'Hirondelle et al., 2006).

Table 4.15. The frost tolerance measuring techniques used, by number of studies.

Combination of measuring techniques	Number of studies
VA	128
VA & Fluorometry	10
VA & DTA	3
VA & Electrical Impedance	3
VA & Tetrazolium assay	1
VA, DTA & LTE	1
EL	80
EL & VA	20
EL, VA & Fluorometry	5
EL & Fluorometry	4
EL & DTA	3
EL, VA & DTA	2
EL, DTA & Electrical Impedance	2
EL, VA & PM-ATPase activity	1
EL, VA & Electrical Impedance	1
EL & Electrical Impedance	1
Fluorometry	7
Fluorometry & Tetrazolium assay	1
Tetrazolium Assay	2
Electrical Impedance	3
Not mentioned	5

4. Discussion

4.1. Sample selection

Most of the studies in this review focused on the most commercially viable conifer species, *i.e.* Norway spruce, Scots Pine and Douglas fir. This was to be expected, as these were some of the most economically important commercially grown species in Europe, the first two species native to Europe and Douglas fir an introduced species (European Environmental Agency, 2006).

Another important aspect of the sample selection was which part of the plant was studied. For the purposes of this review, only above-ground tissues were included, since root frost tolerance is fundamentally different from above-ground tissue tolerance due to the protection of the soil. It was noted that studies performed tests on either the entire plant (only with small plants, either seedlings or stecklings; with the exception of observational field studies, there were no technologies available that can freeze an entire mature tree), or part of the above-ground tissue. When taking a part of a tree, the most common procedure was to take a branch cutting. Branch cuttings contain all the relevant organs: stem, needles, and, depending on the timing, buds. Branch cuttings can be evaluated in their entirety, or each separate part could be evaluated on its own.

Branches have many advantages for sampling: they were small, they contain all relevant organs, and cutting branches allows for measuring the tolerance of the same tree for different test temperatures.

Learning the whole plant freezing resistance was the objective in most cases, as the resistance of branches on its own does not inform the survivability of plants in the tested conditions. But whole-plant freeze testing is inherently destructive, as freezing the plant is likely to kill it or damage it, complicating further tests on the plant. This means that a plant can only be used once for a freezing test when using a whole plant freezing test, while branch samples allow for a plant to be tested multiple times. Besides, whole plant freezing requires larger freezers, and a longer freezing time, as the larger mass of the sample will take longer to equilibrate with the surrounding air. Strategies to slow down the freezing rate, like using thermo flasks, would be harder for whole plants due to size constraints. Despite the complications of whole-plant freeze testing, it was the second most common type, presumably because it allows for conditions that were closer to real-world field results.

Needles, unlike buds, which were formed in autumn and flushed in spring, are present throughout the year. They also lack the protection the stem enjoys, in the form of protective barriers like the bark. Needles are also the most sensitive organ that was present year-round. Visual damage to needles is also more immediately visible, whereas stems and buds are harder to examine and frequently need to be cut for examination (Aitken and Adams, 1996; Bansal et al., 2016; Vangestel et al., 2018), although sometimes a superficial assessment is sufficient (Anekonda et al., 1998; Dirr et al., 1993).

In general, the selected sample for freeze testing will depend on the availability of the biological material, the frequency of testing, the number of replicates for each biological sample, the objective of the study, the available freezing capabilities and the type of measurement used.

4.2. Pre-conditioning

While Keates (1990) assumes plant material will generally come from either field-planted stock or seedlings from nursery or greenhouse culture, this review found more variability in the sources of plant material collected.

The conditions under which the plants were grown before or even during freeze testing depended on the goal of the measurement and were highly variable. The prevalence of studies on field grown material suggest that the most important reason to test frost tolerance was the measurement of frost tolerance in real-world conditions, without the artificial constraints of the lab.

This review focused on the conditions under which the experimental material was grown immediately before or during the freezing tests. This was done because it was common to either grow or obtain seedlings from nurseries, and then move them across different growing conditions as they grew (Colombo et al., 2003; Hodge et al., 2012; Konttinen et al., 2007; Man et al., 2016), or in order to test the effect of growing conditions (Colombo and Raitanen, 1991; Greer et al., 1989; Hawkins and McDonald, 1993; Korhonen et al., 2015).

The pre-conditioning design differed according to the aims of each study. For example, a study performed on indoor grown trees aimed to explore the frost resistance of indoors Christmas trees (Gooch et al., 2009) whereas another study was performed with plants left in cold storage aimed to observe the effect of cold storage, which was commonly used by commercial nurseries, on frost tolerance (Colombo, 1990). Another study used cold storage aiming to measure the decrease in stored carbohydrates and their effect on frost hardiness (Ogren, 1997).

In general, field conditions, which for the purposes of this review included both potted and field planted plants grown outside, offer less control over growing conditions than every other type of pre-conditioning. It was thus the form most similar to real natural conditions. Field conditions differ between each other on the level of control, on whether the plants were watered and fertilized. Open top chambers allow growing plants in the field exposed to the same light, watering and temperatures as field-grown plants, while controlling the gaseous environment in which the plants were grown. This was done to measure things such as the effects of acid mist (Cape et al., 1991; Eamus, 1993; Vann et al., 1992), ozone (Amundson et al., 1990) or increased levels of CO_2 (Repo et al., 1996).

Greenhouses allow for more control of growing conditions, providing heating, watering, and additional lighting when necessary. Some greenhouses also filter the air for particles, thus allowing to control the air. Greenhouses rarely offer the possibility to cool beyond opening windows when outside temperatures and sunlight overheat the greenhouse. While additional lighting can be provided, blackout darkness is rarely available in greenhouses. Humidity control beyond watering is also rarely available in greenhouses.

When control over every aspect of growing conditions is desired (temperature, photoperiod, light intensity, air composition and humidity), growth rooms and phytotrons could be used, which provide the ability to control every aspect of plant growing conditions.

Thus, growing conditions will depend on the objective of the study, and the level of control over growing conditions necessary to achieve these objectives. As each additional level of control will require an additional cost, researchers should focus on the growing conditions that achieve their objectives in the most cost effect manner.

4.3. Freeze testing

4.3.1. Freeze testing techniques

Keates (1990), classified freeze testing into two types: field and laboratory testing. Warrington and Rook (1980) classified freeze testing into three main types: field studies, controlled enclosure studies (equivalent to laboratory freezing according to Keates (1990)), and temperature gradient bars. No studies that used temperature gradient bars were found with the search criteria used in this review.

4.3.1.1. Field testing

The main difference, as both Keates (1990) and Warrington and Rook (1980) note, was that for the field tests, the results of naturally occurring frosts were observed, whereas in laboratory/controlled exposure studies frosts can be controlled.

The simplest method of freeze testing was to observe the results of naturally occurring frost events in field-planted stock. These tests were perceived by scientists and foresters to be the only real measure of frost hardiness (Warrington and Rook, 1980). However, as noted in both reviews by Warrington and Rook (1980) and Keates (1990), field testing has many limitations.

Both reviews note the unpredictability of field conditions. Warrington and Rook (1980) notes that some years plants with different frost tolerances can be killed by a particularly harsh frost, and others none of them would be harmed due to a particularly mild year. This problem can be accounted for by running the observations for a number of years in different sites, which increases cost.

The lack of precision of field testing was another problem, as measuring frost conditions across a site can be a very costly endeavour, due to microsite variation (Warrington and Rook, 1980). Effects of frosts were also hard to distinguish from other effects of the site, such as drying winds or weed competition (Keates, 1990; Warrington and Rook, 1980). These problems could be overcome by increased replication, which was also costly.

The cost itself of field observation was also a factor, as noted by Keates (1990). The costs could be part of the reason why studies that include field testing (n = 16) represent only 5.6% of the total number of studies reviewed. Field testing was rare, and the majority of studies were done in controlled enclosures, where frosts can be done on demand. Furthermore, as shown in the two studies that measured correlation between field observations and controlled enclosure results, field results were strongly correlated with controlled enclosure results (de Waal et al., 2018; Vann et al., 1992).

4.3.1.2. Controlled enclosure testing

Keates (1990) found that three main equipment types were used to administer freezing tests in the lab: freeze chambers, liquid baths, or temperature gradient bars (classified by Warrington and Rook (1980) into a different main category).

Although studies that do freezing tests using temperature gradient bars seemed to be important enough to put in a different category by Warrington and Rook (1980), none of the studies included in this review use temperature gradient bars. This could be because temperature gradient bars were only suitable for extremely small samples (Warrington and Rook, 1980). The latest reference used by Keates (1990) when talking about this technology was from 1983. This technology seems to be old and could have been abandoned as newer technologies became available.

Warrington and Rook (1980) classify laboratory testing into two types: cold rooms and freezer cabinets, and controlled environment rooms. Cold rooms can be lab or field based and use liquid nitrogen to cool the unit, and controlled environment rooms can be either radiation frost chambers or advective frost enclosures. Neither type of controlled environment rooms was found in this search, and they were not described in the later review by Keates (1990). Presumably, these were also old technologies that were abandoned as newer technologies became available.

Evidence from the Warrington and Rook (1980) review suggests that as technologies improved, the techniques used before the eighties were abandoned in favour of machines that can perform controlled freezing tests according to the requirements of the researcher. The reason why radiation frost chambers or advective frost enclosures were abandoned is unknown, but they do not appear in any studies beyond 1978.

The technologies that were used most frequently were freezers and freezing chambers, mostly of the non-programmable type, although programmable versions take a substantial chunk of the equipment. Many of the non-programmable versions were modified in some way to allow for more customized freeze testing. While programmable versions allow for more control over the freeze testing process, non-programmable freezers and freezing chambers were more widely available in most labs, as they were not specialist equipment. Thus, the wide use of non-programmable freezers cannot be used as an argument in their favour, since their widespread use was presumably due to their availability and cost rather than inherent technical advantages.

Liquid baths, while they do allow for a uniform freezing, have the problem of a limit to the coldest temperature achieved, as the liquid becomes solid. It was thus logical that equipment that relies on air freezing, which can achieve extremely low temperatures, were the most common.

In general, programmable specialized equipment can be presumed to better serve the purpose of frost tolerance measurement, since they were used despite the higher cost and the widespread availability of non-specialized freezers.

4.3.2. Freeze testing conditions

4.3.2.1. Freezing rates

Freezing rates were an important factor for the assessment of frost tolerance. High freezing rates that can be artificially achieved are not expected to occur in nature, as large masses of air take time to cool. For example, freezing rates faster than 5 K h^{-1} occurred very rarely in Scotland (Cannell and Sheppard, 1982). Thus, in order to measure frost tolerance that is closer to field values, freezing rates that are closer to natural ones should be used.

Freezing rates that can be achieved will heavily depend on the equipment used, and the modifications made to said equipment (Table 4.10). For some types of programmable equipment, the freezing rate

can be programmed, allowing for this factor to be controlled. However, the most used types of equipment were non-programmable (Table 4.10), and the rate of freezing could only be decreased to a degree by the use of insulation. Many studies, 22.2%, did not mention the type of equipment they used for freezing, thus inspiring doubts about the freezing rates they mention.

Higher freezing rates seem to lead to a warmer frost tolerance temperature in Norway spruce buds, although the difference was only of 2.6 K (Räisänen et al., 2006). It should be noted that this study only used freezing rates between -1 and -5 K h^{-1}, not using rates faster than -5 K h^{-1}.

In a study in Leyland cypress, × *Cupressocyparis leylandii* (A.B. Jacks, & Dallim.) Dallim., a freezing rate of -6 K h^{-1} led to tip browning, while the slower freezing rates of -4 K h^{-1} and -2 K h^{-1} did not cause such damage (Haynes et al., 1992). In a study of radiata pine (*Pinus radiata* D. Don) seedlings, higher freezing rates caused higher levels of damage across different treatments, maintaining temperature, thawing rate and frost duration constant (Warrington and Jackson, 1981).

The majority of studies scored in this review used a freezing rate of -5 K h^{-1} or slower (Figure 4.3), with most studies using a freezing rate of -5 K h^{-1}. This could be because of the increased cost and time of slowing freezing rates from -5 K h^{-1} and the small effect on the measured results at freezing rates slower than -5 K h^{-1} (Räisänen et al., 2006). However, it seems that significant efforts were made in multiple studies to ensure freezing rates slower than -5 K h^{-1} (Figure 4.3), as achieving such a rate would be more costly.

4.3.2.2. Frost exposure time

Frost exposure was an important factor in determining frost tolerance. Increasing the length of frost exposure significantly increases the rate of damage in radiata pine (Warrington and Jackson, 1981).

The majority of studies scored used a frost exposure time up to 4 h (Figure 4.4), with a peak at 1h. This was in line with reports that report similar damages with freezing durations of 1-4 h, with less damage below 1 h (Day and Peace, 1937).

Longer exposure times are probably closer to field exposure results, as physical phenomena that reduce temperatures of large masses of air for such brief periods of time (< 1 h) are not going to be very likely. And, although damage increases linearly with exposure time (Warrington and Jackson, 1981) between 2 and 8 h of exposure time, it seems that the difference between lower exposure times was much higher, possibly non-linear (Day and Peace, 1937).

But flash exposure was still quite prevalent in these studies (Figure 4.4). Flash exposure was usually done by removing samples when the desired test temperature was reached. Its frequent use was speculated to be due to technical constraints; while programmable freezers can be programmed to reach and maintain a certain temperature, most non-programmable freezers can only be set to the lowest temperature setting they have. Thus, keeping samples in such a freezer would lead to a lower

test temperature than the desired one, unless the test temperature was the lowest temperature the freezer could achieve.

It was probably better to use longer exposure time to achieve results that were closer to field results, although it was understandable that cost constraints impede it. But, although the number of studies that test different exposure times was insufficient to determine the optimum exposure time, it seems that the available evidence favours the use of exposure times above 1 h.

4.3.2.3. Thawing rate

Thawing rates are another factor that could affect the measured frost tolerance. Many studies did not measure thawing rates, although they used different thawing times. Higher thawing times would lead to slower thawing rates if the rate of thawing was uniform.

In a study of primordial shoots of Norway spruce, a slower thawing rate leads to lower measured freezing tolerance temperatures (Räisänen et al., 2006), or less frost damage at identical frost temperatures. The study notices large differences in measured frost tolerance between 2 - 18 h of thawing, for the same temperature differential, with faster thawing leading to more damage. This relationship was exponential, with a threshold point of 6 K h^{-1} where thawing rate had an exponential correlation with frost damage at thawing rates faster than 6 K h^{-1}, and a linear relationship at thawing rates slower than 6 K h^{-1} (Räisänen et al., 2006).

Thawing rate increases from 2 K h^{-1} to 10 K h^{-1} also seem to lead to higher degrees of damage in a study in radiata pine, where freezing rates and frost exposure were maintained constant (Warrington and Jackson, 1981). A study in Norway spruce by Floistad and Kohmann (2001), found that increased thawing time (and slowing thawing rates) lead to less freezing damage. However, it should be noted that the study compares a 16 h thawing time to a month-long thawing time.

In addition to the evidence from the aforementioned studies that seem to indicate that slower thawing rates decrease the level of frost damage, it should be noted that high thawing rates are unlikely to occur in the field under a natural frost. Large masses of air take longer to warm than what can be achieved artificially by taking a sample from the freezer and putting it at room temperature. It is thus likely that studies that do not try to control the thawing rate will measure frost tolerance temperatures higher than what they would be in field conditions.

4.3.2.4. Freezing temperatures

While the freezing rate, frost exposure time, and thawing rate used by researchers partly depend on the availability of equipment that allowed for the control of these factors, researchers have more control over the choice of test temperatures, the number and range of temperatures compared and the decrements between chosen test temperatures. Freezing rate, frost exposure time and thawing

rate also change the estimated frost tolerance because it will be contingent on them. Chosen test temperatures do not change the value obtained. The range of temperatures used, the decrements and their number will allow for a more precise and accurate calculation of the frost tolerance.

Using a wide array of freezing temperatures allows for the calculation of the frost tolerance, or temperature at which 50% of the sample was damaged (LT_{50}), the middle value on the frost response curve. Three things will determine the accuracy of the estimated LT_{50}: the range of temperature used, and whether it includes the real value of LT_{50}; the steps between two temperatures, with smaller steps allowing for a more precise value; and the number of temperatures used, as fewer values mean a higher level of freedom on the shape of the frost response curve.

In order to calculate the real frost tolerance, a range wide enough to include the frost tolerance value should be used. If the real value of frost tolerance falls outside the tested range, calculations of the frost tolerance value will be much less precise. Thus, in cases where the real value of frost tolerance was unknown, a range as wide as possible should be used. It should be noted, however, that by using pre-tests, the approximate value of frost tolerance can be estimated, and a narrower range will still lead to informative results (Hannerz and Westin, 2005; Hawkins et al., 1991). If an approximate value could be estimated from the literature or prior knowledge, a wide range of temperatures was also unnecessary. Most studies use a temperature range of 10-19 K or below (Figure 4.6). This means most studies use a narrow window of test temperatures, and if the real value falls below or above the tested range, it would not be possible to estimate it.

It was common for only one test temperature to be used (Figure 4.7). This type of study, however, can only determine whether the real frost tolerance falls above or below this test temperature: if frost damage was above 50%, the test temperature was below frost tolerance, if it was below 50%, it was above. This level of accuracy will be unsatisfactory, which was why the majority of studies use 3-4 test temperatures (Figure 4.7). However, as the use of each additional test temperature increases costs, there was a trade-off between accuracy and cost.

Temperature decrements determine how accurate the test will be, and smaller decrements will allow uncertainty to reduce, giving a better fit of the frost response curve. However, it should be noted that temperature decrements should be above the accuracy level of the freezing equipment, otherwise comparisons could be leading to false conclusions. This only happened in 20.6 % of cases. Most studies used small temperature decrements, which allows to get closer to the real value although more widely space decrements were usually used at lower temperatures.

4.4. Measuring freezing damage

VA was the simplest method, as it requires no equipment, and thus was most commonly used (Table 4.15). In VA the samples were either visually observed and compared to a grading scale (Aitken et al.,

1996; Lucas et al., 1988; Prada et al., 2014), or a damaged/undamaged grading was given (Martinez Meier et al., 2005; Riikonen and Luoranen, 2020). Sometimes microscopes were used (Wulff et al., 1994), although these instances were scored together with the rest.

VA was also the method that was usually used to observe frost damage in the field (Table 4.9), and, as most researchers consider field observations the gold standard, VA should be the technique other techniques were compared to. VA was compared to every other technique, as was the other technique commonly used in the field, EL.

EL was also the second most used technique in controlled enclosure testing (Table 4.15). It consists of placing a treated sample in pure water and measuring the change in conductivity. The level of conductivity was compared to a control, and sometimes the sample was autoclaved in the water to make sure all the electrolytes have leaked (Bachofen et al., 2016; Murray et al., 1989).

VA and EL were well correlated with each other, as shown in the twelve studies that compared the two methods (Gillies and Binder, 1996; L'Hirondelle et al., 2006; Mabaso et al., 2017; Martin et al., 2010; Odlum and Blake, 1996; Prada et al., 2014; Repo et al., 1996; Sutinen et al., 1992), or have similar results (Jalkanen et al., 1998; Man et al., 2017, 2016; van den Driessche, 1976). EL and VA were also used throughout the full historical range of studies reviewed in this map, 1972-2020 for EL, and 1973 and VA. EL was also a relatively simple technique, as it only requires an electrical conductivity machine, commonly available in most labs. EL also avoids the needs for grading scales that were used according to the researcher's criteria, and it was thus easier to produce results comparable between different researchers. The wide range of years across which this technique was used, its correlation with VA, and the simplicity of its use, combined with well-established protocols, makes this a very good technique for a researcher to use.

Fluorometry, the third most used technique (Table 4.15), was introduced in the nineties, its use spanning between 1990-2020. It consists of measuring the *in vivo* fluorescence of chlorophyll, and the effects of freezing on chlorophyll (Neuner et al., 2020). It requires a fluorometer, and it was more complex to use. Its recent development also means the methods were less established. It was well correlated with both VA in all ten studies that compared these two techniques (Adams and Perkins, 1993; Bannister and Lord, 2006; Binder and Fielder, 1996; Fernandez et al., 2003; Gillies and Binder, 1996; Jones and Cregg, 2006; L'Hirondelle et al., 2006; Prada et al., 2014; Puértolas et al., 2005; Rose and Haase, 2002). It was also well correlated with EL, in the three studies that compared these two techniques (Baldi et al., 2011; Prada et al., 2014; Wulff et al., 1994). Overall, fluorometry seems like a solid, well used technique, albeit a slightly more complex one to use than EL or VA.

DTA, the fourth most used technique (Table 4.15), ranges a wide span of years (1985-2011). EIS consists of measuring exotherms during the freezing process, and comparing them to a dead control

(Buchner et al., 2011). It was quite useful for stems, which were much harder to grade by VA, since damage was harder to estimate. The setup for DTA was quite complex, and there was no study comparing its correlation with others, although it seems to be more precise than VA (Burr et al., 1990). Due to the lack of correlation to date, DTA should be considered a less well proven technique.

EIS, which was used almost as frequently as DTA (Table 4.15) was an old technique, ranging between 1970-2017. It is based on the reduced extracellular resistance caused by freezing (Zhang et al., 2017), and the measurement of the electrical impedance of the tissue. EIS and VA were correlated in one study (Toivonen et al., 1991), not correlated in another (Repo et al., 1994), and have similar results in another (van den Driessche, 1973). EIS was also correlated with EL in one study (Zhang et al., 2010). Overall, it does not seem an established, well tested technique, although there was more solid evidence of its usefulness than there was for DTA.

The tetrazolium assay was a technique introduced in the 1990s, it was used between 1992-2004 but rarely (Table 4.15). It consists of measuring the plants' reductive potential (Beck et al., 2004; Buchner and Neuner, 2009; Hansen, 1992; Mellerowicz et al., 1992). It seems like it was an experimental technique which was briefly used for a decade and then abandoned. It was not a well-established technique, and it has not been used within the last decade.

The PM-ATPase activity measurement, which consisted of measuring plasma concentrations of the H^+-ATPase membrane protein (Ryyppö et al., 1998), was only used in one study (Table 4.15). It seems like another an experimental technique which has not been used in the last ten years.

5. Conclusion

Overall, the conditions used in frost tolerance measurement will depend on the researcher's primary objectives. In order to optimize resources, it is optimal to use branch cuttings or needles for testing, as they allow for the repeated testing of the same plant. It is also advisable to use programmable equipment that allows control of the cooling and thawing rate and allows programming to any desired temperatures. In case this was not practical, precautions should be taken to slow the cooling rate. A wide range of temperatures with multiple testing temperatures should be used in testing, in order to obtain the LT_{50} value with any degree of precision.

References

Adams, G.T., Perkins, T.D., 1993. Assessing cold tolerance in *Picea* using chlorophyll fluorescence. *Environ. Exp. Bot.* **33**, 377-382.

Aho, M.L., 1994. Autumn frost hardening of one-year-old *Pinus sylvestris* seedlings: effect of origin and parent trees. *Scand. J. For. Res.* **9**, 17-24.

Aitken, S.N., Adams, W.T., 1996. Genetics of fall and winter cold hardiness of coastal Douglas-fir in

Oregon. *Can. J. For. Res.* **26**, 1828-1837.

Aitken, S.N., Adams, W.T., Schermann, N., Fuchigami, L.H., 1996. Family variation for fall cold hardiness in two Washington populations of coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco). *For. Ecol. Manage.* **80**, 187-195.

Amundson, R.G., Kohut, R.J., Laurence, J.A., 1990. Mineral nutrition, carbohydrate content and cold tolerance of foliage of potted red spruce exposed to ozone and simulated acidic precipitation treatments. *Water. Air. Soil Pollut.* **54**, 175-182.

Andersson, B., 1992. Autumn frost hardiness of *Pinus sylvestris* offspring from seed orchard grafts of different ages. *Scand. J. For. Res.* **7**, 367-375.

Anekonda, T.S., Adams, W.T., Aitken, S.N., 1998. Influence of second flushing on genetic assessment of cold hardiness in coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco). *For. Ecol. Manage.* **111**, 119-126.

Bachofen, C., Wohlgemuth, T., Ghazoul, J., Moser, B., 2016. Cold temperature extremes during spring do not limit the range shift of Mediterranean pines into regions with intermittent frost. *Funct. Ecol.* **30**, 856-865.

Baldi, P., Pedron, L., Hietala, A.M., La Porta, N., 2011. Cold tolerance in cypress (*Cupressus sempervirens* L.): a physiological and molecular study. *Tree Genet. Genomes* **7**, 79-90.

Bannister, P., Lord, J.M., 2006. Comparative winter frost resistance of plant species from southern Africa, Australia, New Zealand, and South America grown in a common environment (Dunedin, New Zealand). *New Zeal. J. Bot.* **44**, 109-119.

Bansal, S., Harrington, C.A., St. Clair, J.B., 2016. Tolerance to multiple climate stressors: a case study of Douglas-fir drought and cold hardiness. *Ecol. Evol.* **6**, 2074-2083.

Beck, E.H., Heim, R., Hansen, J., 2004. Plant resistance to cold stress: Mechanisms and environmental signals triggering frost hardening and dehardening. *J. Biosci.* **29**, 449-459.

Beuker, E., Valtonen, E., Repo, T., 1998. Seasonal variation in the frost hardiness of Scots pine and Norway spruce in old provenance experiments in Finland. *For. Ecol. Manage.* **107**, 87-98.

Bigras, F.J., Colombo, S.J., 2001. Conifer cold hardiness, tree physiology. Springer Netherlands, Dordrecht.

Bigras, F.J., D'Aoust, A.L., 1993. Influence of photoperiod on shoot and root frost tolerance and bud phenology of white spruce seedlings (*Picea glauca*). *Can. J. For. Res.* **23**, 219-228.

Binder, W.D., Fielder, P., 1996. Chlorophyll fluorescence as an indicator of frost hardiness in white spruce seedlings from different latitudes. *New For.* **11**, 233-253.

Buchner, O., Neuner, G., 2009. A low-temperature freezing system to study the effects of temperatures to -70 °C on trees in situ. *Tree Physiol.* **29**, 313-320.

Buchner, O., Neuner, G., Ball, M., 2011. Winter frost resistance of *Pinus cembra* measured in situ at the alpine timberline as affected by temperature conditions. *Tree Physiol.* **31**, 1217-1227.

Burr, K.E., Tinus, R.W., Wallner, S.J., King, R.M., 1990. Comparison of three cold hardiness tests for conifer seedlings. *Tree Physiol.* **6**, 351-369.

Cannell, M.G.R., Sheppard, L.J., 1982. Seasonal changes in the frost hardiness of provenances of *Picea sitchensis* in Scotland. *Forestry* **55**, 137-153.

Cape, J.N., Leith, I.D., Fowler, D., Murray, M.B., Sheppard, L.J., Eamus, D., Wilson, R.H.F., 1991. Sulphate and ammonium in mist impair the frost hardening of red spruce seedlings. *New Phytol.* **118**, 119-126.

Carles, S., Lamhamedi, M.S., Stowe, D.C., Veilleux, L., Margolis, H.A., 2012. An operational method for estimating cold tolerance thresholds of white spruce seedlings in forest nurseries. *For. Chron.* **88**, 448-457.

Climent, J., Costa e Silva, F., Chambel, M.R., Pardos, M., Almeida, M.H., 2009. Freezing injury in primary and secondary needles of Mediterranean pine species of contrasting ecological niches. *Ann. For. Sci.* **66**, 407-407.

Colombo, S.J., 1990. Bud dormancy status, frost hardiness, shoot moisture content, and readiness of black spruce container seedlings for frozen storage. *J. Am. Soc. Hortic. Sci.* **115**, 302-307.

Colombo, S.J., Glerum, C., Webb, D.P., 2003. Daylength, temperature and fertilization effects on desiccation resistance, cold hardiness and root growth potential of *Picea mariana* seedlings. *Ann. For. Sci*. **60**, 307-317. h

Colombo, S.J., Raitanen, E.M., 1991. Frost hardening in white cedar container seedlings exposed to intermittent short days and cold temperatures. *For. Chron*. **67**, 542-544.

Coursolle, C., Bigras, F.J., Margolis, H.A., Hébert, C., 1997. Dehardening and second-year growth of white spruce provenances in response to duration of long-night treatments. *Can. J. For. Res.* **27**, 1168-1175.

Davis, S.D., Sperry, J.S., Hacke, U., 1999. The relationship between xylem conduit diameter and cavitation caused by freezing. *Am. J. Bot.* **86**, 1367-1372.

Day, W.R., Peace, T.R., 1937. The influence of certain accessory factors on frost injury to forest trees. *Forestry* **11**.

de Waal, L., Mitchell, R.G., Hodge, G.R., Chirwa, P.W., 2018. The use of field and artificial freezing studies to assess frost tolerance in natural populations of *Pinus oocarpa*. *South. For. a J. For. Sci.* **80**, 195-208.

Dirr, M.A., Lindstrom, O.M., Lewandowski, R., Vehr, M.J., 1993. Cold hardiness estimates of woody taxa from cultivated and wild collections. *J. Environ. Hortic.* **11**, 200-203.

Eamus, D., 1993. Assimilation and stomatal conductance responses of red spruce to midwinter frosts and the constituent ions of acid mist. *Tree Physiol.* **13**, 145-155.

European Environmental Agency, 2006. European forest types: categories and types for sustainable forest management reporting and policy. *EEA Tech. Rep.* **9**, 1-114.

Fernandez, M., Royo, A., Gil, L., Pardos, J.A., 2003. Effects of temperature on growth and stress hardening development of phytotron-grown seedlings of Aleppo pine (*Pinus halepensis* Mill.). *Ann. For. Sci.* **60**, 277-284.

Fløistad, I.S., Granhus, A., 2010. Bud break and spring frost hardiness in *Picea abies* seedlings in response to photoperiod and temperature treatments. *Can. J. For. Res.* **40**, 968-976.

Frederiks, T.M., Christopher, J.T., Sutherland, M.W., Borrell, A.K., 2015. Post-head-emergence frost in wheat and barley: defining the problem, assessing the damage, and identifying resistance. *J. Exp. Bot.* **66**, 3487-3498.

Fujikawa, S., Kuroda, K., Jitsuyama, Y., Sano, Y., Ohtani, J., 1999. Freezing behavior of xylem ray parenchyma cells in softwood species with differences in the organization of cell walls. *Protoplasma* **206**, 31-40.

Gillies, S.L., Binder, W.D., 1996. The effect of sub-zero temperatures in the light and dark on cold-hardened, dehardened and newly hushed white spruce (*Picea glauca* [Moench.] Voss) seedlings. *New For.* **13**, 91-104.

Gooch, N.J., Nzokou, P., Cregg, B.M., 2009. Effect of indoor exposure on the cold hardiness and physiology of containerized Christmas trees. *Horttechnology* **19**, 72-77.

Greer, D.H., Stanley, C.J., Warrington, I.J., 1989. Photoperiod control of the initial phase of frost hardiness development in *Pinus radiata*. *Plant, Cell Environ.* **12**, 661-668.

Haddaway, N., Macura, B., Whaley, P., Pullin, A., 2018. ROSES for Systematic Review Protocols. Version 1.0. figshare. Online resource.

Hannerz, M., Westin, J., 2005. Autumn frost hardiness in Norway spruce plus tree progeny and trees of the local and transferred provenances in central Sweden. *Tree Physiol.* **25**, 1181-1186.

Hansen, J.M., 1992. Effects of nutritional factors on frost hardening in *Larix leptolepis* (Sieb & Zucc.) *Gord. Scand. J. For. Res.* **7**, 183-192.

Hawkins, B.J., McDonald, S.E., 1993. Photoperiod influences dehardening of *Chamaecyparis nootkatensis* seedlings. *Can. J. For. Res.* **23**, 2452-2454.

Hawkins, B.J., Sweet, G.B., Greer, D.H., Bergin, D.O., 1991. Genetic variation in the frost hardiness of *Podocarpus totara*. *New Zeal. J. Bot.* **29**, 455-458.

Haynes, C.L., Lindstrom, O.M., Dirr, M.A., 1992. Cooling and warming effects on cold hardiness estimations of three woody ornamental taxa. *HortScience* **27**, 1308-1309.

Hodge, G., Dvorak, W., Tighe, M., 2012. Comparisons between laboratory and field results of frost tolerance of pines from the southern USA and Mesoamerica planted as exotics. *South. For. a J. For. Sci.* **74**, 7-17.

Hughes, M.A., Dunn, M.A., 1996. The molecular biology of plant acclimation to low temperature. *J. Exp. Bot.* **47**, 291-305.

Jalkanen, R.., Redfern, D.., Sheppard, L.., 1998. Nutrient deficits increase frost hardiness in Sitka spruce (*Picea sitchensis*) needles. *For. Ecol. Manage.* **107**, 191-201.

Jones, G.E., Cregg, B.M., 2006. Budbreak and winter injury in exotic firs. *HortScience* **41**, 143-148.

Kasuga, J., Takata, N., Yamane, K., Kuroda, K., Arakawa, K., Fujikawa, S., 2007. Larch (*Larix kaempferi*) xylem parenchyma cells respond to subfreezing temperature by deep supercooling. *CryoLetters* **28**, 77-81.

Kathke, S., Bruelheide, H., 2011. Differences in frost hardiness of two Norway spruce morphotypes growing at Mt. Brocken, Germany. *Flora* **206**, 120-126.

Keates, S.E., 1990. Assessing cold hardiness in conifers: a problem analysis and discussion paper. *FRDA Rep.* **106**, 1-20.

Konttinen, K., Luoranen, J., Rikala, R., 2007. Growth and frost hardening of *Picea abies* seedlings after various night length treatments. *Balt. For.* **13**, 140-148.

Korhonen, A., Lehto, T., Repo, T., 2015. Frost hardiness of mycorrhizal and non-mycorrhizal Scots pine under two fertilization treatments. *Mycorrhiza* **25**, 377-386.

L'Hirondelle, S.J., Simpson, D.G., Binder, W.D., 2006. Overwinter storability of conifer planting stock: operational testing of fall frost hardiness. *New For.* **32**, 307-321.

Leinonen, I., Repo, T., Hanninen, H., 1997. Changing environmental effects on frost hardiness of Scots Pine during dehardening. *Ann. Bot.* **79**, 133-137.

Li, C., Junttila, O., Palva, E.T., 2004. Environmental regulation and physiological basis of freezing tolerance in woody plants. *Acta Physiol. Plant.* **26**, 213-222.

Lucas, P.W., Cottam, D.A., Sheppard, L.J., Francis, B.J., 1988. Growth responses and delayed winter hardening in Sitka spruce following summer exposure to ozone. *New Phytol.* **108**, 495-504.

Mabaso, F., Ham, H., Nel, A., 2017. Frost tolerance of various *Pinus* species and hybrids. Stellenbosch Univ.

Man, R., Colombo, S., Lu, P., Dang, Q.-L., 2016. Effects of winter warming on cold hardiness and spring budbreak of four boreal conifers. *Botany* **94**, 117-126.

Man, R., Lu, P., Dang, Q.-L., 2017. Cold hardiness of white spruce, black spruce, jack pine, and lodgepole pine needles during dehardening. *Can. J. For. Res.* **47**, 1116-1122.

Martin, M., Gavazov, K., Körner, C., Hättenschwiler, S., Rixen, C., 2010. Reduced early growing season

freezing resistance in alpine treeline plants under elevated atmospheric CO_2. *Glob. Chang. Biol.* **16**, 1057-1070.

Martinez Meier, A., Mondino, V., Gallo, L., 2005. Evaluación de daños por heladas tardías en ensayos de procedencias de pino oregón introducidos en el norte de la Región Andino Patagónica Argentina. *Bosque* **26**, 113-120.

Mayr, S., Gruber, A., Bauer, H., 2003. Repeated freeze-thaw cycles induce embolism in drought stressed conifers (Norway spruce, stone pine). *Planta* **217**, 436-441.

Mellerowicz, E.J., Coleman, W.K., Riding, R.T., Little, C.H.A., 1992. Periodicity of cambial activity in *Abies balsamea*. I. Effects of temperature and photoperiod on cambial dormancy and frost hardiness. *Physiol. Plant.* **85**, 515-525.

Murray, M.B.B., Cape, J.N., Fowler, D., 1989. Quantification of frost damage in plant tissues by rates of electrolyte leakage. *New Phytol.* **113**, 307-311.

Neuner, G., 2014. Frost resistance in alpine woody plants. *Front. Plant Sci.* **5**, 654.

Neuner, G., Huber, B., Plangger, A., Pohlin, J.-M., Walde, J., 2020. Low temperatures at higher elevations require plants to exhibit increased freezing resistance throughout the summer months. *Environ. Exp. Bot.* **169**, 103882.

Nicoll, B.C., Redfern, D.B., McKay, H.M., 1996. Autumn frost damage: clonal variation in Sitka spruce. *For. Ecol. Manage.* **80**, 107-112.

Nielsen, C.C.N., Rasmussen, H.N., 2009. Frost hardening and dehardening in *Abies procera* and other conifers under differing temperature regimes and warm-spell treatments. *Forestry* **82**, 43-59.

Nilsson, J., Eriksson, G., 1986. Freeze testing and field mortality of *Pinus sylvestris* (L.) in northern Sweden. *Scand. J. For. Res.* **1**, 205-218.

Odlum, K.D., Blake, T.J., 1996. A comparison of analytical approaches for assessing freezing damage in black spruce using electrolyte leakage methods. *Can. J. Bot.* **74**, 952-958.

Ogren, E., 1997. Relationship between temperature, respiratory loss of sugar and premature dehardening in dormant Scots pine seedlings. *Tree Physiol.* **17**, 47-51.

Prada, E., Alía, R., Climent, J., Díaz, R., 2014. Seasonal cold hardiness in maritime pine assessed by different methods. *Tree Genet. Genomes* **10**, 689-701.

Puértolas, J., Gil, L., Pardos, J.A., 2005. Effects of nitrogen fertilization and temperature on frost hardiness of Aleppo pine (*Pinus halepensis* Mill.) seedlings assessed by chlorophyll fluorescence. *Forestry* **78**, 501-511.

Räisänen, M., Repo, T., Lehto, T., 2006. Effect of thawing time, cooling rate and boron nutrition on freezing point of the primordial shoot in Norway spruce buds. *Ann. Bot.* **97**, 593-599.

Repo, T., Hanninen, H., Kellomaki, S., 1996. The effects of long-term elevation of air temperature and

CO_2 on the frost hardiness of Scots pine. *Plant, Cell Environ.* **19**, 209-216.

Repo, T., Zhang, G., Ryyppö, A., Rikala, R., 2000. The electrical impedance spectroscopy of Scots pine (*Pinus sylvestris* L.) shoots in relation to cold acclimation. *J. Exp. Bot.* **51**, 2095-2107.

Repo, T., Zhang, M.I.N., Ryyppö, A., Vapaavuori, E., Sutinen, S., 1994. Effects of freeze-thaw injury on parameters of distributed electrical circuits of stems and needles of Scots pine seedlings at different stages of acclimation. *J. Exp. Bot.* **45**, 823-833.

Riikonen, J., Luoranen, J., 2020. An assessment of storability of Norway spruce container seedlings in freezer storage as affected by short-day treatment. *Forests* **11**, 692.

Rodrigo, J., 2000. Spring frosts in deciduous fruit trees — morphological damage and flower hardiness. *Sci. Hortic. (Amsterdam).* **85**, 155-173.

Rose, R., Haase, D., 2002. Chlorophyll fluorescence and variations in tissue cold hardiness in response to freezing stress in Douglas-fir seedlings. *New For.* **23**, 81-96.

Ryyppö, A., Repo, T., Vapaavuori, E., 1998. Development of freezing tolerance in roots and shoots of Scots pine seedlings at nonfreezing temperatures. *Can. J. For. Res.* **28**, 557-565.

Sakai, A., Malla, S.B., 1981. Winter hardiness of tree species at high altitudes in the East Himalaya, Nepal. *Ecology* **62**, 1288-1298.

Sakai, A., Wardle, P., 1978. Freezing resistance of New Zealand trees and shrubs. *N. Z. J. Ecol.* **1**, 51-61.

Sheppard, L.J., 1994. Causal mechanisms by which sulphate, nitrate and acidity influence frost hardiness in red spruce: review and hypothesis. *New Phytol.* **127**, 69-82.

Sperry, J.S., Nichols, K.L., Sullivan, J.E.M., Eastlack, S.E., 1994. Xylem embolism in ring-porous , diffuse-porous , and coniferous trees of Northern Utah and interior Alaska. *Ecology* **75**, 1736-1752.

Sperry, J.S., Sullivan, J.E.M., 1992. Xylem embolism in response to freeze-thaw cycles and water stress in ring-porous, diffuse porous, and conifer species. *Plant Physiol*. **100**, 605-613.

Strimbeck, G.R., Schaberg, P.G., Fossdal, C.G., Schröder, W.P., Kjellsen, T.D., 2015. Extreme low temperature tolerance in woody plants. *Front. Plant Sci.* **6**, 884.

Sutinen, M.-L., Palta, J.P., Reich, P.B., 1992. Seasonal differences in freezing stress resistance of needles of *Pinus nigra* and *Pinus resinosa*: evaluation of the electrolyte leakage method. *Tree Physiol*. **11**, 241-254.

Toivonen, A., Rikala, R., Repo, T., Smolander, H., 1991. Autumn colouration of first year *Pinus sylvestris* seedlings during frost hardening. *Scand. J. For. Res.* **6**, 31-39.

van den Driessche, R., 1976. Prediction of cold hardiness in Douglas Fir seedlings by index of injury and conductivity methods. *Can. J. For. Res.* **6**, 511-515.

van den Driessche, R., 1973. Prediction of frost hardiness in Douglas Fir seedlings by measuring electrical impedance in stems at different frequencies. *Can. J. For. Res.* **3**, 256-264.

Vangestel, C., Eckert, A.J., Wegrzyn, J.L., St. Clair, J.B., Neale, D.B., 2018. Linking phenotype, genotype and environment to unravel genetic components underlying cold hardiness in coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii*). *Tree Genet. Genomes* **14**, 10.

Vann, D.R., Strimbeck, G.R., Johnson, A.H., 1992. Effects of ambient levels of airborne chemicals on freezing resistance of red spruce foliage. *For. Ecol. Manage.* **51**, 69-79.

Warrington, I.J., Jackson, A.K.H., 1981. Injury to radiata pine as influenced by freezing and thawing rate, and low temperature duration. *New Zeal. J. For. Sci.* **11**, 37-44.

Warrington, I.J., Rook, D.A., 1980. Evaluation of techniques used in determination of frost tolerance of forest planting stock: A review. *New Zeal. J. For. Sci.* **10**, 116-132.

Welling, A., Palva, E.T., 2006. Molecular control of cold acclimation in trees. *Physiol. Plant.* **127**, 167-181.

Wulff, A., Sheppard, L., Leith, I., 1994. Evaluation of electrolyte leakage, chlorophyll fluorescence and ultrastructural techniques for detecting effects of acid mist on frost hardiness of Sitka spruce shoots. *Environ. Exp. Bot.* **34**, 261-273.

Zhang, G., Li, Y. Q., Dong, S. H., 2010. Assessing frost hardiness of Pinus bungeana shoots and needles by electrical impedance spectroscopy with and without freezing tests. *J. Plant Ecol.* **3**, 285-293.

Zhang, G., Sun, B., Li, Z., Di, B., Xiang, D., Meng, Y., 2017. Effects of soil temperature on bud break, shoot and needle growth, and frost hardiness in *Pinus sylvestris* var. *mongolica* saplings during dehardening. *Acta Physiol. Plant.* **39**, 169.

Chapter 5. Phenotyping for frost tolerance in Sitka spruce (*Picea sitchensis* [Bong.] Carr.)

Author contributions: AA, KS and AS conceived and planned the experiment. Field sampling was conducted by AA and AS in one trip and by AA and Dr Dave Buenavista in a second trip. Forest Managers at Tilhill Forestry Ltd. provided assistance in the field collection. Maelor Forest Nurseries Ltd. provided the commercial varieties that were used in the analysis, and the substrate used for rooting, as well as guidance on rooting and growing trees. AA did the phenotyping work and analysed the data. Writing was done by AA with feedback and guidance from KS and AS.

Abstract

Genetic improvement of frost tolerance in Sitka spruce (*Picea sitchensis* [Bong.] Carr.) could be conducted faster with reliable frost tolerance markers. Identifying phenotypic differences in frost tolerance between Sitka spruce trees which could then be associated with genetic markers is thus key for improving breeding efforts. This study aimed to find out whether phenotypic differences detected in experiments which measured electrolyte leakage in response to freezing damage correspond to differences in frost damage recorded in natural conditions for the same individuals. The data for 19 undamaged and 18 frost damaged trees affected by a particularly severe 2015 frost showed that there was no relationship that could be found between relative electrolyte leakage and frost damage. No clear pattern of relative electrolyte leakage following freezing could be observed between the different commercial cultivars and breeding lines either.

1. Introduction

Sitka spruce (*Picea sitchensis* [Bong.] Carr.) is the main species used in softwood production in the UK, occupying 51% of the total conifer area (Forestry Commission, 2019). Sitka spruce was first introduced into Great Britain by David Douglas in 1831, and the first major seed imports occurred in 1852. Sitka spruce is native to a narrow coastal strip of North America ranging from Alaska, at 61 °N, to California, at 39 °N, with a maximum development on Haida Gwaii islands (formerly known as Queen Charlotte Islands, QCI). The range has rather uniform climatic conditions, due to mild temperature changes at low altitudes. The frost-free season is long, humidity high, with annual rainfall 1000-3000 mm, and an absence of severe frosts (Lines, 1987). In its native range, Sitka spruce grows at low elevation, and does not extend further than a 100 miles inland from the coast (Forestry Commission, 1957). When grown commercially it is used for structural timber, due to its strength and stiffness (Moore, 2011). The typical rotation length of Sitka spruce plantations is of 40 years with the planting stock used in the UK (Herbert et al., 1999).

This study was initiated after severe damages were observed in some trees in the spring of 2015 in Sitka spruce plantations under 4 years old across the UK. It was presumed that frost damage had occurred due to the minimum air temperature being below the frost hardiness threshold of the trees (Chapter 3). Many young tree plantations across the UK were damaged (Figure 5.1) including leader death, in some cases the dead tissue exceeding a meter of new and old growth (Figure 5.2). Damage to side branches also occurred in more mature trees, as side branches get damaged much more frequently than leaders, although it is of lesser importance because it does not affect wood quality.

Figure 5.1. Photo of the Hewisbridge forest exhibiting severe frost damage in many young Sitka spruce (*Picea sitchensis* [Bong.] Carr.) trees. Damaged trees indicated with white arrows.

This level of damage may delay the growth of the plantation by several years, as the apical meristem will need to be re-established. Side branches will try to establish apical dominance, and it could lead to the tree having multiple competing leaders (Figure 5.3). Poor tree form resulting from such problems reduces the economic value of the forest. Sitka spruce trees in the UK are mostly used for timber, the highest value type of wood. Wood that exhibits knots or crooked form may not be suitable for structural timber, thus reducing the value of the plantation (Moore, 2011). Not all trees exhibit the same level of frost damage and this may be due to both environmental and genetic effects.

Frosts, defined as days during which the minimum daily air temperature goes below 0 °C, can vary in their degree of harmfulness depending on both internal and external factors, as well as their

interaction (Chapter 3). The main internal factor inherent to the plant is their frost hardiness, the physiological and morphological adaptations plants acquire to resist sub-freezing temperatures.

External factors like drying winds, weed competition (reviewed by Warrington and Rook, 1980), high freezing rates (Räisänen et al., 2006), air humidity (Jeong et al., 2018), snow cover (Wipf et al., 2009), acid mist (Sheppard, 1994), can all affect how harmful a given frost will be to a plant. External factors interact with the physiological status of the plant. Biotic factors are also known to affect frost hardiness. For example, ice-nucleation-active (INA) bacteria such as *Pseudomonas syringae* (van Hall), can act as a nucleating centre for ice formation (Lindow, 1983), affecting the level of frost damage experienced by plants.

1.1. Mechanisms influencing frost damage and tolerance

External ice formation can be harmful, or even lethal to the cell by destroying the cell membrane or by the impact on the extracellular environment such as changes in pH and the denaturation of proteins (Kakac et al., 2003). Internal ice formation, however, is more lethal as it can cause the rupture of the cellular membrane and intracellular structures.

When air temperatures decrease below 0 °C, ice can start forming inside and outside plant cells. But while water melts at 0 °C and ice can form at 0 °C, water can remain liquid below 0 °C through the production of osmolytes and antifreeze proteins, using a mechanism known as supercooling. The threshold temperature at which ice formation in pure water becomes unavoidable is -40 °C, also called the homogeneous ice nucleation point (Bigras and Colombo, 2001). This occurs because ice formation needs a small ice crystal embryo to form from which to grow, and the formation of a crystal in pure water at temperatures above -40 °C is not highly probable.

Various molecules can act as ice nucleation points. Some plants, such as Taiwan cypress (*Chamaecyparis taiwanensis* Masam. & Suzuki) produce antinucleation compounds such as hinokitiol, which, thanks to its effect in neutralizing ice-nucleating activity, decreases ice nucleation temperature (Kawahara et al., 2000).

Of course, water in plant cells is not pure. But plants can still decrease the ice formation temperature inside the cell by increasing the concentration of osmolytes (Charra-Vaskou et al., 2012), lowering ice formation temperatures by a mechanism known as freezing point depression.

Highly viscous cytosol can undergo a process known as vitrification, where it avoids the formation of ice crystals and acquires a glassy amorphous state without ice crystals (Kakac et al., 2003). The type of ice formation can also differ in its lethality. Optimal cooling rates lead to a frozen state where the structure and function of the cell is conserved in a frozen state. Faster or slower cooling rates can lead to lethal alterations in the cell structure. Thawing can also cause problems, if fast cooling is followed

by slow thawing or if slow cooling is followed by fast thawing, by the induction of migratory recrystallization, where smaller ice crystals melt and the bigger crystals grow.

Figure 5.2. Image that exhibits the leader frost damage in a Sitka spruce (*Picea sitchensis* [Bong.] Carr.) tree from the 2015 frost in the Blazehill forest, showing how more than a meter of new and old growth died as a result of the frost.

Figure 5.3. A Sitka tree (*Picea sitchensis* [Bong.] Carr.) in the Hewisbridge forest with a dead leader, and thus no main apical meristem. It has two side branches competing to establish themselves as the main apical meristem.

Internal ice formation not only damages cell membranes and walls, but it also leads to the denaturation of proteins and dehydration. Dehydration is caused by the freezing of extracellular liquid, which increases the osmotic pressure on the water inside the cell, forcing water to move out of the cell, causing cell shrinkage (Fujikawa et al., 1999; Kasuga et al., 2007). As the extracellular liquid has a lower concentration of osmolytes (salts, sugars and amino acids that cause freezing-point depression), it freezes earlier than cytoplasm. This causes dehydration as water rushes out of the cell into the extracellular space, thus increasing intracellular concentration and shrinking the cell. Ice then slowly forms inside the cell with high osmolyte concentration, until the cell is completely frozen, leading to

the conservation of the cell (Kakac et al., 2003). However, when the freezing rate is faster than the rate of water loss by osmosis, dehydration does not occur, and freezing occurs both inside and outside the cell. This increases the speed of ice formation and leads to greater damage.

A common method of measuring frost damage consists of measuring changes in the conductivity of pure water when in contact with frost damaged tissue. This technique, known as electrolyte leakage (EL), is based on the increased permeability of the cell membrane that occurs after frost injury (Bigras and Colombo, 2001). This is due to both physical damage of the cell membrane and its loss of ability to regulate the ion transport across the membrane (Wulff et al., 1994). Damaged plant tissues release electrolytes, mainly K^+, increasing the electrical conductivity of the water in which they are placed, which can be measured and compared to the changes in electroconductivity of pure water in contact with intact (undamaged) tissues. This technique can be used for phenotypic analysis. In studies that compared visually observable levels of frost damage in the field with the results of EL tests in closed enclosure conditions they were well correlated (de Waal et al., 2018; Vann et al., 1992) (Chapter 4).

1.2. Frost tolerance of Sitka spruce

Sitka spruce is a species that is highly variable in its frost tolerance, and replicated experiments with large sample sizes are required, otherwise significant differences will be harder to detect (Sheppard et al., 2003). Within the origins of Sitka spruce, Alaska is the most frost tolerant genotype, followed by QCI and then Washington (Nicoll et al., 1996). Trees of different origins also differ in their hardening speed, with Alaska origin hardening the fastest, followed by QCI and Oregon origin (Cannell et al., 1985).

Some of this variability is not entirely genetic, as shown by the significant variability in frost tolerance of different clones of Washington origin Sitka spruce, although QCI and Alaska clones did not significantly differ from each other (Nicoll et al., 1996). It is hard to separate environmental and genetic effects in such studies.

Differences in frost tolerance also depend on the tissue sampled. Sitka spruce seems to have a significantly smaller difference between the LT_{50} of apical and lateral buds than other species, such as red fir (*Abies procera* Rehder), which showed an average of 4 K difference between the LT_{50} of lateral and apical buds (Nielsen and Rasmussen, 2009). This difference in LT_{50} between apical and subapical buds remained stable over the year for Sitka spruce, as hardening and dehardening did not change the difference in sensitivity between the tissues, unlike the changes that occur in other species such as Norway spruce (*Picea abies* (L.) H. Karst.). Sitka spruce also exhibits much higher sensitivity in autumn and spring than would be expected based on its baseline level of frost tolerance. Sitka spruce exhibits seasonal patterns of bud damage, with damage more common in autumn and spring. Even when compared with the less winter hardy species Nordmann fir (*Abies nordmanniana* (Steven)

Spach), Sitka spruce exhibited similar levels of damage in spring and autumn and much lower levels in winter. Tissue age also affects frost tolerance; 1 year old needles are less hardy than 2 year old needles (Jalkanen et al., 1998). Although age is a factor, there are no differences in frost tolerance between juvenile and mature acid-treated Sitka spruces (Sheppard et al., 1994), indicating that it is the age of the tissue and not the stage of the plant that is the important factor.

Sitka spruce frost tolerance is affected by multiple external factors. Acid mist can reduce the frost hardiness of Sitka spruce (Sheppard et al., 1994; Wulff et al., 1994), although one study only found an effect in small (< 4 m tall) Sitka spruce trees (Sheppard et al., 1997). While adding nutrients to deficient trees did not seem to affect the frost hardiness of Sitka spruce (Sheppard et al., 2003), artificially induced nutrient deficiency seemed to improve frost tolerance. Nitrogen, phosphorus and especially potassium deficiency seemed to improve frost hardiness of Sitka spruce (Jalkanen et al., 1998). This could be because nutrient deficient tissues did not respond to warm periods by dehardening. Also, since Sitka spruce that experienced a warm spell in winter did not recover to the baseline winter level of hardiness (Nielsen and Rasmussen, 2009), any dehardening during the winter can lead to lower frost hardiness. Sitka spruce is also immune to the effects of UV-B on frost tolerance, unlike some other conifer species, such as Engelmann spruce (*Picea engelmannii* Parry ex Engelm.), Douglas fir (*Pseudotsuga menziesii* (Mirb). Franco), lodgepole pine (*Pinus contorta* Douglas ex Loudon) and grand fir (*Abies grandis* (Douglas ex D.Don) Lindl.) (L'Hirondelle and Binder, 2005).

Sitka spruce has a frost tolerance below -20 °C in winter, with a wide range of variation in frost tolerance during the hardening-dehardening cycles (Cannell and Sheppard, 1982). The warmest temperature at which damage was noted was - 3 °C in spring, in a study performed in Scotland by Cannel and Sheppard (1982). Mid-summer hardiness fluctuated between -5 °C and -10 °C, while around bud burst, newly emerging shoots dehardened to between -3 °C and -5 °C, while older tissues dehardened to between -6 °C and -8 °C.

1.3. Measuring frost tolerance

There are a range of traits associated with the responses of different tissues to freezing that have been used for phenotyping. The gold standard of frost tolerance measurement is usually considered to be the field observation study, where naturally planted trees are monitored for damage across a range of years during which daily temperature and weather conditions are measured. This method, however, as pointed out in Chapter 4, is costly and requires a long time, as natural frosts do not happen on demand.

Thus, many techniques to simulate field conditions and measure the damage have been developed, an overview of which was made in Chapter 4. Visual assessment (VA), the one that was most frequently used, consisted in observing the samples after freezing exposure and evaluating damage.

Frost damage can be scored by either comparing to a grading scale (Aitken et al., 1996; Lucas et al., 1988; Prada et al., 2014), or giving a damaged/undamaged score (Martinez Meier et al., 2005; Riikonen and Luoranen, 2020). Observations could be made by the naked eye or by using microscopes (Wulff et al., 1994), which allowed for a closer examination of the cell. VA was the method used to measure frost damage in most field studies (Chapter 4, Table 5.9).

Electrolyte leakage, the second most common technique, is based on changes in cell wall and membrane permeability after frost exposure, which leads to a higher degree of changes in the electroconductivity of the water in which the sample is placed. Other techniques, such as fluorometry and differential thermal analysis, make use of changes in chlorophyll *in vivo* fluorescence and exotherms released during freezing, respectively.

Of these techniques, EL is the one that does not require the researcher's subjective evaluation of the treated material and does not require complex equipment. It also permits the measurement of frost tolerance with very small samples. Electrolyte leakage is also a robust technique that seems to correlate well with the results for the other techniques (Chapter 4), including field observations (de Waal et al., 2018; Vann et al., 1992).

1.3.1. Measuring the frost tolerance of Sitka spruce

The systematic search conducted in Chapter 4 found a total of 12 studies that measured the frost tolerance of Sitka spruce. The majority of these studies (n = 10) used Sitka spruce cuttings as a source material, while the other two used either needles or the whole plant. Sitka spruce trees that were growing in the field were used in 75% of these studies with modifications such as open top chambers or pots, while the others used trees grown in greenhouses (n =1) or growth chambers (n = 2). Trees grown in the field become too large for whole plant testing after 4-5 years, hence the need to use branch cuttings. The majority of these studies used programmable equipment to simulate frosts and managed to maintain freezing rates of 5 K h^{-1} (n = 8), while the other four studies did not mention the freezing rate. Most studies used a thawing rate of 10 K h^{-1} (n = 8), with one using thawing at 4 °C, one using the natural thawing rate in the field, and two studies didn't mention the thawing rate used.

The studies mostly used a wide range of temperatures, with 5-6 testing temperatures, and long (3 h or more) exposure times. The majority used EL to measure frost tolerance (n = 7).

1.4. Aims and objectives

In addition to its widespread use for measuring frost tolerance of Sitka spruce, as well as its robustness, reliability and simplicity, EL was selected for this study as being the most appropriate phenotyping method because it provided an objective measure that did not require the researcher's subjective grading, and it could be used with little test material.

This project was initially developed with the goal of determining whether there is a genetic component to frost damage in Sitka spruce, which could be used to breed more frost tolerant trees. This was based on a hypothesis that the differences in field damage that were observed across the commercial forests in the UK were caused by underlying genetic differences. These genetic differences could be directly controlling frost tolerance or be upstream genes that control the expression of frost tolerance genes. While tolerance in nature is the objective of this project, reliable determination of frost tolerance based on field measurements requires a field observation program where trees are measured and observed on a regular basis, in order to find whether the differences are stable over time or not. Thus, in order to find whether these differences remained even when the plants were grown in a more controlled, uniform environment, the samples were brought back to the lab and phenotyped. A secondary hypothesis assumed that if differences in damage could be detected, colder temperatures would lead to higher degrees of damage in the same sample.

The objectives of the phenotyping study were thus to:

i) measure the frost tolerance of individual genotypes and identify populations that exhibited different patterns of frost tolerance;
ii) check whether the results of the field observations correlate with the frost tolerance measured in the field.

2. Materials and Methods

2.1. Field observations

In order to assess whether the damages observed by frost managers in the spring of 2015 were caused by genetic or environmental factors, five sites in Dumfries & Galloway, Scotland (Figure 5.4) where these damages occurred were observed two years later, in the summer of 2017. The sites were examined with the help of forest managers from TillHill Forestry, Ltd. The objective of this examination was to see whether there were clear environmental factors that caused different patterns in damages across these forests.

Three factors, the slope of the site, the direction it was facing, and elevation, were measured, in the five forests assessed (in the Hewisbridge forest, three different sites were examined, see Table 5.1). The ground was also observed for things like frost hollows (low-lying areas that experience local temperature minima).

Table 5.1. Environmental information recorded for the sites observed in August 2017.

Forest	Compartment[1]	Facing (direction)	Slope (%)	National Grid Reference	Cultivar[2]	Elevation (m)	Degree of damage[3]	Leader damage[4]
Minnygryle	Cpt 12	NE	37	NX71095 BNG88326	A17	268	25	No
Blazehill		SE	14	NY13483 BNG94397		203	90	Yes
Kirtleton	Cpt 6	S	17	NY28437 BNG81413	A13 and M0065	220	75	Yes
Billholm	Cpt 52	S	15	NY26059 BNG93498		257	63	Yes
Hewisbridge	Site 1	N	10	NY53905 BNG92036		180	27	No
Hewisbridge	Site 2	S	5	NY53819 BNG92403		175	13	No
Hewisbridge	Site 3	S	5	NY52833 BNG92813		155	12	No

[1] Part of the forest that was examined, as named on the official maps used by the forest managers.

[2]For the forests there were records for, the cultivar that was planted was noted. However, unfortunately, due to poor record keeping and forests exchanging hands, it was only possible to do so in two sites.

[3]On a 150-point scale. 50 points were added if there was leader damage to the overall percentage damage. Percentage estimated by forest managers (Chapter 4, Table 5.4.S1).

[4]Whether it was noted by forest managers

Figure 5.4. Map of locations in Dumfries & Galloway, Scotland, marked by stars, assessed for frost damage in spring 2017 for the damages that occurred in spring 2015. © OpenStreetMap contributors.

Planting records were also examined, to determine which variants were planted which year. However, due to poor record keeping, it was only possible to determine the commercial variants in two cases. Records of the degree of damage, made by the forest managers at TillHill Forestry, were also examined. In order to convert field observations into a single, numerical value, the degree of damage (measured in %) was added with +50 if there was leader damage observed in that site, and the numerical scale could go to a maximum of 150 points. Damages were not severe enough to exceed 50%.

2.2. Phenotyping samples

2.2.1. Field collection and rooting

Six forests, the five in Dumfries & Galloway, Scotland (Figure 5.4), Minnygryle, Blazehill, Kirtleton, Billholm, and Hewisbridge, and one in Wales, Llyn Brenig (latitude and longitude: 53.123535N, 3.551604W, UK-NGR: SH962596), were used for sampling.

Branch cuttings of around 20 cm were taken from damaged and undamaged trees on the 7-8th August 2017. For the purposes of this study, damage was defined as leader damage and significant side branch damage, and undamaged trees showed no signs of neither leader nor side branch damage. Sampled trees were chosen by walking a transect and selecting the clear representatives of each category, ignoring those that were in-between (*i.e.*, no leader damage but some subapical frost damage).

Forty cuttings were taken of each tree, and ten damaged and ten undamaged trees were sampled for each forest, each sample in each category named by the site name, the damage level, and the number assigned at sampling (Table 5.2) giving 4800 cuttings representing all 120 trees. Branch cuttings were transported to the lab in a ziplock bag, which was placed inside a thick plastic bag with water regularly sprinkled inside to maintain humidity, with one freezer pack in each bag, within a maximum of 48 h.

The bags were kept in a cold room at 2 °C until potted for rooting. The samples were then placed in a soil mixture for rooting provided by our company partner Maelor Forest Nurseries, between the 9th and 11th of August 2017, within 2-3 days of sampling (Table 5.3).

Due to space restrictions, growth chambers could not fit all samples. At least 50% of samples from damaged and undamaged trees for each forest were placed in the growth chambers, with the rest being placed in the greenhouse as described in Table 5.4. The growth chamber (Conviron A1000) was programmed to keep conditions of under 70% humidity, a 16 h light and 8 h dark cycle (500 µmol light intensity), at 20 °C day and 18 °C night and watered regularly. The pots that did not fit in the growth chamber were left in a greenhouse in trays with an inch of water, covered by transparent plastic to maintain humidity, under natural light and photoperiod, at 15-20 °C day and 5-10 °C night. Samples were left in their growth conditions until they rooted, which took 5 months.

Table 5.2. Explanation of naming conventions used for samples collected from the field. It should be noted that not all samples were successfully rooted, and some of the rooted samples died before they could be phenotyped; the sample numbers are thus not ordered, because only those that survived were phenotyped.

Site Name	Abbreviation	Damaged/Undamaged*
Minnygryle	M	X
		O
Hewisbridge	H	X
		O
Llyn Brenig	W (for Wales)	X
		O
Billholm	BH	X
		O
Blazehill	BZ	X
		O
Kirtleton	K	X
		O

*X was used to indicate damage, O to indicate lack of damage.

2.2.1.1. Growing conditions

After growing the samples for four months, the cuttings both in the growth chamber and in the greenhouse suffered from an aphid infestation (Figure 5.5) and were sprayed with 0.05 g L^{-1} of the insecticide acetamiprid. Three weeks later, an application of another insecticide, abamectin at 0.018 g L^{-1} (Dynamec at 1 mL L^{-1} dilution) and the fungicide propiconazole at 0.5 g L^{-1} (Bumper 250 EC at 2 mL L^{-1} dilution) was made.

Table 5.3. Dates when each forest was sampled and when the samples were potted for rooting.

Site Name	Country*	Date of Cutting	Date of Potting
Llyn Brenig	Wales	07/08/2017	09/08/2017
Minnygryle	Scotland	07/08/2017	10/08/2017
Billholm	Scotland	08/08/2017	10/08/2017
Hewisbridge	Scotland	08/08/2017	11/08/2017
Blazehill	Scotland	08/08/2017	11/08/2017
Kirtleton	Scotland	08/08/2017	11/08/2017

*UK country.

After transplanting the rooted material to pots, all material was grown in a Constant Temperature Room at 20°C, controlled by a Clwyd Refrigeration Ltd (Conwy, UK) system, at ambient humidity with a 16 h daylight under artificial light (cool white fluorescent tubes 4000K) conditions and watered as necessary. The plants were grown in peat (0-14mm 65% 6-12mm 35%). Watering was kept constant, and the soil in the pots was kept well hydrated. The exception to the growing conditions happened when the plants suffered a prolonged (3 day) heat event in January 2018 because the temperature control in the growth room broke over the weekend.

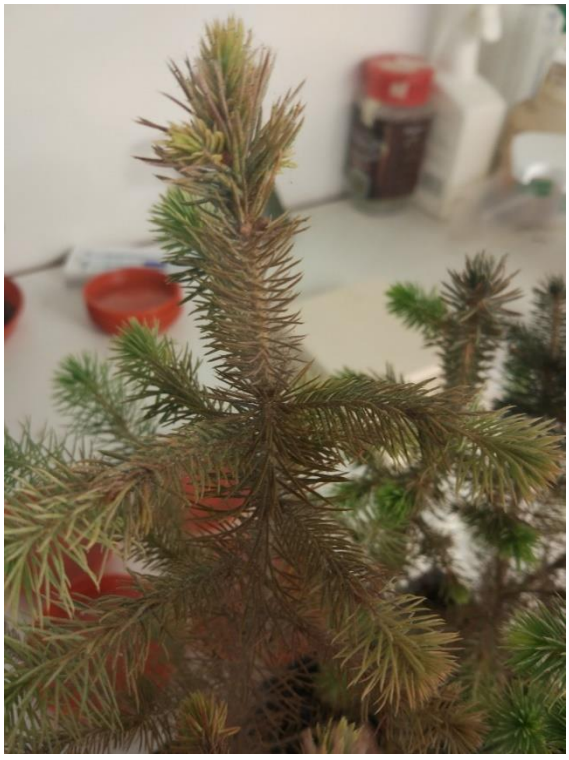

Figure 5.5 The aphid infestation severely damaged many of the young Sitka spruce (*Picea sitchensis* [Bong.] Carr.) seedlings and stecklings. Aphids are visible as small black dots at the crown, with the fungal infection visible by the whitish film on the stem. The discoloration of needles, which acquired a yellow-brownish colour, indicate the severity of damage.

Material for EL analysis was collected two-three months after flushing, once fresh branches had grown to 5 cm, and branches of that length were cut. Material was only collected from healthy-looking branches, not infected ones. Needles from these branches were collected and mixed, to get a uniform mix of needles of all parts of the branch tested. Visibly damaged needles were discarded, only intact whole needles were used.

Table 5.4. Number of genotypes from each forest left for rooting in the growth chamber or in the greenhouse.

Site Name	Growth Chamber		Greenhouse	
	Damaged	Undamaged	Damaged	Undamaged
Billholm	9	5	1	5
Blazehill	6	8	4	2
Hewisbridge	10	9	0	1
Kirtleton	7	10	3	0
Llyn Brenig	8	6	2	4
Minnygryle	6	8	4	2

2.2.2. Commercial full sibs and half sibs

In addition to the material collected from the field, four different individuals of six commercial varieties provided by our company partner, Maelor Forest Nurseries Ltd., of seed orchard Sitka spruce trees were tested (sold commercially under the names A12, A13, A17, A18, A21, A22). The naming conventions used for these samples can be seen in Table 5. These commercial varieties were most commonly sold by the nursery, including in the forests that were examined for which we have the documentation (Table 5.1). These were seedlings around 50 cm height, grown in a controlled environment room at 20°C, ambient humidity with a 16 h daylight under artificial light (cool white fluorescent tubes 4000K) conditions and frequently watered to maintain a constant soil moisture. The plants were grown in peat (0-14 mm 65%, 6-12 mm 35%). In addition to the full sibs, 5 different individuals of two different Sitka spruce origins, Washington and Queen Charlotte Islands/Haida Gwaii (QCI), which were used to breed the commercial varieties, were tested (Table 5.5). These individuals were about 70 cm in height and were grown in the same conditions as the commercial varieties.

In an attempt to test the heritability of frost tolerance 5 individual full sibs plus their 6 parental trees were also phenotyped for frost tolerance (Table 5.5). These were rooted cuttings of 20 cm in height, rooted by our company partner Maelor Forest Nurseries.

Due to a spider mite infestation five months after potting, the plants had to be treated by the application of insecticide lambda cyhalothrin at 0.05 g L^{-1} (Hallmark Zeon at 0.5 mL L^{-1} dilution) and chlorothalonil at 2.5 g L^{-1} (Bravo 500 at 5 mL L^{-1} dilution).

Material was collected two months after flushing, once branches had grown by 5 cm. All needles from these branches were collected and mixed, to get a uniform mix of needles from all parts of the branch tested. Visibly damaged needles were discarded, only intact whole needles were used.

Mature needles from a control tree that grows in Henfaes Research Centre, Bangor University's field research centre, selected due to convenience of obtaining material, were used as a control, by collecting a branch of old growth every time the EL was conducted, from a Sitka spruce tree. The branch was kept in water for two weeks, at which point a fresh branch would be collected.

2.2.3. Naming conventions

In order to keep track of the different biological samples, rooted cuttings and seedlings were named with a system that distinguished between the samples while keeping the naming system short enough to fit on a 1.5 ml Eppendorf tube. For the samples collected from the field, the naming conventions in Table 5.2 were used. The list of all samples collected from the field used for freeze testing and EL analysis can be seen in Table 5.6. The reason why the numbering of tested samples is so different is because names were assigned at collection and kept throughout the experiment, and only those that rooted and survived were tested on.

For the commercial species, alphabet characters were added to the name, to separate those that came from the same origin/full sib family. For example, the four different samples of the commercial full sib species A12 were named A12A, A12B, A12C and A12D (Table 5.5). The five full sibs were named 1, 2, 3, 4, and 5, whereas their parents were named A, B, C, D, E and F.

Table 5.5. Explanation of the naming conventions used for commercial varieties.

Variety	Individual	Sample name (example)
Commercial seed orchard variety (half-sib)		
A12	A-H	A12A
A13	A-H	A13A
A17	A-H	A17A
A18	A-H	A18A
A21	A-H	A21A
A22	A-H	A22A
Full sibs		
1	-	1
2	-	2
3	-	3
4	-	4
5	-	5
Parentals of the full sibs		
A	-	A
B	-	B
C	-	C
D	-	D
E	-	E
F	-	F
Wild origins		
DK4*	A-H	DK4A
QCI	A-H	QCIA

*Name used for the Washington variety, which came to the UK via importation from Denmark.

Table 5.6. List of samples collected from the field that were freeze tested after rooting.

Name*	Damage level	Forest
BHO8	Undamaged	Billholm
BZO10	Undamaged	Blazehill
BZO9		
BZX1	Damaged	
BZX10		
BZX7		
BZX8		
BZX9		
HO1	Undamaged	Hewisbridge
HO10		
HO3		
HO7		
HO9		
HX10	Damaged	
KO9	Undamaged	Kirtleton
KX2	Damaged	
KX5		
KX7		
KX9		
M010	Undamaged	Minnygryle
MO5		
MO9		
MX1	Damaged	
MX10		
MX5		
MX7		
MX8		
MX9		
WO10	Undamaged	Llyn Brenig
WO3		
WO4		
WO8		
WO9		
WX1	Damaged	
WX6		
WX8		

*Naming system explained in Table 5.2.

2.3. Freezing treatment

The frost tolerance quantification by rate of electrolyte leakage method by Murray et al., (1989) was used for the measurement of frost tolerance levels. This method was chosen because it was a widely cited method used by many researchers studying frost tolerance of trees, and the simplicity of the method.

Needles were placed in 1.5 mL Eppendorf tubes at a rate of 10 needles per tube. 5 technical replicates (Eppendorf tubes) were used for each treatment, placed in a vacuum flask, to a total of five separate vacuum flasks. It took around three hours to prepare each batch of needle samples for freezing experiments. This was done the day before the samples were exposed to the freezing treatment. Five replicates for each test temperature were placed in each vacuum flask, with five separate flasks, one for each temperature treatment. The flasks were placed in a cold room kept by a cooling system made by Clwyd Refrigeration Ltd (Conwy, UK) at +2 °C overnight (Figure 5.6).

After overnight cooling at 2 °C in the cold room, all the vacuum flasks except the 2 °C control treatment were transferred to a -20 °C Proline (Comet, UK) standing freezer and taken out when the temperature inside the flasks was estimated to have reached the treatment temperature (Figures 6 and 7). The estimation was made by measuring the rate of cooling using the vacuum flasks at the -20 °C temperature and calculating the amount of time it would take a sample at +2 °C to cool to the desired temperature. Once the estimated time had passed, the samples were taken out of the freezer and placed to thaw at a rate not exceeding 5K h^{-1} at the +2 °C cooling room. This method was accurate enough to reach and keep the desired treatment temperatures ± 1 °C for a 30 min period inside the vacuum flask, as measured by the iButton data logger (Figures 6 and 7). Four freezing treatments, flash exposures (which lasted around 30 min) to temperatures of -3 °C, -6 °C, -10 °C, -20 °C, were used. If the treatment temperature went below the desired temperature as measured by the iButton datalogger by more than 1 °C, the samples were discarded.

Two different types of vacuum flasks (stainless steel Dewar flasks, as used for liquid nitrogen, and off-the shelf vacuum flasks (George brand) from the supermarket chain Asda (Leeds, UK) were tested to find out which gave the optimum the rate of freezing. For the main experiment the rate of cooling and thawing was slowed more by using the Asda vacuum flasks, which slowed the rate of freezing from 120 K h^{-1} to 5 K h^{-1}.

The rate of cooling inside the flasks was measured with an iButton (Thermochron, Wisconsin, USA) temperature data logger, placed inside the vacuum flask at the same time as the Eppendorf tubes with the needles. The iButton was configured to measure and record the temperature every five minutes.

2.4. Electrolyte leakage tests

After freezing treatment of needles, 1.5 mL of miliQ water, filtered with the Millipore Direct-Q 3 purification system (Merck, Darmstad, Germany), were added to each Eppendorf tube, after which conductivity was measured with a Jenway 4520 Conductivity Meter (Cole-Parmer, Stone, UK) used with a Jenway Micro-volume Conductivity probe. Hourly measurements were made for the first 5 hours, after which a final measurement was made at 24 h. Time was calculated from the moment the water was added, once all samples were thawn.

As the conductivity of the samples was measured every hour, and each individual measurement took an estimated minute, no more than two genotypes per day could be analysed, as fifty replicates would take an estimated 50 min. In order to verify that the treatments were identical across samples, the matured needles used as a control were used in multiple occasions, to verify there were no changes due to differences between days.

After the final measurement, the samples were autoclaved for 30 minutes at 121 °C. The total conductivity level was then obtained by measuring the level of conductivity in the autoclaved sample. Relative Electrolyte Leakage (REL) was calculated as the ratio of the electroconductivity (measured in µS, micro Siemens, a Siemens being equivalent to $s^3 \cdot A^2 \cdot kg^{-1} \cdot m^{-2}$) at a time point to total electroconductivity after autoclaving ($\mu S\ \mu S^{-1}_{TOTAL}$).

2.5. Data analysis

In order to analyse data, R 3.6.3 (R Foundation for Statistical Computing and R Core Team, 2019) with the RStudio IDE 1.3.959 (RStudio Team, 2019) was used. In order to determine whether the data distribution was normal, density plots were drawn using the e1071 1.7.3. R package (Meyer et al., 2019), and qqplots using the ggplot2 3.3.1. R package (Wickham et al., 2020). Homoscedasticity was checked with a Levene's test with the leveneTest() function in the car 3.0-8 R package (Fox et al., 2020). The same procedure was used to check the normality and homoscedasticity of all datasets. ANOVA comparisons were conducted using the standard aov() function in R. For data that did not follow a normal distribution or did not have equal variance across groups, the non-parametric Kruskal-Wallis H test was used to compare the indices by different factors, with a post-hoc Bonferroni-Dunn test used for pairwise comparisons, using the PMCMRplus 1.4.4 R package (Pohlert, 2020). Interactions between factors were analysed by using an extension to the Kruskal-Wallis H test, the Scheirer-Ray-Hare test, using the rcompanion 2.3.27 R package (Mangiafico, 2021).

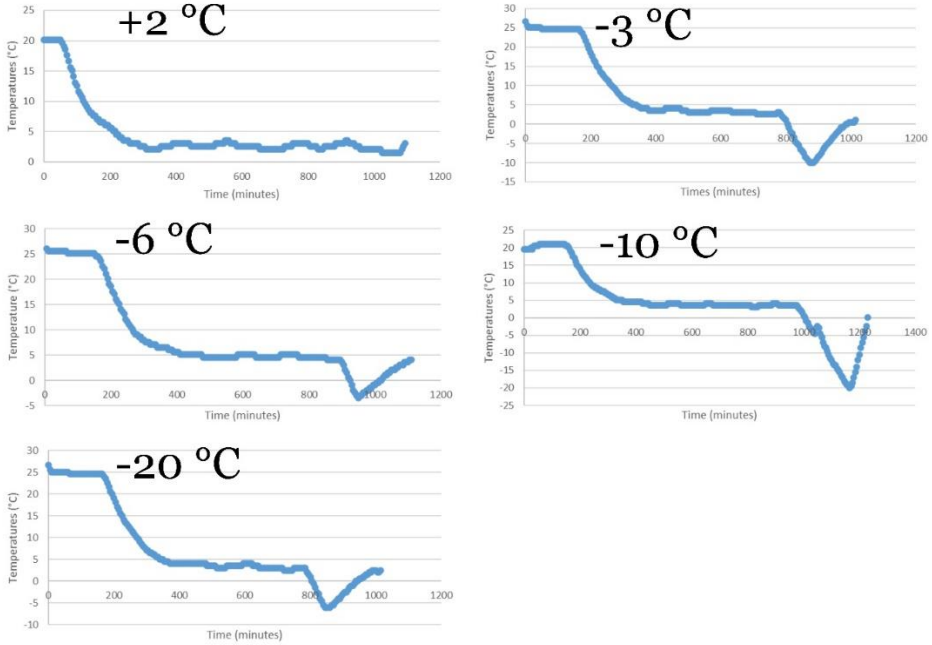

Figure 5.6. Time curve of temperatures for the treatments after the collection of needles, from the time they were placed in the cold room to the moment they finished thawing. From top to bottom, left to right: time curve for the +2 °C control and the -3 °C, -6 °C, -10 °C and -20 °C treatments. Representative example of one experiment. All iButton logs for the different dates look similar to this one, as the length of the overnight placement of samples varied day by day.

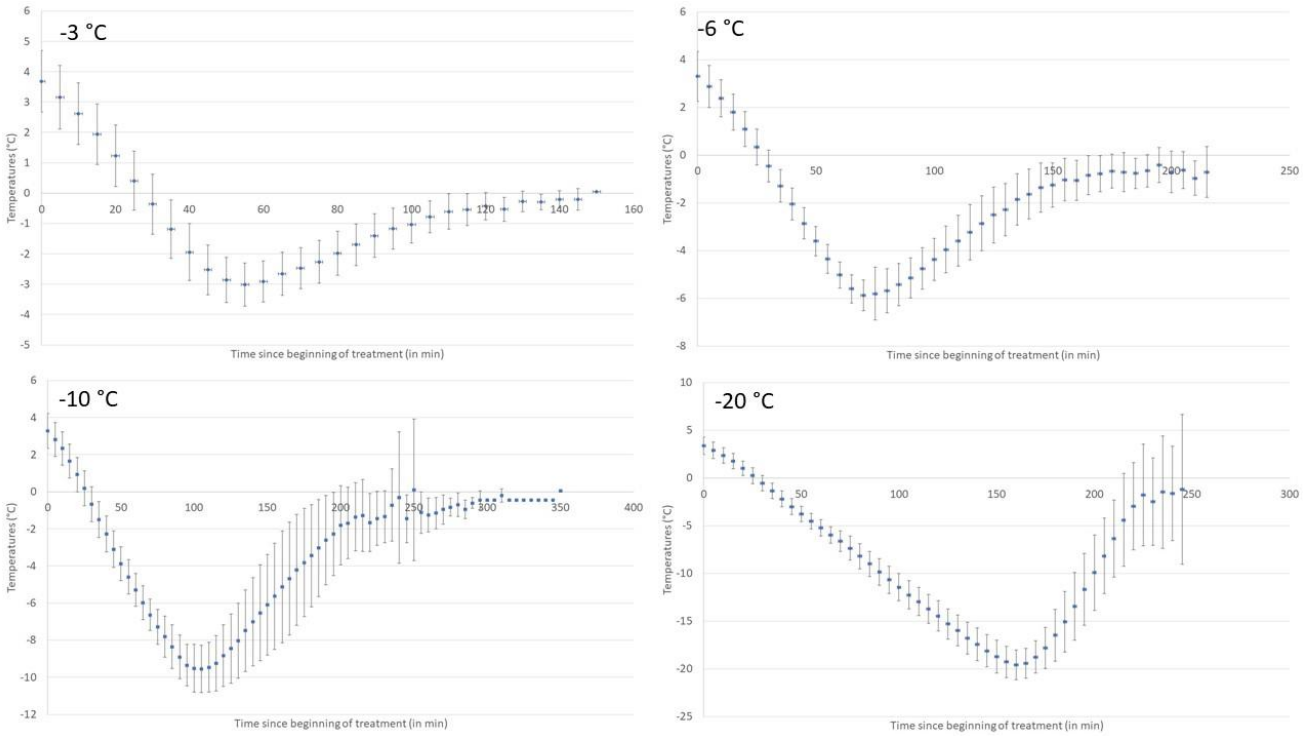

Figure 5.7. Time curve of temperatures with time for the treatment -3 °C, -6 °C, -10 °C, -20 °C, after the transfer of the vacuum flasks from the cold room to the freezer. Average of all experiments, with error bars indicating standard deviation.

2.5.1. Field data analysis

Data from the field observations was examined to determine whether there was an environmental factor that affected the degree of damage. Since the data did not have equal variance across groups, three separate Kruskal-Wallis tests were run with the approximate level of damage as the dependent variable and direction, altitude and slope as independent factors.

2.5.2. Rooting data analysis

After checking whether the data for rooting success followed a normal distribution, the data was log-transformed so it would conform to a normal distribution. The success of rooting by the growth conditions used for rooting was compared with a one-way ANOVA, using the aov() function in R, using the growth conditions as the independent variable and the growing success as the dependent variable. The rooting success by the forest from which the sample was collected and whether the sample was damaged or undamaged was also studied, with rooting success as the dependent variable and the forest and degree of damage of the original sample as the independent variable.

2.5.3. Electrolyte leakage data analysis

2.5.3.1. Calculating the rate of electrolyte leakage

The data for electrolyte leakage relative to time did not follow a normal distribution, but the method of ordinary least squares does not require normality for regression.

The methodology used in this analysis was taken from Murray et al. (1989), who suggest using the model in equation 1 for the loss of tissue electrolytes when added to water. In this equation, Q is the total electrolyte content of the tissue at time t, *k* is the first-order rate constant with unit time^{-1}, while Q_0 is the initial electrolyte content. The change in cell electrolyte content at time t, which would be equal to the change in the water electrolyte content at time t (since the electrolytes go from the tissue to the water), can then be calculated by equation 2. The proportion of change of electrolyte content Q_0 - Q, to the total initial electrolyte content, Q_0, can be seen in equation 3. Since autoclaving the solution will get most of the initial electrolyte content into the solution, it is presumed that the relation of the conductivity of the water at time t, C_t, to the conductivity of water after autoclaving, C_{auto}, will be equal to the rate of the value of electrolyte loss of the tissue to the total initial electrolyte content, equation 4. The proportion of C_t to C_{auto} will be called Relative Electrolyte Leakage (REL) (equation 5).

$$Q = Q_0 e^{-kt} \quad (3)$$

$$Q - Q_0 = Q_0 e^{-kt} - Q_0 \quad (4)$$

$$\frac{Q_0 - Q}{Q_0} = (1 - e^{-kt}) \quad (5)$$

$$\frac{C_t}{C_{auto}} = \frac{Q_0 - Q}{Q_0} = (1 - e^{-kt}) \quad (6)$$

In order to analyse the non-linear model, which could not be analysed in R using the nonlinear model function, nlm(), the model was linearized by a log transformation into equation 6.

$$REL = (1 - e^{-kt}) \quad (7)$$

$$\ln(1 - REL) = -kt \quad (8)$$

This model was compared to two other models for fit, evaluating how many of the total number of genotypes fit the model. Equation 7 shows a simple linear model of REL in relation to parameter t, whereas equation 8 uses the Michaelis-Menten model.

$$REL = a + bt \quad (9)$$

$$REL = \frac{at}{b + t} \quad (10)$$

Each biological sample was fitted to a curve that calculated the relationship between REL and time, to calculate the parameter of the rate of electrolyte leakage change with time. The models in equations 6, 7 and 8 were evaluated for fit. The model which fit the most biological samples best (an R^2 above 0.4 and a p value < 0.05) was selected. The same model had to be used for all biological samples so the value of the electrolyte leakage rate could be compared across samples. The value of electrolyte leakage would then be compared between samples and be used as a measure of frost hardiness.

2.5.3.2. Comparing samples

As the data did not have equal variance across groups, a Kruskal-Wallis test was run with the slopes, *k*, calculated by equation 6 in section 2.5.3.1, as the dependent variable and the treatment as the independent variable. The distribution of slopes respective to the genotype did not follow a normal distribution but could be log-transformed to follow a normal distribution. Since the variance between the groups was equal, ANOVA analyses were performed with *k* as the dependent variable and genotype, site of material collection and degree of damage of collected material as independent variables. A post-hoc Tukey's HSD test using the HSD.test() function in R was run on the data of ANOVA slope by genotype and treatment to see the comparisons between the genotypes and the treatments. The distribution of REL across samples was bimodal and could not be transformed into a normal distribution. Samples were thus compared with a Kruskal-Wallis test with REL at 24 h as the dependent variable and genotype and treatment as independent variables. Interactions were analysed with the Scheirer-Ray-Hare test. For the subset of data that came from rooted field material, Kruskal-Wallis tests with the REL at 24 h as the dependent variable and the forest from which the sample was collected and the degree of damage as the independent variable were run. A post-hoc Bonferroni-Dunn test was run on the data of the Kruskal-Wallis REL 24 h by genotype, treatment, and forest from which the material was collected to see the comparisons between the genotypes, treatments, and site of material collection.

2.5.4. Figures

After calculating the rate of REL change with time, k, for each genotype, the values for each individual genotype were drawn in a bar graph. All three groups of genotypes mentioned in section 2.2. were drawn into separate figures, and all three included the control sample which consisted of mature needles. The genotypes were ordered in the order of the rate of change in REL at -20 °C. This ordering was used to maintain the same ordering in all charts so they could be visually compared.

3. Results

3.1. Field observations

No obvious differences in environmental conditions between damaged and undamaged trees, such as frost hollows, could be observed. Differences in damage patterns did not seem to be caused by microsite variation, either, as trees that were located in close proximity to each other exhibited different degrees of damage (Figure 5.8). There was no significant effect of slope ($p = 0.32$), direction ($p = 0.5$) or elevation ($p = 0.52$) on the degree of damage.

Figure 5.8. Example of field observations in Hewisbridge forest the summer of 2017, comparing a frost damaged (right) and undamaged Sitka spruce tree (left), both in close enough proximity to each other to discard environmental variation as the main reason for the difference.

3.2. Rooting and growing

The distribution of rooting success by genotype did not follow a normal distribution and a log transform was used. The ANOVA test shows no significant difference between the rooting success of samples collected from different forests ($p = 0.3$) or between samples collected from damaged or undamaged trees ($p = 0.81$). The technique used for rooting had a significant effect on rooting success ($p < 0.01$). A much higher proportion of cuttings rooted in the greenhouse than in the growth chamber, despite the higher number of samples placed in the growth chamber (Table 5.7). However, many of the rooted field samples did not later survive to the phenotyping phase (Table 5.8).

3.3. Freezing tests and electrolyte leakage analysis

3.3.1. Rate of electrolyte leakage

When comparing the three models for the relationship of REL to time given in equations 6, 7 and 8, it was found that the exponential (equation 6) and linear (equation 7) model were quite similar in fit while the Michaelis-Menten model (equation 8) did not fit the curve at all. For the Michaelis-Menten model, the data fit the model significantly in 415 out of the 541 biological replicates, 77%. The data fit the linear model significantly in 98.9 % of the cases, with an R^2 above 0.4 in 83.3% of the cases, while the data fit for the exponential model was significant in 98.9% of the cases, with an R^2 above 0.4 in 83.7% of the cases. It was decided to use the exponential model because the data seemed to conform to a non-linear shape and most of the studies that analyse the changes in REL with time use this model. The variance of slopes was not equal between the treatments.

The Kruskal-Wallis test of the slopes, k, h^{-1} in relation to treatment also found the treatment to be a significant factor ($p < 0.001$), while the ANOVA test on the slopes in relation to the genotype found the genotype was not a significant factor ($p = 0.20$). Among field collected samples, the forest where the material was collected was not a significant factor ($p = 0.17$), and neither was the level of damage ($p = 0.59$).

The Dunn-Bonferroni test shows that both the -20 °C and the -10 °C treatments differ significantly from every other treatment ($p < 0.001$), while the -6 °C, -3 °C and +4 °C treatments do not differ from each other. This can also be observed in Figures 9, 10 and 11, where we can see that the -6 °C, -3 °C and +4 °C treatments show very similar rates of leakage.

Trees that were not damaged in the field showed a high rate of leakage, showing a lack of agreement between field observations with higher degree of damage in that group and lab experiments that found no differences between the two groups (Figure 5.9). The ordering of the bars by the rate of leakage at -20 °C also shows a lack of agreement in the ordering of the genotypes by rate of leakage at different treatments.

It was not possible to group genotypes into damaged and undamaged groups based on the rate of electrolyte leakage after the lab freezing test. Not only was there no effect of whether the sample came from the damaged or undamaged population on the slope, but there was no observable trend, either (Figure 5.9).

The lack of significance of the genotype on the rate of leakage also meant no clear differences could be detected between the different commercial seed orchard varieties of Sitka spruce, or the half sibs (Figure 5.10). There was no clear trend regarding the provenance varieties, QCI and Washington, either, with both QCI and Washington having rates of leakage at both the high and the low end.

Although the parentals and the full-sibs seem to group into two groups (Figure 5.11, Pane D), the absolute difference is too small, and not significantly different.

Table 5.7. Rooting success in the greenhouse.

Site Name	Growth Chamber				Greenhouse			
	Damaged		Undamaged		Damaged		Undamaged	
	Rooted	Not rooted	Rooted	Not rooted	Rooted	Not rooted	Rooted	Not rooted
Billholm	7	2	2	3	1	0	3	2
Blazehill	2	4	4	4	3	1	2	0
Hewisbridge	5	5	8	1	0	0	1	0
Kirtleton	7	0	8	2	2	1	0	0
Llyn Brenig	6	2	3	3	2	0	3	1
Minnygryle	6	0	5	3	3	1	2	0

Table 5.8. Number of rooted cuttings that survived to the phenotyping phase.

Forest	Damage	#Number successfully rooted	#Number survived to phenotyping
Billholm	Undamaged	5	1
	Damaged	8	0
Blazehill	Undamaged	6	2
	Damaged	5	5
Hewisbridge	Undamaged	8	5
	Damaged	3	1
Kirtleton	Undamaged	8	1
	Damaged	9	4
Llyn Brenig	Undamaged	6	4
	Damaged	8	3
Minnygryle	Undamaged	7	3
	Damaged	9	6

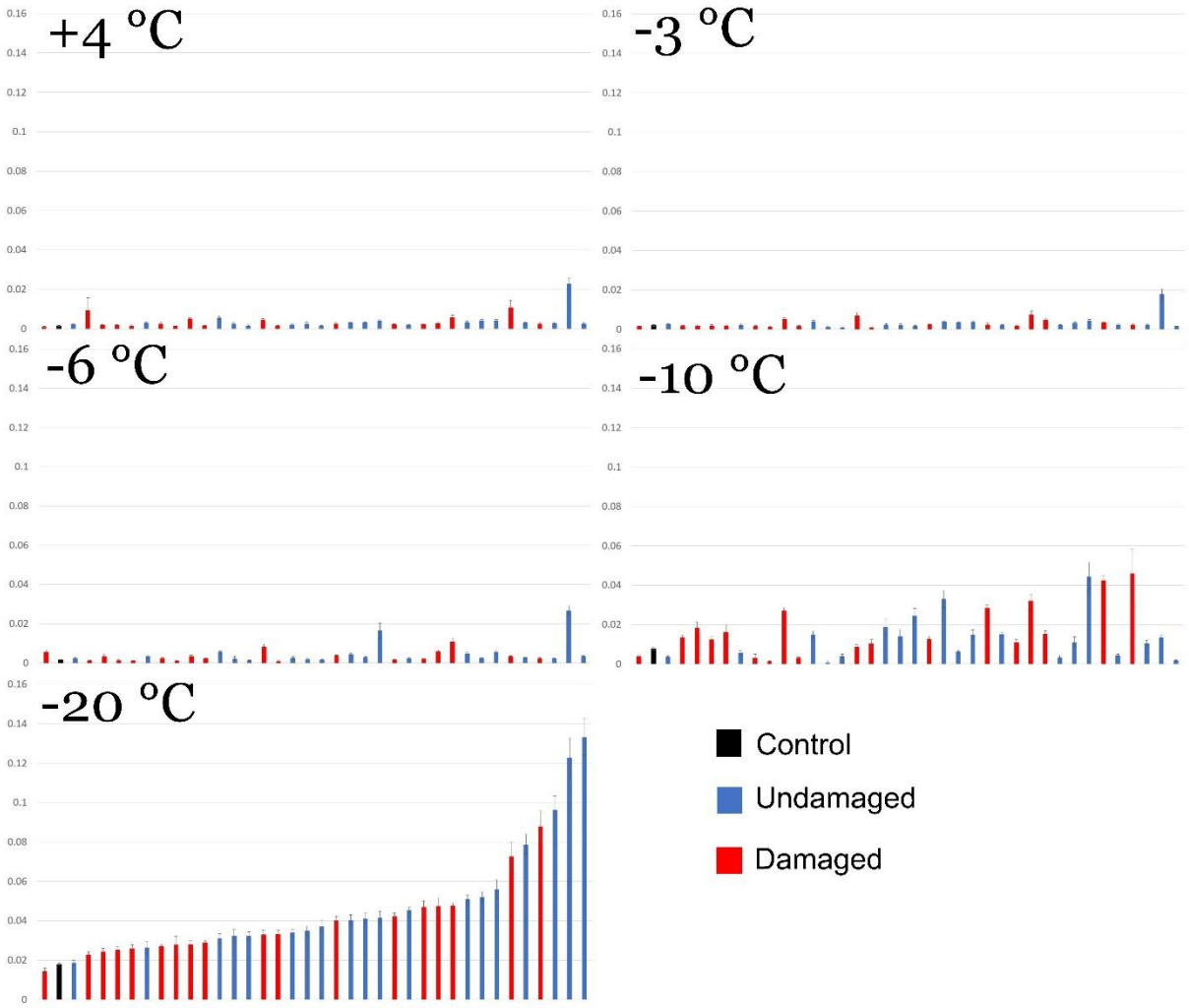

Figure 5.9. Rate of the REL (Relative Electrolyte Leakage) changes with time, *k*, fitted to equation 6. after treatment of 18 damaged and 19 undamaged genotypes of Sitka spruce collected in five different forests across the UK. Samples ordered by the slopes at -20 °C. Mature needles of a single tree were used as a control. The same control that was used in Figures 10 and 11. Order: KX7, Control, BZO9, BZX9, WX6, MX7, MX9, HO1, MX10, BZX7, HX10, BZX10, KO9,HO7, BHO8, WX8, BZX8, WO9, HO3, BZX1, KX9, HO10, WO3, WO4, WX1, BZO10, KX2, MX8, MX5, MO9, WO4, HO9, MX1, MO5, KX5, WO8, WO10 and MO10. Refer to Tables 4 and 5 for the naming system.

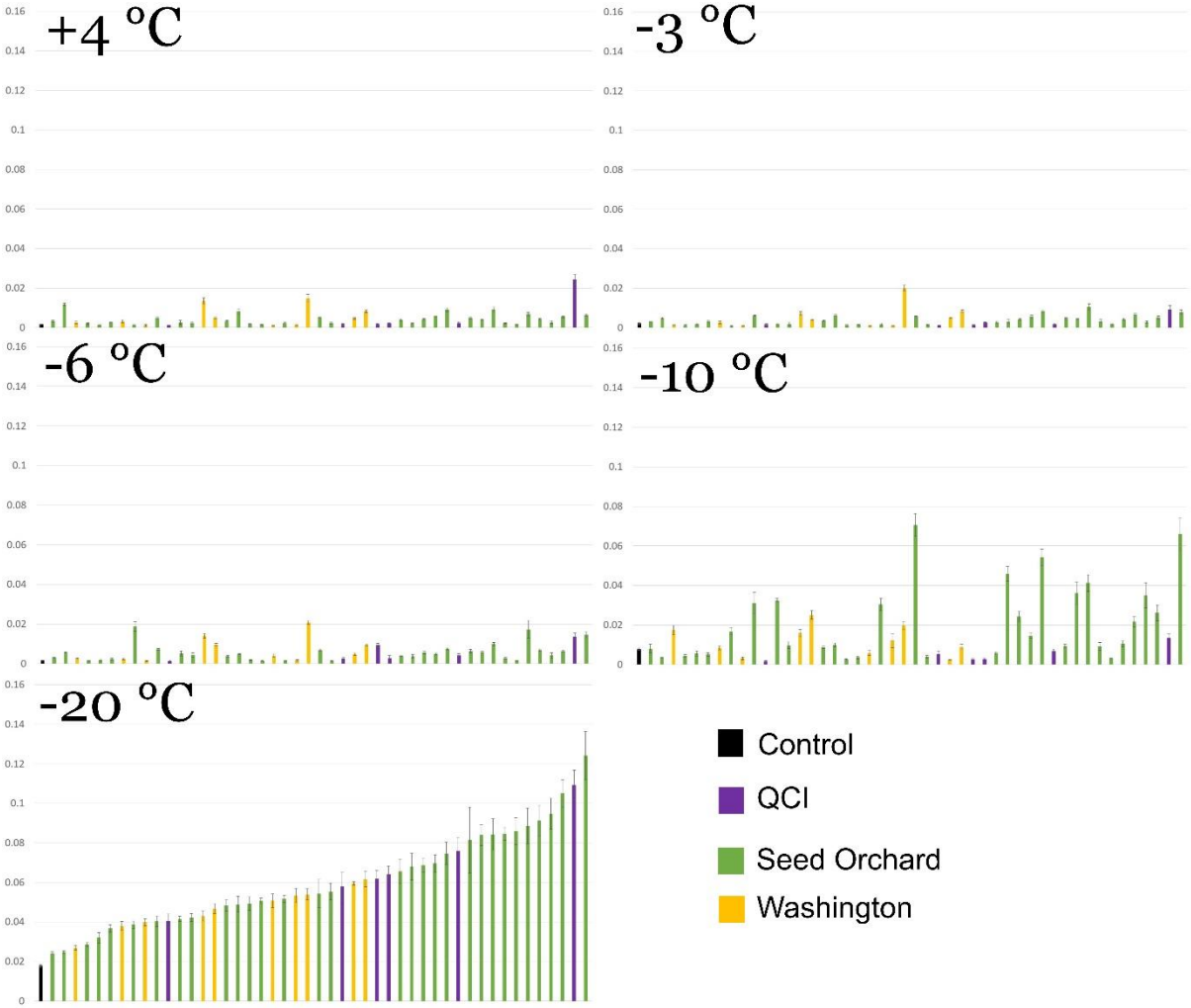

Figure 5.10. Slopes of REL (Relative Electrolyte Leakage) changes with time after treatment of different individual genotypes of several commercial seed orchard Sitka spruce lines, as well as wild origin trees of Washington and QCI/Haida Gwaii origin. Samples ordered by the slopes at -20 °C. Mature needles of a single tree were used as a control. The same control that was used in Figures 9 and 11. Order: Control, A13B, A22B, QCIJ, A17H, A12D, A17G, QCIK, A22E, QCIE, A17A, DK4D, A13E, QCIC, A13A, A21B, A22C, A22D, QCIA, A13G, QCIG, QCIB, A13C, A17D, DK4B, QCIH, QCID, DK4C, DK4F, A18A, A21A, A22A, A12C, A12B, DK4E, A17C, A21D, A21E, A18D, A13D, A21C, A18B, A18C, A17C, A17B, D4A and A12A. Refer to Tables 4 and 5 for the naming system.

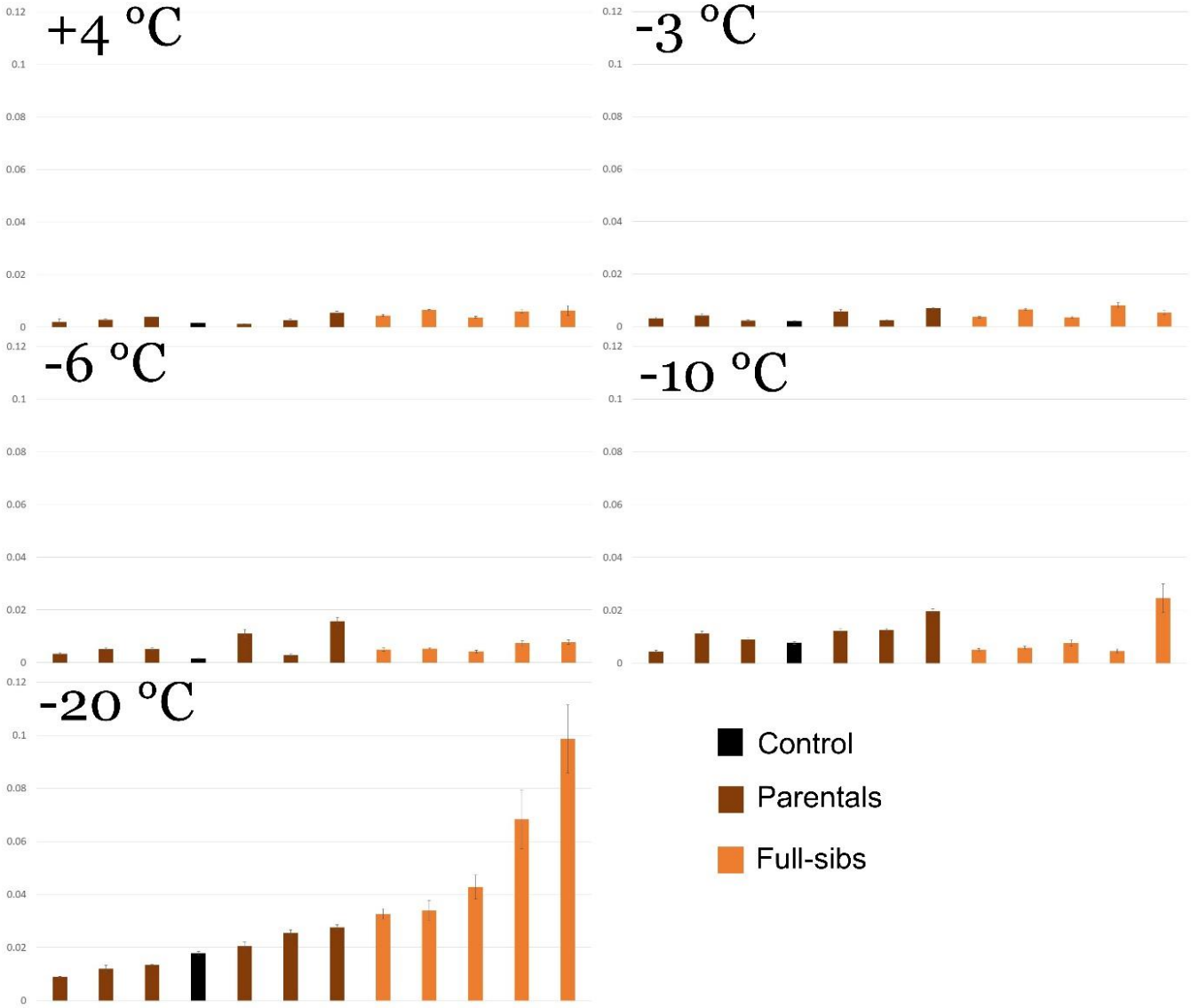

Figure 5.11. Slopes of REL (Relative Electrolyte Leakage) changes with time after treatment of individual full sib genotypes of Sitka spruce, as well as their parents. Samples ordered by the slopes at -20 °C. Mature needles of a single tree were used as a control. The same control that was used in Figures 9 and 10. Order: D, A, F, Control, C, B, E, 4, 2, 1, 5 and 3. Refer to Tables 2 and 5 for the naming system.

3.3.2. Differences in REL

A Kruskal-Wallis test showed that the genotype and the treatment are significant factors that affect REL at 24 h ($p < 0.001$). The Scheirer-Ray-Hare test shows no significant genotype : treatment interaction ($p = 0.78$).

The treatments at the -20 °C and the -10 °C were significantly different from every other treatment ($p < 0.001$), as shown by the post-hoc Dunn-Bonferroni test. The -6 °C, -3 °C and +4 °C treatments do not differ from each other, with the exception of the -6 °C and the -3 °C treatment, which differ significantly from each other ($p < 0.01$).

As for the comparisons between individual genotypes, the Dunn-Bonferroni test showed no significant difference between most genotypes at REL 24 h. However, there were differences between some samples collected in different forests; WO10, collected in Llyn Brenig, differed significantly ($p < 0.05$) in REL at 24 h from BH08, BZ09, BZX1, BZX10, BZX7, KX2, KX7, MO10, MO9, MX0, and MX9, while BZX7 differed significantly from MX8. It can be noted that WO10, the second from the right bar in each pane, seems to differ from the others in REL 24 h at treatments at +4 °C, -3 °C and -6 °C. This seems to parallel the similar trend in slope differences for WO10 at +4 °C, -3 °C and -6 °C, although the level of difference does not reach significance in this case. There were other significantly different pairs between samples collected from forests, commercial seed varieties and origins.

Commercial seed orchard varieties varied wildly, and many pairs were significantly different from each other in the REL 24 h value. There were significant differences even within pairs from the same commercial variety, although the different seed orchard variety could not be grouped separately based on cultivar. Washington and QCI origin trees differed from other samples in their REL 24 h value, with many significantly different pairs. No general trend could be observed in REL 24 h. Although there is a slight trend for the Washington origin trees to have lower electrolyte leakage rate values than QCI, this trend did not rise to the level of significance.

Neither full sibs nor their parentals differed significantly from each other in REL 24 h. Full sibs 5 and 3 differed significantly from the control ($p < 0.05$), and it can be observed that these two have the highest values for the rate of electrolyte leakage.

Electrolyte leakage rates did not in most cases differ from the control.

For the subset of the data that came from field-collected material, there was a significant effect of the forest from which the material was collected ($p < 0.001$), as well as the degree of damage ($p < 0.05$). There was also a significant interaction between the forest and tolerance ($p < 0.001$), although the Scheirer-Ray-Hare test shows the forest as a significant factor ($p < 0.001$) while frost tolerance was not a significant factor according to the test ($p = 0.32$).

More pairs of forests were significantly different on their REL at 24 h value, as shown by the Dunn-Bonferroni post-hoc test. Billholm differed significantly from both Hewisbridge ($p < 0.01$) and Llyn Brenig ($p < 0.01$); Blazehill differed from Hewisbridge, Llyn Brenig and Minnygryle ($p < 0.001$). No other pair differed from each other.

4. Discussion

4.1. Field observations

The environmental factors in the five forests that were examined, were not associated with the observed frost damage. It should be noted that the estimates to the degree of damage were made by a visual % estimation by experienced forest managers, not by counting the number of trees damaged as a % of the overall population. A more systematic approach could have found environmental factors across different forests that cause the frost damage, although, as it was noted in the introduction, field studies are notoriously costly, and proxies are used most of the time. The lack of clear environmental causes for the frost damage, both the ones that were directly measured and those that could be observed visually, indicated that there could be underlying genetic differences between the trees that caused the differences in the pattern of observed frost damage.

It should be noted that field observation studies require multiple observations over several years, as was done in field observation studies by Barney et al. (2013). Differences in frost damage caused by sporadic occurrences of frosts could be caused by underlying environmental or genetic differences. In order to distinguish between environmental and genetic factors, experiments need to be conducted in either controlled conditions or genetic duplicates should be planted in different sites and observations over different years should be collected, in order to distinguish between the roles genetics and the different environmental factors play.

4.2. Rooting

The original design sampled 120 trees in total, 60 damaged and 60 undamaged, from six different forests. In order to grow viable plants from each tree sample in uniform conditions from which to take needles for EL analysis, branches collected from the field were potted and placed under two contrasting conditions to encourage rooting. It was observed that the conditions in the growth chamber were sub-optimal since the level of successful rooting was lower than the plants placed in the greenhouse for rooting. This was probably caused by sub-optimal light in the growth chamber, as it is extremely hard to replicate natural sunlight artificially.

Subsequent problems, such as an aphid infestation, further reduced the pool of plants available for experimentation (Table 5.8). It is advisable to preventively spray the plants to avoid any similar

incidents in future work. Due to these setbacks a final sub-set of only 37 individual trees were available for experimentation, however this sub-set were evenly split according to damaged and undamaged.

Rooting might not be the optimal strategy to obtain plants from field collected branches. Even after successful rooting (a small, 1 cm long root growing out of the cutting in 4 months), it takes a very long time to grow from that initial length (3-4 months to get a cm long root), and even more time to establish themselves and provide optimum nutrition and water to the plant. Furthermore, the rate of rooting is not very high. In the study on frost tolerance by Sheppard et al. (1994) mature Sitka spruce branches were grafted into rootstock. It took 8 months for the Sitka cuttings to be re-potted in that study, after which they were overwintered for another 8 months until they were experimented on. There does not seem to be much of a time saving between rooting and grafting, so the decision of which technique to use should be based on the material conditions and the skills available to the researchers. There is a lot of skill and knowledge to both grafting and rooting which need to be acquired from more experienced growers, as a lot of it cannot be well described in text form.

4.3. Electrolyte leakage phenotyping

4.3.1. Data structure

Three different models, described in equations 5 (log-transformed into equation 6), 7 and 8 were tested for their fit to the REL in respect to time data. The linear (equation 7) and exponential (equation 6) model fit the data similarly well, as described in the results, with the Michaelis-Menten equation (equation 8) fitting the data poorly.

This study uses the frost tolerance measuring technique by (Murray et al., 1989), who use the exponential model (equation 5) to calculate the rate of leakage. Multiple other studies that measure frost tolerance also use this model to calculate the rate of electrolyte leakage, such as Wulff et al. (1994); Sheppard et al. (2003), who use REL measurements at 1 h, 24 h and 120 h; Sheppard et al. (1994), with REL measurements at 24 h and 120 h, or Sheppard et al. (1997), who use measurements of REL at 120 h. Although there is little difference in data fit between the exponential and linear model, the exponential model was selected because it was used more widely. Both the linear and exponential model fit the data much better (98.9 % of samples with significant fit vs. 77%).

This study used measurements at 0 h, 1 h, 2 h, 3 h, 4 h, 5 h and 24 h to produce sufficient data to calculate the rate of electrolyte leakage, k, in a short time frame. Extending the time frame would add complications, such as the rotting of the needles in water. The highest changes in EL, that is, the highest rate, is achieved in the first hours, with not much change between the 24 h and 48 h mark (pre-test result, data not available).

However, this setup could have been improved if fewer measurements had been taken more widely spaced out and spread over a longer time period (up to a week in refrigerated conditions, the time at

which the sample would begin to rot). This would increase the number of samples that could be tested, as the requirement to measure the electrolyte leakage hourly decreased the number of samples to those that could be tested in an hour.

4.3.2. Comparisons in rates of EL

The trees that were damaged and undamaged in the field by frost seemed to be equally likely to show either high or low rate of electrolyte leakage (Figure 5.9). As can be observed in Figure 5.9, both damaged and undamaged samples appeared at both ends of the distribution, with no apparent grouping. There did not seem to be any agreement between field frost damage and the lab EL test. There are three possible reasons for this lack of agreement: i) damage in the field is due mainly to environmental factors, and once rooted cuttings are grown in uniform environmental conditions, these environmental differences disappear; ii) the lab test does not simulate the same physiological processes that influence the field results; iii) the number of samples used was too small and noisy to find a correlation. In addition, there could be a combination of all three reasons, hence further studies should be designed to take them all into account.

While there was no agreement between field and lab results, and differences between genotypes were hard to classify, there were significant differences in damage between the different temperature treatments. This was confirmed by the ANOVA test, which showed treatment to be a significant factor affecting both the rate of electrolyte leakage, k, h^{-1}, and REL at 24 h. This shows that, while the lab experiment failed at finding similarity between field and lab results, lab based frost tolerance measurement did identify differences in frost tolerance, as the differences in treatment went in the direction that was predicted *a priori* (*i.e.*, lower temperatures would lead to a higher degree of damage).

4.3.3. Limitations of phenotyping methods

The small sizes of the rooted trees provided a limited amount of fresh material for collecting the necessary number of branches (25 in total, 5 replicates for each treatment and 5 temperature treatments). Most studies that measure frost tolerance use branches (Chapter 4), and there are good reasons for that, since a branch has all the tissues that may suffer from frost: needles, stems, and (if present at the time of the year) buds. Whole plant tests were rarely used for Sitka spruce studies, although they might give a closer approximation to real world results. Needles were used in our study, despite this being the less commonly studied type of sample in most studies (Table 5.8) to enable replication and due to the lack of sufficient plant material, as well as the lack of suitable programmable freezing units that could be used to test whole plants.

Most studies used a programmable freezing cabinet or some other type of programmable freezing equipment to adequately control the freezing rate. It could be observed that a lot more care was

exerted by the experimenters on controlling the freezing rate, slowing it to 5 K h^{-1}, than the thawing rate 10 K h^{-1}. If programmable freezing equipment that could control the freezing rate, exposure time and thawing rate was available, that would have been most advisable to use. However, we did not have such equipment available, and there was not sufficient funding to acquire it. Hence, attempts were made to control the freezing rate by using a thermo flask, and cooling from 2 °C instead of room temperature, reducing the temperature differential. This was successful in reducing the freezing rate to 5 K h^{-1} (Figures 6 and 7). Thawing rate was also slowed by thawing samples at 2 °C.

While most studies used a long exposure time to measure frost tolerance in Sitka spruce, and it would be best to use a longer time than the flash exposure used in this study, as it is highly unlikely frost events in the field would only last 15 minutes, such exposure length are only possible in programmable equipment that can be set to a certain temperature. That is why in this study flash exposure, lasting 15 min at the target temperature ± 1 °C was used, even though this might not be the most optimal exposure time.

The lack of detected significant differences in frost tolerance between both individual genotypes and Sitka spruce varieties could be because of problems in the study design, such as insufficient replication, inadequate sampling, not using the appropriate exposure time and not using the right treatment temperatures. It could also be because the growing conditions severely affected all trees removing some of the detected differences. Previous stresses, such as the aphid infestation the plants suffered from can affect membrane properties (Bigras and Colombo, 2001).

Increasing the number of tested phenotypes that have been genotyped could improve the data available if time and funding was not a constraint.

4.3.3.1. Effects of soil and nutrition

The samples were all grown in the same type of soil, with the same light and the same watering regime. This would remove differences in frost tolerance caused by environmental factors such as soil nutrients. Many nutrients, such as Cu, Mg, P, N affected the frost hardiness of Norway spruce (Jonsson et al., 2001). Nitrogen is a very important nutrient that affects plant growth and height as well as frost tolerance (Fløistad and Kohmann, 2004). Nitrogen has an effect at lower levels of supplementation, while an excess of N seems to have a negative effect on frost tolerance in Norway spruce.

Differences in the field could be caused by different levels of nutrients or micronutrients in the soil, or by different levels of irrigation. The level of hydration is very important for frost tolerance. Slightly lower levels of hydration in needles improved frost hardiness in white spruce (*Picea glauca* (Moench) Voss) and yellow pine (*Pinus banksiana* Lamb.) (Kedrowski, 1980). Long-term frost tolerance, however, is worsened with water stress, as carbohydrates get depleted faster due to the stress.

4.3.3.2. Field studies vs lab measurements

The greatest number of the studies found in the systematic search (Chapter 4) used field-grown material for the measurement of frost tolerance after artificial chilling in laboratory conditions. The present study used 37 plants grown from cuttings taken from field-grown trees that were identified as tolerant and not tolerant to actual naturally occurring frost damage, which is the ultimate objective of measuring frost damage (since artificial lab conditions do not adequately reflect the conditions trees encounter in nature). In this study, the rooted trees were grown in controlled conditions and used a single field-grown control tree for comparison. Growing material in the field and testing it in the lab is quite different from the field observation study, as the field observation study is based on observing the results of naturally occurring frosts. There are multiple problems with field observation studies for measuring frost tolerance, reviewed in detail by Warrington and Rook (1980). Field conditions are unpredictable; conditions can be too harsh, killing all trees, or too benign, causing no damage to any tree. Both conditions mean that no difference can be detected between the trees, the first one causing further damage by eliminating the tested population.

Microsite variation, which can cause large differences in frost conditions (Warrington and Rook, 1980), can create large differences in frost damage by *e.g.* frost hollows. The effect of temperatures on frost damage in the field is further confounded by other environmental factors (winds, drought, weed competition). Thus, when evaluating field damage, precautions have to be taken to avoid observational error, seeing phenotypic differences between trees that differ in their environmental conditions, but not in the genes relevant to frost tolerance.

During this study, field observations were made in the sites where samples were collected (Table 5.1). Microsite variation was not clearly visible to the naked eye, and these were even, if sloped, fields (Figure 5.8). There did not seem to be any consistent environmental factor that would cause the damage, although there were too few datapoints to calculate any meaningful correlations.

There are ways to make field tests more predictable: using multiple testing locations, increasing replication and controlling for environmental factors (Warrington and Rook, 1980). Multiple sites were used in this study, with multiple biological replicates collected (although not all rooted), and observation was used to try to eliminate a clear case of environmentally caused variation.

The systematic assessment of the literature in Chapter 4 found 3 studies that did both field and lab measurements, among which two found a correlation between lab EL tests and field observations, one in *Pinus oocarpa* Schiede ex Schltdl. (de Waal et al., 2018) and another in red spruce (*Picea rubens* Sarg.) (Vann et al., 1992), and another study on 14 exotic pine species found agreement between EL tests and field observations (Hodge et al., 2012). However, laboratory tests are not always consistent

with field observations, as was the case in a study in olive trees (*Olea europaea* L.) (Bartolozzi and Fontanazza, 1999).

It could be that the lack of agreement between field and lab results was due to the EL test not being sufficiently similar to real-world field conditions. Another problem that could have caused this was the bottleneck between the collections of the cuttings and the rooting, was that many samples did not root at all (Tables 1 and 7). The reduced number of samples tested for EL could have reduced the statistical power of the analysis, making the detection of effects not possible. Further problems with the cultivation, where many of the plants died due to an aphid infestation and fungal infection, could have selectively killed trees that were less prone to frost damage. Resistance to pests is correlated to frost tolerance; some pest resistance proteins are also protective against frost damage (Liu et al., 2003).

Field results from a one-time event are also far from reliable, as multiple observations in different test sites across different years will give a more reliable result. If frost tolerance changes year-to-year, this could be presumed to be due to environmental effects. In the case of lasting patterns of frost tolerance across sites and time, the differences could be presumed to be due to underlying genetic differences between the samples, instead of environmental effects.

However, such quality data is not available, as field studies across sites and years are very costly in both time and money. Growing samples in a controlled, uniform environment should provide an avenue toward distinguishing between environmental and genetic causes for the variation in frost tolerance.

This study failed at an attempt to calibrate lab measured frost tolerance to actual underlying differences in frost tolerance found in the field. It has been noted by Bigras and Colombo (2001) that interpreting injury curves can be difficult because there is no distinct killing point. Differences in EL between species, genotypes and treatments may not indicate underlying differences in cold hardiness. The lack of agreement between the rank order of the rate of electrolyte leakage and the presence or absence of damage in the field (Figure 5.11) agrees with the ANOVA test, which showed that damage levels were not a significant factor in respect to the rate of electrolyte leakage, k, h^{-1}. It could be because the observations it the field are one-off observations and could be confounded by multiple factors. Another factor could be that the tests were performed, for practical reasons, with needles, whereas in general, whole plant test give results closer to field results (de Waal et al., 2018).

5. Conclusion

This study attempted to find whether there were underlying differences between Sitka spruce that suffered from frost damage and those that did not in a severe frost. Attempts at growing the plants in

uniform conditions to replicate the patterns of frost response found in the field were inconclusive. No significant differences could be found between most of the sample types tested, and no underlying pattern could be found among the differences found.

In order to improve a future study, better rooting techniques would need to be used for field material, or grafting should be used to get a more reliable production of plants. An improved design, that takes into account all factors described in Chapter 4, would aid in finding whether there are underlying differences in the frost tolerance of Sitka trees. A longer freeze exposure time and a more controlled freezing rate, aided by the use of a programmable freezer where temperature can be controlled, would make the conditions in the experiment more similar to field conditions.

While a minimum treatment temperature of -20 °C was used in this study, and Sitka spruce fresh growth is unlikely to have such a low frost tolerance, using lower temperatures could help distinguish between samples that have a frost tolerance close to -20 °C. The improved study would thus include temperatures below -20 °C, with a wider temperature range. A longer exposure time might also obtain results in the lab that more closely resemble field observations.

References

Aitken, S.N.N., Adams, W.T.T., Schermann, N., Fuchigami, L.H.H., 1996. Family variation for fall cold hardiness in two Washington populations of coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco). *For. Ecol. Manage.* **80**, 187-195.

Barney, D.L., Bauer, M., Jensen, J., 2013. Survival, frost susceptibility, growth, and disease resistance of corkbark and subalpine fir grown for landscape and Christmas trees. *Horttechnology* **23**, 194-200.

Bartolozzi, F., Fontanazza, G., 1999. Assessment of frost tolerance in olive (*Olea europaea* L.). Sci. *Hortic. (Amsterdam).* **81**, 309-319.

Bigras, F.J., Colombo, S.J., 2001. Conifer cold hardiness, Tree Physiology. Springer Netherlands, Dordrecht.

Cannell, M.G.R., Sheppard, L.J., 1982. Seasonal changes in the frost hardiness of provenances of *Picea sitchensis* in Scotland. *Forestry* **55**, 137-153.

Cannell, M.G.R., Sheppard, L.J., Smith, R.I., Murray, M.B., 1985. Autumn frost damage on young *Picea sitchensis* 2. Shoot frost hardening, and the probability of frost damage in Scotland. *Forestry* **58**, 145-166.

Charra-Vaskou, K., Charrier, G., Wortemann, R., Beikircher, B., Cochard, H., Ameglio, T., Mayr, S., 2012. Drought and frost resistance of trees: a comparison of four species at different sites and altitudes. *Ann. For. Sci.* **69**, 325-333.

de Waal, L., Mitchell, R.G., Hodge, G.R., Chirwa, P.W., 2018. The use of field and artificial freezing

studies to assess frost tolerance in natural populations of *Pinus oocarpa*. *South. For. a J. For. Sci.* **80**, 195-208.

Fløistad, I.S., Kohmann, K., 2004. Influence of nutrient supply on spring frost hardiness and time of bud break in Norway spruce (*Picea abies* (L.) Karst.) seedlings. *New For.* **27**, 1-11.

Forestry Commission, 2019. Chapter 1: woodland area and planting, in: Forestry Statistics 2019. pp. 1-37.

Forestry Commission, 1957. Exotic forest trees in Great Britain. *For. Comm. Bull.* **30**, 1-167.

Fox, J., Weisberg, S., Price, B., Adler, D., Bates, D., Baud-Bovy, G., Bolker, B., Ellison, S., Firth, D., Friendly, M., Gorjanc, G., Graves, S., Heiberger, R., Krivitsky, P., Laboissiere, R., Maechler, M., Monette, G., Murdoch, D., Nilsson, H., Ogle, D., Ripley, B., Venables, W., Walker, S., Winsemius, D., Zeileis, A., R-Core, 2020. car: Companion to Applied Regression. Accessed: 01/03/2021. URL https://cran.r-project.org/web/packages/car/index.html

Fujikawa, S., Kuroda, K., Jitsuyama, Y., Sano, Y., Ohtani, J., 1999. Freezing behavior of xylem ray parenchyma cells in softwood species with differences in the organization of cell walls. *Protoplasma* **206**, 31-40.

Herbert, R., Samuel, S., Patterson, G., 1999. Using local stock for planting native trees and shrubs. *For. Comm.* 1-8.

Hodge, G., Dvorak, W., Tighe, M., 2012. Comparisons between laboratory and field results of frost tolerance of pines from the southern USA and Mesoamerica planted as exotics. *South. For. a J. For. Sci.* **74**, 7-17. https://doi.org/10.2989/20702620.2012.683637

Jalkanen, R.. E., Redfern, D.. B., Sheppard, L.. J., 1998. Nutrient deficits increase frost hardiness in Sitka spruce (*Picea sitchensis*) needles. *For. Ecol. Manage.* **107**, 191-201.

Jeong, Y., Chung, U., Kim, K.H., 2018. Predicting future frost damage risk of kiwifruit in Korea under climate change using an integrated modelling approach. *Int. J. Climatol.* **38**, 5354-5367.

Jonsson, A.M., Kivimaenpaa, M., Stjernquist, I., Sutinen, S., 2001. Frost hardiness in bark and needles of Norway spruce in southern Sweden. *Trees - Struct. Funct.* **15**, 171-176.

Kakac, S., Smirnov, H.F., Avelino, M.R., 2003. Low temperature and cryogenic refrigeration. Springer Netherlands, Dordrecht.

Kasuga, J., Takata, N., Yamane, K., Kuroda, K., Arakawa, K., Fujikawa, S., 2007. Larch (*Larix kaempferi*) xylem parenchyma cells respond to subfreezing temperature by deep supercooling. *CryoLetters* **28**, 77-81.

Kawahara, H., Masuda, K., Obata, H., 2000. Identification of a compound in *Chamaecyparis taiwanensis* inhibiting the ice-nucleating activity of *Pseudomonas fluorescens* KUIN-1. *Biosci. Biotechnol. Biochem.* **64**, 2651-2656.

Kedrowski, R.A., 1980. Changes in cold hardiness of introduced and native interior Alaskan evergreens in relation to water and lipid content during spring dehardening. *Physiol. Plant.* **48**, 438-442.

L'Hirondelle, S.J., Binder, W.D., 2005. Temperature stress tolerance of conifer seedlings after exposure to UV-B radiation. *Photochem. Photobiol.* **81**, 1094-1100.

Lindow, S.E., 1983. The role of bacterial ICE nucleation in frost injury to plants. *Annu. Rev. Phytopathol.* **21**, 363-384.

Lines, R., 1987. Choice of seed origins for the main forest species in Britain. For. Comm. Bull. 66, 1-61.

Liu, J.J., Ekramoddoullah, A.K.M., Yu, X., 2003. Differential expression of multiple PR10 proteins in western white pine following wounding, fungal infection and cold-hardening. *Physiol. Plant.* **119**, 544-553.

Lucas, P.W., Cottam, D.A., Sheppard, L.J., Francis, B.J., 1988. Growth responses and delayed winter hardening in Sitka spruce following summer exposure to ozone. *New Phytol.* **108**, 495-504.

Mangiafico, S., 2021. rcompanion: Functions to Support Extension Education Program Evaluation. Accessed: 01/03/2021. URL http://rcompanion.org/

Martinez Meier, A., Mondino, V., Gallo, L., 2005. Evaluación de daños por heladas tardías en ensayos de procedencias de pino oregón introducidos en el norte de la Región Andino Patagónica Argentina. *Bosque* **26**, 113-120.

Meyer, D., Dimitriadou, E., Hornik, K., Weingessel, A., Leisch, F., 2019. e1071: Misc Functions of the Department of Statistics, Probability Theory Group (Formerly: E1071), TU Wien. Accessed: 01/03/2021. URL https://cran.r-project.org/package=e1071

Moore, J., 2011. Wood properties and uses of Sitka spruce in Britain. For. Commision Res. Rep. 1-60.

Murray, M.B.B., Cape, J.N., Fowler, D., 1989. Quantification of frost damage in plant tissues by rates of electrolyte leakage. *New Phytol.* **113**, 307-311.

Nicoll, B.C., Redfern, D.B., McKay, H.M., 1996. Autumn frost damage: clonal variation in Sitka spruce. *For. Ecol. Manage.* **80**, 107-112.

Nielsen, C.C.N., Rasmussen, H.N., 2009. Frost hardening and dehardening in Abies procera and other conifers under differing temperature regimes and warm-spell treatments. *Forestry* **82**, 43-59.

Pohlert, T., 2020. Calculate Pairwise Multiple Comparisons of Mean Rank Sums Extended. Accessed: 01/03/2021. URL https://cran.r-project.org/package=PMCMRplus

Prada, E., Alía, R., Climent, J., Díaz, R., 2014. Seasonal cold hardiness in maritime pine assessed by different methods. *Tree Genet. Genomes* **10**, 689-701.

R Foundation for Statistical Computing, R Core Team, 2019. R: A language and environment for statistical computing. Accessed: 01/03/2021. URL http://www.r-project.org/index.html

Räisänen, M., Repo, T., Lehto, T., 2006. Effect of thawing time, cooling rate and boron nutrition on

freezing point of the primordial shoot in Norway spruce buds. *Ann. Bot.* **97**, 593-599.

Riikonen, J., Luoranen, J., 2020. An assessment of storability of Norway spruce container seedlings in freezer storage as affected by short-day treatment. *Forests* **11**, 1-16.

RStudio Team, 2019. RStudio: Integrated Development for R. Accessed: 01/03/2021. URL http://www.rstudio.com/

Sheppard, L.J., 1994. Causal mechanisms by which sulphate, nitrate and acidity influence frost hardiness in red spruce: review and hypothesis. *New Phytol.* **127**, 69-82.

Sheppard, L.J., Crossley, A., Harvey, F.J., Wilson, D., Cape, J.N., 1997. Field application of acid mist to a single clone of sitka spruce: effects on foliar nutrition and frost hardiness. *Environ. Pollut.* **98**, 175-184.

Sheppard, L.J., Leith, I.D., Cape, J.N., 1994. Effects of acid mist on mature grafts of Sitka spruce. Part I. Frost hardiness and foliar nutrient concentrations. *Environ. Pollut.* **85**, 229-238.

Sheppard, L.J., Rosengren, U., Emmett, B.A., 2003. Do nitrogen additions change the sensitivity of detached shoots from Sitka and Norway spruce to freezing temperatures? Evidence from three field manipulation studies. *Scand. J. For. Res.* **18**, 487-498.

Vann, D.R.R., Strimbeck, G.R.R., Johnson, A.H.H., 1992. Effects of ambient levels of airborne chemicals on freezing resistance of red spruce foliage. *For. Ecol. Manage.* **51**, 69-79.

Warrington, I.J., Rook, D.A., 1980. Evaluation of techniques used in determination of frost tolerance of forest planting stock: A review. *New Zeal. J. For. Sci.* **10**, 116-132.

Wickham, H., Chang, W., Henry, L., Pedersen, T.L., Takahashi, K., Wilke, C., Woo, K., Yutani, H., Dunnington, D., RStudio, 2020. ggplot2: Create Elegant Data Visualisations Using the Grammar of Graphics. Accessed: 01/03/2021. URL http://ggplot2.tidyverse.org

Wipf, S., Stoeckli, V., Bebi, P., 2009. Winter climate change in alpine tundra: plant responses to changes in snow depth and snowmelt timing. *Clim. Change* **94**, 105-121.

Wulff, A., Sheppard, L., Leith, I., 1994. Evaluation of electrolyte leakage, chlorophyll fluorescence and ultrastructural techniques for detecting effects of acid mist on frost hardiness of Sitka spruce shoots. *Environ. Exp. Bot.* **34**, 261-273.

Chapter 6: Identifying potential frost tolerance genetic markers in Sitka spruce (*Picea sitchensis* [Bong.] Carr.) *in silico*

Author contributions: AA, KS and AS conceived and planned the experiment. DNA extraction was conducted by AA. The genotyping chip used was designed for our partners at the University of Oxford led by Prof. John MacKay. The quantification of DNA by using the fluorometry method was conducted in the lab in Canada that was hired for the genotyping. Data analysis was conducted by AA. Writing was done by AA with feedback and guidance from KS and AS.

Abstract

Sitka spruce (*Picea sitchensis* [Bong.] Carr.) frost tolerance is a complex trait controlled by many different genes that have an additive effect. In order to perform a Genome-Wide Association Study (GWAS), the 93 individual genotypes phenotyped in Chapter 5 were sent to a lab in Canada for genotyping with a custom SNP microarray designed by our partners in the University of Oxford led by Prof. John MacKay. Due to Covid-19 analysis of the microarray was delayed and no genotyping data has been received at the time of writing. Subsequently a literature-based approach was used to identify 4 conifer studies revealing 165 genes associated with frost tolerance. Of these, 82 possible candidate genes represented in Sitka spruce could be analysed to determine their function in relation to frost tolerance. Stress-related genes were found to be the most common category of genes identified by biological process ontology. Most genes were expressed in the cytoplasm and nucleus, and catalytic activity and protein binding were the main categories of molecular functions of the frost tolerance related genes. Overall, frost tolerance in conifers seems to be a very complex trait controlled by many genes with a wide variety of functions and roles.

1. Introduction

1.1. Frost tolerance

Plant tolerance to frost is affected by multiple physiological and morphological adaptations the plant undergoes to withstand sub-freezing temperatures. For the purposes of this thesis, the temperature at which 50% of the exposed plant tissue dies (LT_{50}) is considered the minimum sub-freezing temperature Sitka spruce (*Picea sitchensis* [Bong.] Carr.) can tolerate.

It should be noted that cold and frost tolerance are not the same thing, as one involves cool temperatures and the other involves sub-freezing temperatures (for the purposes of this work, air temperatures below 0 °C will be considered sub-freezing), and the response mechanisms are different

(Wellburn, 1995). The process of developing frost tolerance is called frost hardening and is triggered by either changes in photoperiod, decreases in ambient air temperatures, or both, depending on the species and population (Holliday et al., 2008).

The process of hardening in trees, which happens in autumn (although plants may re-harden as temperatures drop throughout the year), is accompanied by changes in tree phenology, as trees generate bud tissue to prepare for the following spring. The process of dehardening that occurs in spring is also accompanied by changes in bud phenology.

Many studies examine phenology and frost tolerance together, and consider them parts of the same trait (De La Torre et al., 2021; Eckert et al., 2009; Vangestel et al., 2018). Many efforts toward frost tolerance are directed solely at improvements in bud phenology (Goto et al., 2017; Prunier et al., 2011). It is true that in some plant species frost damage occurs due to phenology, when budburst occurs before frosts stop occurring in spring (Jermstad et al., 2001; Ma et al., 2019). This, however, is by no means a universal phenomenon, as examined in Chapter 3, which shows that phenology-related frost damage is unlikely to occur in Sitka spruce grown in the UK. While it may be reasonable to focus on phenology to avoid frost damage for other species, it is advisable to evaluate the evidence on a species-by-species basis. Thus, for the purposes of identifying relevant frost tolerance markers in Sitka spruce, phenology-related markers that do not correlate with actual frost tolerance will not be considered in this study.

1.2. Genetics of frost tolerance in conifer trees

Most of the current knowledge about plant genes or QTLs associated with frost tolerance are based on studies of herbaceous annual plants such as *Arabidopsis thaliana* (L.) Heynh. and winter rye (*Secale cereale* L.) (Holliday et al., 2008). However, woody perennials will adapt to their environment differently from herbaceous annuals, as they need to survive even through the harshest of winters. This manifests in many different genes in woody perennials that have evolved for frost tolerance: in a study of gene expression during frost hardening in Sitka spruce, 549 upregulated genes with no homology in *Arabidopsis* were found (Holliday et al., 2008).

It is also important to note that spring and autumn frost tolerance, as measured by the degree of frost damage, while both important to the ability of trees to withstand freezing temperatures, rarely correlate, or correlate negatively with each other (Aitken and Adams, 1996; Jermstad et al., 2003; O'Neill et al., 2001). This indicates these two processes are controlled by different molecular mechanisms, with different genes controlling each mechanism (Jermstad et al., 2003). These mechanisms are not completely separate, though, as autumn and spring cold hardiness traits share one QTL in a study in Douglas fir (*Pseudotsuga menziesii* (Mirb). Franco) (Jermstad et al., 2003).

Cold hardiness levels between tissues, on the other hand, correlate positively between tissues (O'Neill et al., 2001), although they seem to correlate more frequently in the case of spring frost tolerance than autumn frost tolerance (Jermstad et al., 2003).

In a genetic association study that associated single nucleotide polymorphisms (SNPs) to cold hardiness in Douglas fir, most identified genes explain a minor part of the variation, pointing at the polygenic quantitative model. The effect of genes can add up, though, with 6 genes explaining 17% of the phenotypic variance in stem frost damage (individual r^2 values between 0.019 and 0.034) (Eckert et al., 2009). This indicates that, although most genes that affect frost hardiness have a minor effect, and are thus harder to use for breeding, some potential genes could be used for selective crossing when combined, as they can still add up to a significant effect.

1.3. Identifying QTLs or genes

Quantitative Trait Locus (QTL) mapping is a powerful tool that consists of measuring segregation of traits and alleles within multiple generations of crosses in either F2 populations or Recombinant Inbred Line (RIL) families, which allows for the identification of all major QTLs involved in one trait that are segregating in that population (reviewed by Korte and Farlow, 2013). This approach has two fundamental problems: i) the resolution, limited by the amount of recombination that can occur within two generations, which can be improved by more generations of intercrossing in RILs, and ii) the limited number of alleles that segregate due to the selection of the parents, which can be increased by the use of Multi-parent Advanced Generation Inter-Cross (MAGIC) populations.

Genome-Wide Association Studies (GWAS) do not have the limitations of QTL mapping, as natural populations are used to associate a particular phenotypic trait of interest to a genotype (reviewed by Korte and Farlow, 2013). The use of natural populations avoids the problem of the reduced number of alleles, and natural populations are the product of many generations of natural crosses, which will exhibit the level of recombination that could be expected from the degree of closeness of the loci in which the genes are located.

GWAS is limited by the genetic architecture of traits; if the effect size (the phenotypic variance explained by a SNP) of a SNP is small, or if the allele is rare, GWAS may not be able to detect the allele (reviewed by Hou and Zhao, 2013; Korte and Farlow, 2013). Rare alleles, present in a few individuals, will also be associated with other SNPs present in those individuals due to linkage disequilibrium (LD), and the association between the two will seem stronger than it actually is, creating synthetic associations (reviewed by Korte and Farlow, 2013). This problem can be avoided by using QTL analysis by elevating the frequency of the rare allelic variant through crossing. GWAS is also a species-specific method; it cannot be used for comparisons across species (reviewed by Nagy et al., 2020).

Genes with small effect sizes can be detected by using customized chips that target specific genomic regions with a high density of markers to finely map all phenotype-associated variants, in cases when the genes are co-located in the same region (reviewed by Hou and Zhao, 2013). Rare allele detection can be improved by using whole exome sequencing or by including extreme versions of the phenotype in the GWAS study, thus enriching it with rare variants.

Multiple GWAS studies can be combined, increasing the power to identify more SNPs (reviewed by Begum et al., 2012; Hou and Zhao, 2013). When the studies used the same genotyping platform or chip, they are combined into a mega-analysis. They can still be combined when they use different chips and genetic platforms, or when the raw data is not available, into a meta-analysis.

Although most SNP datasets used in array-based testing represent only a minority of SNPs present in that species, significant associations with the SNPs can still be detected because causative alleles are in some degree of LD with the genotyped markers (reviewed by Korte and Farlow, 2013). As long as the distance at which LD decays to 50 % is smaller than the distance between the SNPs, causative alleles can be detected.

Even if SNPs correlated with a phenotypic trait are identified, this does not mean that the underlying genes influencing the trait can also be identified, as many SNPs associated with phenotypes are found in non-coding regions, or, when found in coding regions, are in close linkage with various similar candidate genes, making it difficult to establish the causative allele (reviewed by Hou and Zhao, 2013). These problems can be minimized by prioritizing SNPs in genomic areas that are likely to be functional, such as exon and regulatory regions of genes.

1.3.1. Identifying gene function

Identifying gene function can be done by traditional biological experiments, such as gene knockout and targeted mutations, but those methods tend to be quite costly (reviewed by Chen et al., 2021). These techniques also have the problem of identifying only one gene at a time, which quite limits their application. Expression studies that can isolate expressed genes for further analysis have been developed. Techniques range from cDNA libraries through to differential gene expression arrays, which measure RNA levels from specific tissues under different conditions and can help identify gene function (reviewed by Hou and Zhao, 2013). Differential gene expression arrays identify a subset of the RNA transcribed in the different tissues of the organism at different moments in time. The study of the entire set of RNA transcribed in an individual is called transcriptomics (reviewed by Wang et al., 2009). Identifying gene function from such studies requires observing different gene expression profiles. Differential gene expression can be studied by using expressed sequence tags (ESTs).

ESTs are short sequences of cDNA obtained from the reverse transcription of mRNA at specific moments of time in specific tissues(reviewed by Bouck and Vision, 2007; Gill and Sanseau, 2000). ESTs

are obtained by sequencing cDNA libraries obtained from the reverse transcription of expressed mRNA by RNAse H and DNA polymerase 1. They are typically only partial, very short sequences (500-800 bp), as they are limited by the read length available with Sanger sequencing. ESTs are inexpensive genomic resources that can be developed for most organisms, and they are among the most phylogenetically diverse and abundant type of sequence (reviewed by Bouck and Vision, 2007). This process can be automated and can serve for the expression profiling of tissues. ESTs can also be used to develop molecular markers, whether they are simple sequence repeats (SSRs), SNPs, or Exon-primed Intron-crossing (EPIC) markers. ESTs can also be used to develop microarrays containing cDNA probes that measure mRNA abundance in a sample of cDNA obtained from reverse transcription from a specified tissue under investigation. Microarray chips can be made by either affixing oligonucleotides or cDNA to glass beads, or by synthesizing DNA directly on the chip.

There are several problems with ESTs. Transcripts that are low in abundance may not be sequenced; ESTs give no information about genomic position, gene order, or regulatory motifs; and ESTs represent only partial sequences of the original transcript (reviewed by Bouck and Vision, 2007). The problem of low abundance can be dealt with by using normalization procedures. However, this means ESTs will not be useful for expression profiling. Full-length transcripts can be obtained by a technique known as capping during library construction, although it is costly and biases the library toward short transcripts. Capping involves producing full-length cDNAs, hybridized to mRNAs, with the mRNA biotinylated at both ends and digested with RNase I, to remove the 5' UTR and the 3' UTR regions in both the mRNA and the cDNA, leaving only the coding sequence (Carninci et al., 2000). The mRNA would then be captured with magnetic beads, and hydrolysed, while the full-length cDNA would be processed for cDNA libraries and sequenced.

Microarrays are a high-throughput and relatively inexpensive technology to quantify gene expression by hybridizing cDNA with microarrays (reviewed by Wang et al., 2009). However, microarrays can only be used for relatively well studied species, as they rely on already existing knowledge of genome sequence. The range of expression detection is also limited due to high cross-hybridization and a saturation of signals. Microarrays do not solve the problems of EST use for gene identification, although they are cheaper, as Sanger sequencing is relatively more expensive.

RNA-Seq is a newer technology than microarrays and ESTs that can be used for differential gene expression analysis (reviewed by Wang et al., 2009). RNA is converted into a library of cDNA with adaptors at one or both ends, and each molecule is sequenced on either one end or both. Sequences can be mapped to a genome or assembled *de novo*. RNA-Seq can use the more affordable Illumina sequencing platform instead of Sanger. Although Illumina only offers 2 x 75 bp length for transcriptome sequencing (Illumina, 2021), these reads can be assembled into gene sequences. RNA-

Seq has multiple advantages over ESTs, such as much higher throughput, a dynamic range of expression detection, and lower costs (reviewed by Wang et al., 2009).

Gene expression is not synonymous with gene function, as leaks in the regulatory system lead to expression of mRNAs that may not be functionally required in that particular tissue under the conditions in which it was sampled (reviewed by Rodríguez-Trelles et al., 2005). Spectrometry-based protein co-expression measurement, on the other hand, is driven mainly by functional similarity between the genes (Wang et al., 2017). Proteomics data thus outperforms transcriptomics in identifying gene function.

Both GWAS, expression studies, and proteomics are limited for their application in gene function identification by the costs of the phenotype measurement, which even in high-throughput wet lab experiments in proteomics cannot produce the same volume of data as whole-genome sequencing (reviewed by Chen et al., 2021).

Pure bioinformatics methods can be used to further analyse data accumulated from the studies described above. Bioinformatics is becoming more useful due to a larger knowledge base of gene ontology and a greater number of gene expression databases. Bioinformatics can analyse gene regulatory networks through gene co-expression and genetic interactions, serving to identify gene function without having to repeat the costly experiments or design new ones. Interactions between genes and proteins can be taken into account by the integration of data for both genes and proteins through bioinformatics analyses. Phylogenetic comparative methods can be used to identify gene function by comparing traits and genes across different species (reviewed by Nagy et al., 2020).

1.3.2. Gene ontology

Once gene function is identified the functional properties need to be classified to systematize that knowledge. Gene Ontology (GO) and the MIPS Functional Catalogue are the most used paradigms to describe functional properties of gene products in a rigorous and species neutral way (reviewed by Zhao et al., 2020). Of the two, GO is more comprehensive, includes more annotations, and is more frequently used and updated. Gene Ontology includes all categories for all organisms. A reduced, cut down version of GO is available for plants, in the form of the PlantSlimGO. This ontology is related to the general ontology, but only includes categories that can be found in plants.

GO consists of three ontologies: molecular functional ontology (MFO), biological process ontology (BPO), and cellular component ontology (CCO) (reviewed by Zhao et al., 2020). MFO describes the molecular activity of a gene product (the reactions a protein catalyses and the molecules to which it is bound). BPO describes the biological process, such as biochemical pathways in which the gene product acts. CCO describes the cellular component in which the gene product is found. Each ontology is then organized into a hierarchy.

1.4. Genetics of Sitka spruce cold tolerance

Selective pressure to adapt to cold temperatures is very powerful in Sitka spruce, with the genetically close populations of British Columbia and California having a higher level of divergence in frost tolerance markers than the more genetically distant populations of British Columbia and Alaska (Mimura and Aitken, 2007). Many genes upregulated during frost hardening in British Columbia and Alaska varieties of Sitka spruce were not upregulated in Californian varieties (Holliday et al., 2008). Such selective pressures can also be observed in Scots Pine (*Pinus sylvestris* L.) (Wachowiak et al., 2009).

Differences in Sitka spruce populations manifest not only in clinal patterns of gene expression, but also in their regulation. The southern California population does not seem to respond to photoperiod as a cue to harden, requiring cold temperatures, whereas the northernmost Alaskan population hardens with just the photoperiod, requiring no cold temperatures (Holliday et al., 2008).

1.5. Aims and objectives

The over-arching goal of this thesis was to associate phenotypic traits for frost tolerance with SNP variants across the Sitka spruce genome which could then be used to identify candidate genes influencing frost tolerance. However, due to Covid-19 related unavoidable delays, the SNP data for the phenotyped Sitka spruce (Chapter 5) trees is not yet available, therefore, this chapter focuses on identifying potential genes *in silico*, by comparing the sequences of known potential frost tolerance proteins or genes to the Sitka spruce genome. The primary objectives are to:

i) generate SNP calls for the 93 phenotyped Sitka spruce samples which could subsequently be used for GWAS;
ii) identify which published frost-tolerant candidate genes/proteins have a homologue in Sitka spruce;
iii) identify the predicted role of candidate genes (whether they are up or downregulated, and whether they play a positive or negative role in Sitka spruce frost tolerance).

2. Methods

2.1. DNA extraction and SNP genotyping

Needles from the 93 trees phenotyped in Chapter 5 (Figures 9, 10 and 11) were used for DNA extraction.

A Qiagen (Qiagen, Hilden, Germany) TissueLyser was used to homogenise the needles, and the standard manufacturer's protocol for DNA purification from plant tissue was used. Approximately 100 mg of needles were placed in a 2 mL safe-lock microcentrifuge tube with a 5 mm stainless steel bead. The tube containing the sample and the bead was immersed in liquid nitrogen for 30 seconds to freeze

the needles, after which it was placed in the TissueLyser at 30 Hz for 1 minute. To ensure complete disruption of the needles the freezing and TissueLyser procedure was conducted twice.

A Bioline (Meridian Bioscience, Cincinatti, USA) Isolate II Plant DNA extraction kit was used following the manufacturer's recommended protocol, using the version of the protocol that performed cell lysis by adding 400 µL of Lysis Buffer PA1 instead of using the PA2 Lysis Buffer. 10 µL of RNase A were added, and the vortexed sample was incubated for 10 min at 65 °C.

After incubation, the steel bead was taken out of the tube so it would not interfere with the centrifugation. The mix was loaded onto an ISOLATE II Filter column, and centrifuged for 2 min at 11,000 g. The flow through liquid was collected and transferred into another tube, to get rid of any precipitate. The liquid was then thoroughly mixed with 450 µL of Binding Buffer PB, by pipetting up and down. Up to 700 µL of the mix were then loaded onto a Plant DNA Spin Column, and centrifuged for 1 min at 11,000 g.

The DNA was washed by adding 400 µL of Wash Buffer PAW1 and centrifuging for 1 min at 11,000 g. For another washing, 700 µL of Wash Buffer PAW2 were added, and centrifuged for 1 min at 11,000 g. For the last step of the washing, 200 µL of Wash Buffer PAW2 were added, and centrifuged for 2 min at 11,000 g to get rid of all excess liquid in the column.

For the DNA collection, 50 µL of Elution Buffer PG, preheated at 65 °C were loaded onto the column, and incubated at 65 °C for 5 min. The DNA was removed from the column by centrifugation into a 1.5 mL Eppendorf tube.

DNA concentration was measured by placing 2 µL on a Thermo Scientific NanoDrop spectrophotometer (Waltham, Massachusetts, United States), and another measurement was done in the Canadian lab in charge of genotyping using the Thermo Scientific Qubit Fluorometer (Waltham, Massachusetts, United States).

The extraction process was repeated in 50 extractions where the concentration of DNA as measured by spectrophotometry was below 50 ng μL^{-1}, until the desired concentration was obtained. In the 9 cases where even after five extractions the concentration of DNA obtained was insufficient, the extracted DNA was pooled and centrifuged at 11,000 g for five minutes. The liquid was then discarded and resuspended in 50 µL of Elution Buffer PG.

Since only 93 genotypes were phenotyped, and the microtiter plate had 96 wells, one of which was reserved for the tester used by our collaborators at University of Oxford, who designed the chip, two genotypes for which extra material was available were repeated.

Genotyping of the samples was contracted out to an external service that used an Infinium HTS iSelect chip by Illumina (San Diego, California, USA) with 15,000 custom SNP targets designed for Sitka spruce for our collaborators at the University of Oxford, headed by Prof. John Mackay.

Due to the Covid-19 pandemic the contracted out Infinium HTS iSelect analysis pipeline was shut down during 2020 and the results of the genotyping had not been received by the time of writing.

Correlation between the concentrations obtained by the Qubit and the Nanodrop was measured using the ggpubr 0.3.0 package in R (Kassambara, 2020), using, R 3.6.3 (R Foundation for Statistical Computing and R Core Team, 2019) with the RStudio IDE 1.3.959 (RStudio Team, 2019).

2.2. Search strategies

The peer reviewed literature searching was conducted using 'topic' for a basic search in Web of Science (Clarivate Analytics, USA) on the 24th March 2021, using the entire time span available, "all years" (1970-2021). A basic search was also conducted using the 'keywords' entry in the Cab Direct database (CAB International) on the 24th March 2021, which includes articles between 1968-2021. The search used the terms outlined in Table 6.1. No additional attempt at retrieving grey literature was made.

The search strategy was optimized during a scoping phase, in order to find an appropriate balance of depth (number of papers found) and specificity (how well the papers found fit the search criteria). This was achieved through an explorative search. The search terms were given a broader range by using the asterisk wildcard, which allows the matching of a word with multiple beginnings or endings. For example, the *freezing term will find words such as freezing or subfreezing, and the test* term will find words such as test, tests, testing. Search terms were connected using the Boolean operators 'AND' and 'OR'.

Papers were accessed through the Bangor University access portal and through green literature access. Only one article was found to be not available (Figure 6.1).

2.3. Article screening and inclusion criteria

Literature search results were exported into Excel (Microsoft Corporation, USA), and duplicates deleted. Results were screened based on the inclusion/exclusion criteria listed on Table 6.2.

Three rounds of selection were carried out. In the first selection round search results were excluded based on the title, and more leeway was given with the criteria in Table 6.2 than in the second round. Ambiguity as to the species studied or the part of the plant studied was interpreted favourably, leading to inclusion. In the second round, where the articles were included based on the abstract, the criteria in Table 6.2 were adhered to strictly.

Table 6.1. Definition of the main components of the search and the search terms used.

	Definition	Search terms[1]
Population	All gymnosperms	All gymnosperm Latin species names: (*Cycas OR Dioon OR Bowenia OR Macrozamia OR Lepidozamia OR Encephalartos OR Stangeria OR Ceratozamia OR Microcycas OR Zamia OR Ginkgo OR Welwitschia OR Gnetum OR Ephedra OR Cedrus OR Pinus OR Cathaya OR Picea OR Pseudotsuga OR Larix OR Pseudolarix OR Tsuga OR Nothotsuga OR Keteleeria OR Abies OR Araucaria OR Wollemia OR Agathis OR Phyllocladus OR Lepidothamnus OR Prumnopitys OR Sundacarpus OR Halocarpus OR Parasitaxus OR Lagarostrobos OR Manoao OR Saxegothaea OR Microcachrys OR Pherosphaera OR Acmopyle OR Dacrycarpus OR Dacrydium OR Falcatifolium OR Retrophyllum OR Nageia OR Afrocarpus OR Podocarpus OR Sciadopitys OR Cunninghamia OR Taiwania OR Athrotaxis OR Metasequoia OR Sequoia OR Sequoiadendron OR Cryptomeria OR Glyptostrobus OR Taxodium OR Papuacedrus OR Austrocedrus OR Libocedrus OR Pilgerodendron OR Widdringtonia OR Diselma OR Fitzroya OR Callitris OR Actinostrobus OR Neocallitropsis OR Thujopsis OR Thuja OR Fokienia OR Chamaecyparis OR Callitropsis OR Cupressus OR Juniperus OR Xanthocyparis OR Calocedrus OR Tetraclinis OR Platycladus OR Microbiota OR Austrotaxus OR Pseudotaxus OR Taxus OR Cephalotaxus OR Amentotaxus OR Torreya)*. And ordinary names for the most common gymnosperms (*OR cedar OR celery-pine OR cypress OR fir OR juniper OR larch OR pine OR redwood OR spruce OR yew)*. Also the common name for the largest division among gymnosperms *(OR conifers)*, as well as the common name for conifer wood (*OR softwood)*.
Trait	Frost resistance	Synonyms for **frost** (frost OR *freezing OR subzero OR cold*), joined with synonyms for resistance (toleran* OR hard* OR resistan*), by the AND Boolean operator.
Genes	Genes/molecular markers for frost resistance	Synonyms for genes and molecular markers (gen* OR QTL* OR marker* OR SNP* OR microsatellite OR RAPD OR AFLP)

2. Separate strings in brackets and joined by the AND Boolean operator.

Table 6.2. Inclusion and exclusion criteria for entries to be included in the study (decided *a priori*).

Inclusion criteria	Exclusion criteria
• Studies done on gymnosperms.	• No gymnosperm species studied.
• Mentions frost tolerance.	• Does not mention frost tolerance or uses cold tolerance instead of frost tolerance.
• Mentions molecular markers, DNA, genes, genomes, genetics, or anything plausibly related to DNA.	• Does not mention anything that could be plausibly related to genes.
• Studies on specific proteins related to frost tolerance	• Is not about any protein.
• Research article.	• Review, book article, or other non-research article

In the final selection round, selected papers were excluded if they were not research articles, did not measure frost tolerance or expression under frost hardening, measured cold but not frost tolerance (used cold temperatures above 0 °C, not measuring frost tolerance), or did not identify any protein or gene that could be correlated with frost tolerance (Figure 6.1). In addition, the studies that did not provide database accession codes that could be useful for download could not be used for the blasting part of this study, because gene or protein names are too ambiguous and give too many hits, unlike accession numbers, which give one unique sequence.

2.4. Download the sequences

From the selected papers that provided database accession codes that could be used for downloading, the only selected genes or proteins were those up or downregulated with frost tolerance or those associated with more frost tolerant varieties.

Sequences were bulk downloaded from the NCBI Batch Entrez website, when NCBI accession numbers were available, for both DNA and protein files (https://www.ncbi.nlm.nih.gov/sites/batchentrez) and from the TAIR database for representative *Arabidopsis* gene model entries (https://www.arabidopsis.org/tools/bulk/sequences/). Queries downloaded from the information provided were not the genes identified by the study, as their database entry codes were not provided (Table 6.3), but genes from other species which were identified as similar enough to the genes identified in the target species. Total number of entries of each category can be seen in Table 6.3.

Table 6.3. Databases and formats used, showing the number of gene or protein sequences that could be downloaded.

Study	Number	Database used	Format	Species studied*
De la Torre et al. 2021	33	NCBI	Protein FASTA	*Pseudotsuga menziesii*
Holliday et al. 2008	73	TAIR	DNA FASTA	*Picea sitchensis*
Joosen et al. 2006	32	TAIR	DNA FASTA	*Pinus sylvestris*
Kjellsen et al. 2010	27	TAIR	DNA FASTA	*Picea obovata*

*The species studied in the article, which is not necessarily the species corresponding to the downloaded genes.

2.5. Comparing the genes/proteins to the Sitka spruce database

For the genes for which DNA sequences were obtained, the BLASTN search algorithm (Zhang et al., 2000) was used through the NCBI web portal (https://blast.ncbi.nlm.nih.gov/Blast.cgi), against the nucleotide collection database (nr/nt) and the 'organism' box was set to "*Picea sitchensis* (taxid:3332)". The discontinuous megablast setting was used, as these were queries of different species to the target. For the protein sequences, the TBLASTN search algorithm (Altschul et al., 1997) was used through the NCBI web portal (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The search was against the nucleotide collection database (nr/nt) and the 'organism' box was set to "*Picea sitchensis* (taxid:3332)".

2.6. Evaluating sequence matches

After running the queries against the Sitka spruce database, queries with a BLAST e-value (the number of expected hits of identical quality that could be obtained in a random database of the same size) of $< 10^{-5}$ were filtered, as was done in a similar study in *Pinus koraiensis* Siebold & Zucc. (Wang et al., 2020). After filtering for e-value, a threshold of at least 70% identity was used to filter the relevant entries, similar to the study of *Mentha* sp. by Roy et al. (2011).

2.7. Gene ontology

Once the queries that had a homologue in Sitka spruce were identified, their gene ontology was evaluated. Since the GO database does not recognize GenBank accession numbers, the GenBank accession numbers were related to UniProt Accession numbers using the bioDBnet (https://biodbnet-abcc.ncifcrf.gov/db/db2dbRes.php) database to database (db2db) web conversion tool (Mudunuri et al., 2009). Many accessions did not have an equivalent, as can be observed in Table 6.4.

The QuickGO web browser (https://www.ebi.ac.uk/QuickGO/slimming) (Binns et al., 2009) was used to search the entries with Uniprot Accession numbers, while the TAIR *Arabidopsis* website's GO web search tool was used for the *Arabidopsis* queries, clicking on the "Get all GO annotations" button (Table 6.4) (https://www.arabidopsis.org/tools/bulk/go/index.jsp) (Berardini et al., 2004).

After finding the GO annotations for the genes for which it could be found, the annotations were grouped by functional category, identifying the number of annotations and the number of genes in each functional category, for the three major ontologies. Equation 1 was used to estimate the percentage of genes in each category, in proportion to the total number of genes annotated to that ontology category.

Functional categorization was performed using the TAIR *Arabidopsis* website's GO web search tool.

$$\% = \frac{Number\ of\ genes\ annotated\ to\ ontology\ \times 100}{Total\ number\ of\ genes\ from\ input\ list\ annotated\ to\ ontology} \qquad (11)$$

Table 6.4. Number of query entries at each stage of the identification process, from downloading to matching to Sitka spruce (*Picea sitchensis* [Bong.] Carr.), to translating to a Uniprot Accession number, and the number of Gene Ontologies found (for all genes queried).

Study	Downloaded	Match Sitka spruce	Translated[1]	Found GO[2]
De la Torre et al. 2021	33	10	4	48
Holliday et al. 2008	73	39	39	509
Joosen et al. 2006	32	21	21	260
Kjellsen et al. 2010	27	18	18	527
Total	165	88	82	1344

[1]Accession numbers had to be translated from GenBank entries to Uniprot queries; TAIR entries could be used as is, so did not require translation.

[2]All GO found, for all three major ontologies.

3. Results

3.1. DNA extraction

DNA was successfully extracted from 83 genotypes, plus a duplicate of BZO10, out of the total 93 unique genotypes that had been phenotyped (Chapter 5) and 95 samples (Table 6.5). The other duplicate, BZO9, was only successfully extracted in one case. A concentration below 20 ng μL^{-1} as measured by Qubit is below optimal for genotyping by the external services that handles the genotyping, while a concentration below 10 ng μL^{-1} is too low for genotyping in general. Of the 95 samples sent for genotyping, 7 had concentrations that were below 10 ng μL^{-1} (one of which was the BZO9 duplicate), while 4 had concentrations below 20 ng μL^{-1}. The concentrations measured by the Qubit and the Nanodrop were significantly correlated (Pearson's $r = 0.22$, $p < 0.05$).

3.2. Summary of the literature

In total, 1,336 publications were found, of which 307 duplicates were deleted (Figure 6.1). After screening by title, 120 articles passed the inclusion criteria. Of these, 45 were included after examining the abstracts, and 1 was unavailable. Upon examination of the entire paper, 14 were selected, but only 4 could be used to download query sequences.

The four papers selected all used different techniques to identify genes or proteins that were associated with frost tolerance in four different conifer species.

The study by Holliday et al. (2008) measured the frost tolerance of different Sitka spruce populations with electrolyte leakage (EL) tests for shoot cuttings. They extracted RNA at various timepoints of the hardening process and in different populations, associating RNA to frost tolerance, measuring whether it was up or downregulated, and whether it was upregulated in more frost tolerant populations.

De La Torre et al. (2021) tried to identify frost tolerance genes by conducting a GWAS study, in which they performed multiple generations of crossings in a Douglas fir population. Frost tolerance was measured by observing the visual percentage of damage to buds, needles and stems, while molecular markers were identified by a custom Illumina Infinium SNP array comprising of 80k SNP markers. While the study also measured phenology, only the genes associated with frost damage were selected.

In a study in Scots pine Joosen et al. (2006) used ESTs to identify frost tolerance genes. Frost tolerance was measured with EL tests on shoot cuttings, while the mRNA was isolated form apical buds, and prepared for subtraction genetic libraries. After several enrichment steps, large fragments were sequenced, and the ESTs were assembled. Transcript abundance was compared between the samples, keeping a common reference for comparison.

The study by Kjellsen et al. (2010) used proteomics to measure which proteins were upregulated or down-regulated in frost tolerant Siberian spruce (*Picea obovata* Ledeb.). EL was used to measure frost tolerance of needles, while proteins were identified by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS).

3.3. Identified Sitka spruce DNA sequences

A total of 132 nucleotide sequences (one sequence for each of 132 genes) and 33 protein sequences (representing the products of a further 33 genes), representing 165 different genes in total, were downloaded for querying (Tables 3 and 4). Using the $< 10^{-5}$ threshold e-value and a ≥ 70% identity, a total of 88 unique queries found hits in the Sitka spruce genome that satisfied those criteria. For 82 of them, accession numbers that could be used for a GO search were identified, and 1344 entries were found for GO.

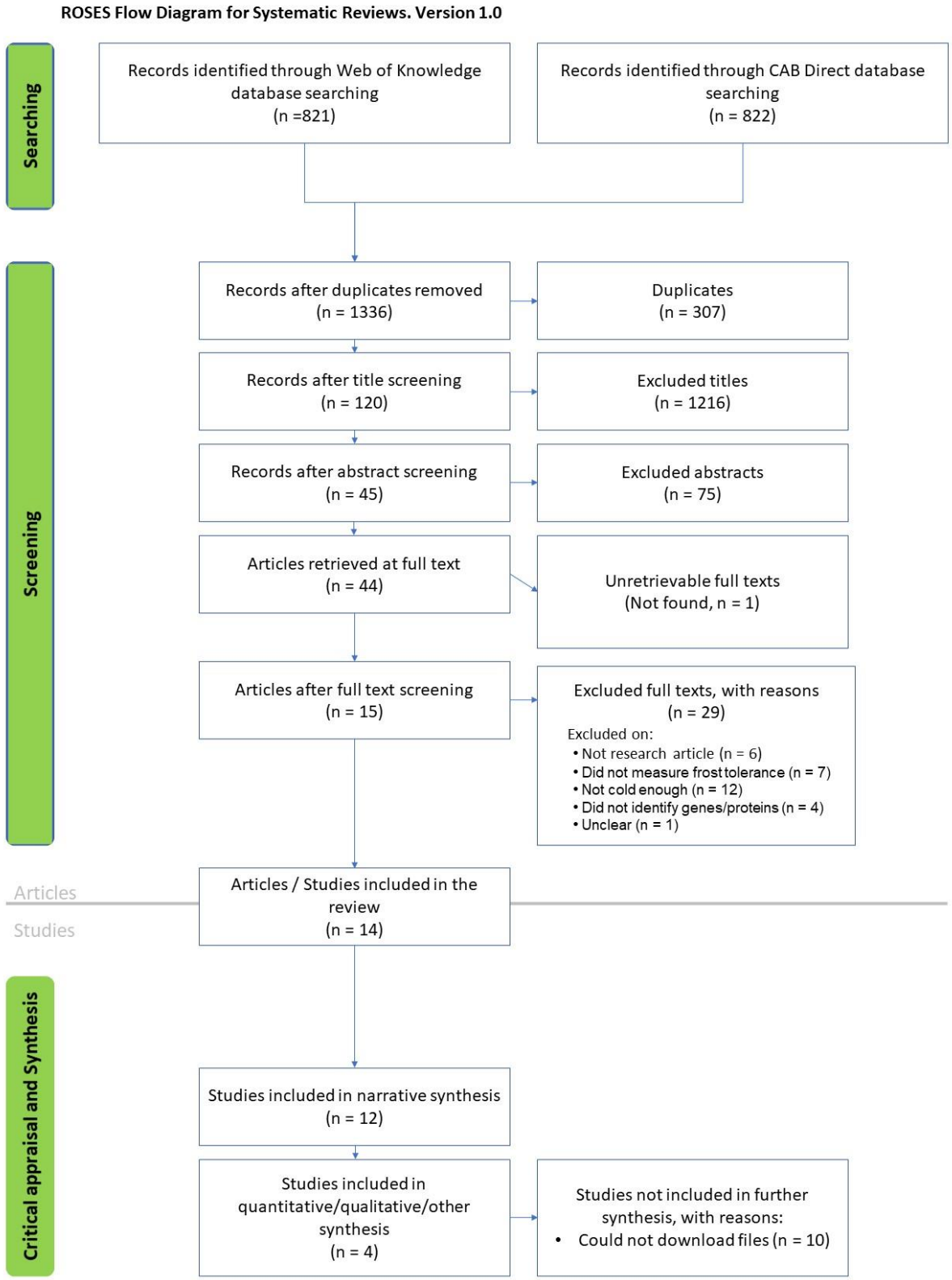

Figure 6.1. ROSES diagram outlining the search, screening and critical appraisal stages. Adapted from (Haddaway et al., 2018).

Table 6.5. DNA concentration, in ng μL^{-1} after extraction, as measured by the Nanodrop and the Qubit.

Name	Nanodrop	Qubit
A	324.6	69.8
A12A	157.4	53.1
A12B	77.8	13.3
A12C	79.9	57.7
A12D	171.1	58.0
A13B	123.9	67.9
A13C	125.5	20.9
A13D	87.8	55.6
A13F	229.0	46.0
A13G	184.2	61.5
A17A	57.9	56.4
A17B	109.6	74.3
A17C	71.5	69.1
A17D	167.8	72.6
A17G	79.5	23.0
A17H	147.3	23.6
A18A	81.4	80.4
A18B	57.2	81.0
A18C	125.2	59.0
A18D	58.9	33.3
A18E	334.1	56.8
A21A	82.5	44.7
A21B	36.2	11.8
A21C	125.5	47.6
A21D	74.0	73.4
A21E	215.0	50.9
A22A	294.0	4.8
A22B	159.8	70.5
A22C	173.4	43.3
A22D	69.8	50.5
A22E	205.4	40.8
B	210.6	43.4

Name	Nanodrop	Qubit
BHO8	70.8	0
BZO10	139.4	74.7
BZO10	174.6	69.7
BZO9	81.1	2.6
BZO9	151.9	46.3
BZX1	55.5	32.2
BZX10	69.7	12.3
BZX7	66.0	80.1
BZX8	178.7	82.0
BZX9	139.0	66.4
C	70.5	37.1
CONTROL	109.1	20.6
D	55.1	47.8
DK4A	80.4	63.1
DK4B	54.7	75.9
DK4C	127.8	34.0
DK4D	120.4	69.0
DK4E	88.8	68.8
DK4F	114.3	41.8
E	87.3	45.6
F	123.9	53.5
HO1	152.0	29.7
HO10	161.0	84.3
HO3	177.1	89.4
HO7	74.3	7.0
HO9	65.0	73.7
HX10	106.9	67.2
KO9	138.5	29.9
KX2	56.3	34.5
KX5	157.1	61.9
KX7	118.1	69.9
KX9	191.8	48.6

Name	Nanodrop	Qubit
MO10	54.1	53.7
MO5	101.3	0.8
MO9	150.1	64.2
MX1	110.4	77.7
MX10	57.5	31.7
MX5	99.0	23.0
MX7	15.0	0.4
MX8	82.7	72.3
MX9	75.9	51.6
1	311.2	60.6
2	71.7	60.5
3	83.0	50.9
4	67.4	75.0
5	54.6	62.7
QCIA	273.4	57.2
QCIB	53.9	47.8
QCIC	62.9	20.1
QCID	80.1	22.0
QCIE	121.3	0
QCIF	85.0	61.6
QCIG	99.7	55.1
QCIH	68.2	22.9
QCIJ	148.8	75.5
QCIK	229.7	66.3
Tester	61.0	7.4
WO3	59.0	14.3
WO4	54.9	55.6
WO8	211.2	81.7
WO9	53.2	50.8
WX3	158.3	75.4
WX6	93.1	61.5
WX8	112.2	65.8

When looking at the overall functional categorization of all 82 genes, they seem to be most expressed in the cytoplasm and nucleus (Figure 6.2). The most common molecular function ontology are catalytic activity and protein binding (Figure 6.3), and the most common biological processes are “other” and stress and chemical response categories (Figure 6.4).

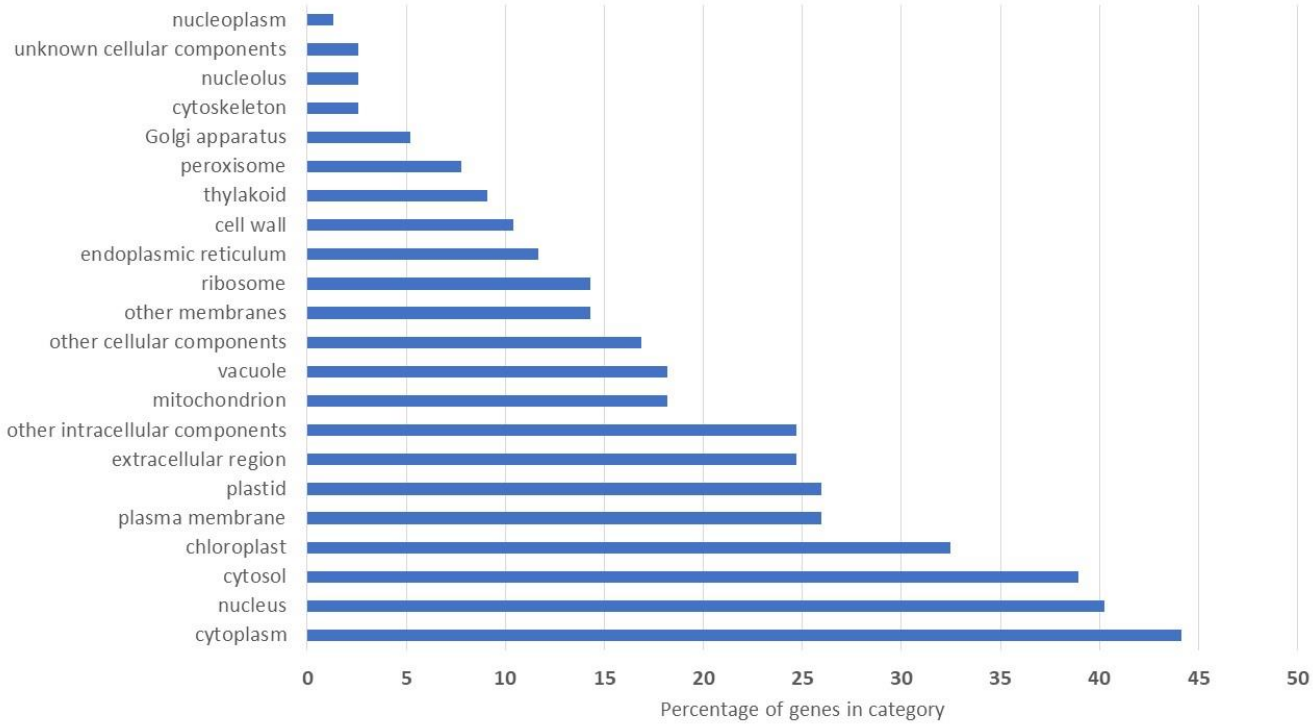

Figure 6.2. Percentage of genes in each category of CCO, calculated by equation 1, for all 82 queries that had a homologue in Sitka spruce (*Picea sitchensis* [Bong.] Carr.).

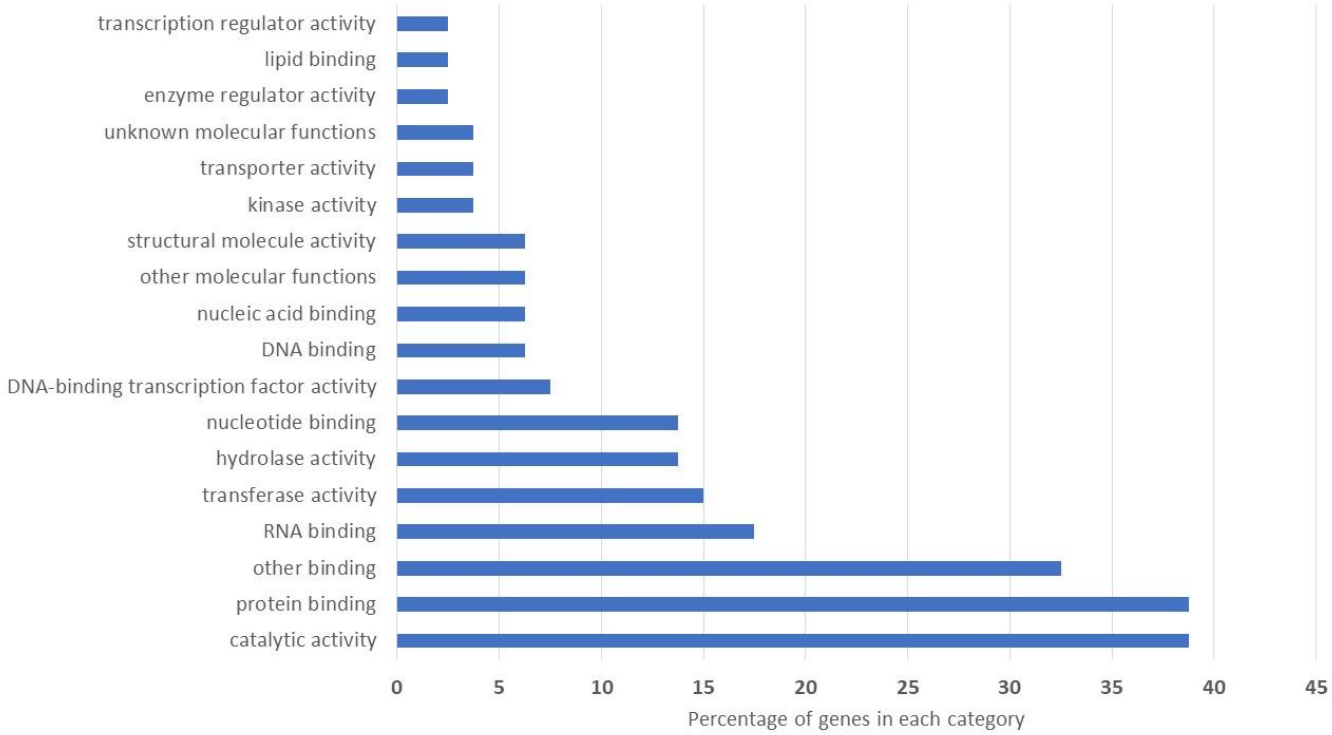

Figure 6.3. Percentage of genes in each category of MFO, calculated by equation 1, for all 82 queries that had a homologue in Sitka spruce (*Picea sitchensis* [Bong.] Carr.).

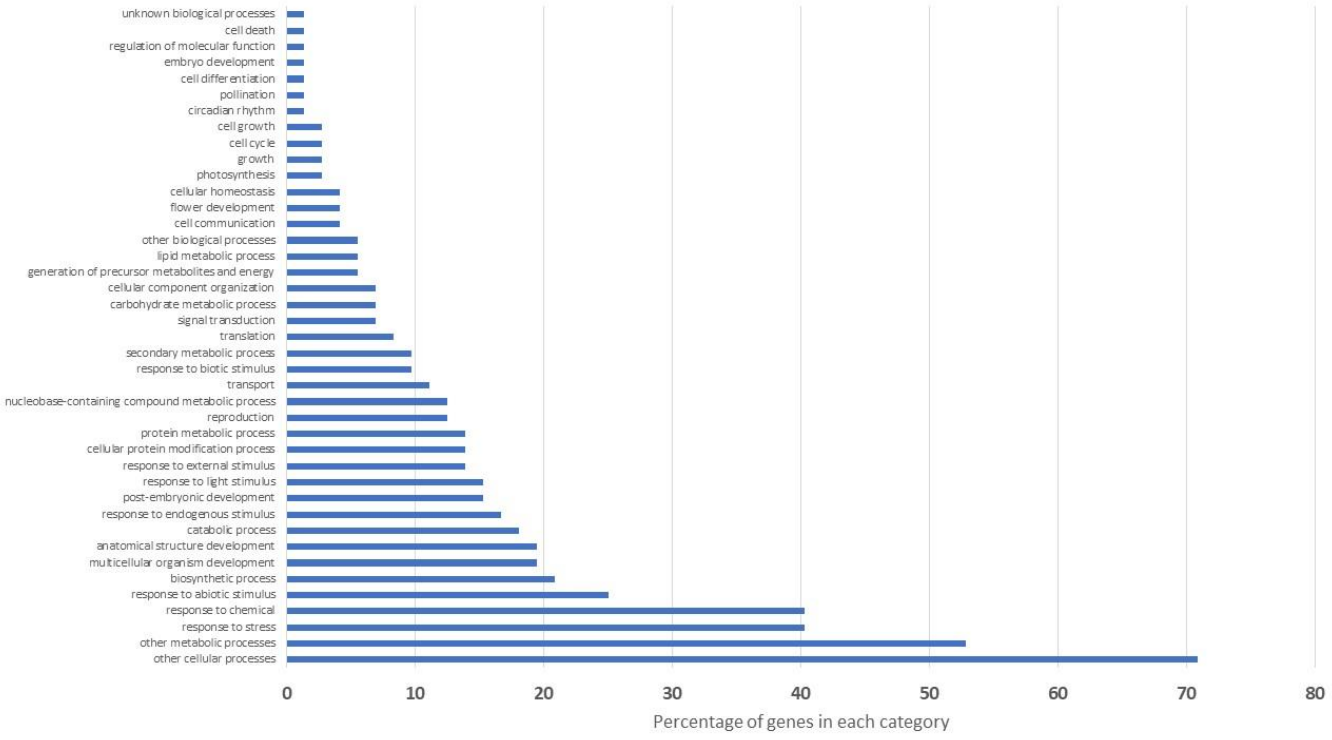

Figure 6.4. Percentage of genes in each category of BPO, calculated by equation 1, for all 82 queries that had a homologue in Sitka spruce (*Picea sitchensis* [Bong.] Carr.).

4. Discussion

4.1. Sitka spruce DNA

The DNA for the 93 samples phenotyped in Chapter 5 was successfully extracted for 83 of the samples (Table 6.5). The samples were analysed with a custom array designed for Sitka spruce by our collaborators at the University of Oxford, led by Prof. John MacKay. The data has not been received yet, so it cannot be analysed for this thesis. The analysis of the data is hypothesised to lead to association of SNPs with the EL phenotypes (although the sample size is low so this may not be achieved), and it could be hypothesised that some of the SNPs may be in the regions that contain genes identified in the literature.

While there was correlation between the concentration of DNA measured by the Qubit and the Nanodrop, this correlation was quite weak. This is probably because the Qubit uses fluorometry, a technique based on detecting fluorescent dyes that bind to the DNA, and the Nanodrop is based on spectrophotometry, which measures UV absorbance at 260 nm, which is much less specific, as DNA, RNA, proteins, free nucleotides, and excess salts all can absorb UV at 260 nm (ThermoFisher Scientific, 2021).

4.2. Literature on conifer frost tolerance genetics

A very limited selection of the frost tolerance literature was systematically selected in this study (Figure 6.1). Many (12 out of 29 rejected papers) were rejected because they used experimental settings that were not cold enough to measure frost tolerance. This comes from a confusion of terms surrounding frost tolerance in the literature.

While a definition is given in the beginning of this chapter for frost tolerance, clearly defining it as referring to temperatures below 0 °C, and distinguishing it from cold tolerance, which could include cold temperatures above 0 °C, such distinctions are not made in the literature. Holliday et al. (2008), for example, use the term ‘cold hardiness’ when referring to frost hardiness, as they use treatment temperatures below 0 °C (ranging between -8 °C and -25 °C) to measure the level of hardiness. La Porta et al. (2015), meanwhile use the term ‘cold acclimation’ to refer to above 0 °C treatments.

‘Frost hardiness’ is an unambiguous term that refers to freezing temperatures, while terms such as ‘cold’ and ‘low temperature’, used when defining search terms (Table 6.1), could mean both freezing and not freezing temperatures. Nevertheless, the frequent use of the term ‘cold’ to refer to ‘frost’ or ‘freezing’ means it cannot be excluded as a search word.

More clarity and adherence to strictly defined terms for frost tolerance and cold tolerance is therefore recommended for all future publications in this field, as these terms each refer to distinct phenomena. Frost involves ice and other physical changes that happen below the freezing temperature of water,

while cold causes damage through other mechanisms (which will also be experienced during frosts, unless hardiness is developed).

4.3. Ontology of imputed Sitka spruce frost tolerance genes

Since this study does not measure gene expression, and the genotyping data is not available, we cannot establish which of the candidate genes identified from the systematic literature survey were present or differentially expressed in our samples. Nevertheless, the comparison with the Sitka spruce genotype allows for the identification of genes that are present in the public NCBI nucleotide (nt) database. Some of the Sitka spruce genes are not present in this database, since even using the queries that have been positively identified by Holliday et al. (2008) to have a homologue in Sitka spruce, no homologue was found in the public database (only 39 out of the 73 queries from this paper were found in the database, Table 6.4). This indicates that a great deal of published data is not available in public datasets. Holliday et al. (2008) did not have a data availability section, nor did they provide any accession numbers that could be used to download the Sitka spruce sequences from any other database.

Thus, the absence of any gene, or the lack of homology between a query and any gene in the Sitka spruce database, is not indicative of the absence of this gene in Sitka spruce. But the genes that are present in the public nucleotide database, and their ontology, could still be interesting to examine.

In a study of gene expression during cold hardening in Sitka spruce, many genes were upregulated, of which the highest proportion encoded various membrane remodelling functions (Holliday et al., 2008). Membrane remodelling is not a category present among the genes identified in this study, neither in the MFO nor in the BPO (Figures 3 and 4). Plasma membrane related genes make up only 26% of the identified genes within the CCO ontology, where the cytoplasm, at 44%, and the cytosol, at 39%, are the main categories within the CCO (Figure 6.2).

Transporter activity genes were also upregulated in Sitka spruce, particularly calcium signalling genes (Holliday et al., 2008). Among the genes identified here, transporter activity genes represented 4% of genes within the MFO, and 11% of genes in the BPO (Figures 3 and 4).

Stress response genes, such as dehydrins and pathogenesis-related genes, were also significantly upregulated in Sitka spruce (Holliday et al., 2008). The study by (Kjellsen et al., 2010) found freezing stress proteins were significantly upregulated in frost-hardened Siberian spruce. Freezing stress proteins, like dehydrins, heat shock proteins, glycine-rich RNA binding proteins, redox-involved cyclophilins, and plasma-membrane related lipocalins were significantly upregulated. Reactive oxygen species proteins were also upregulated. In the identified sample, stress response genes make up 40% of genes in the BPO, coming only after the “other” category of cellular processes and metabolic processes (Figure 6.4). Response to chemicals (40%) and abiotic stimuli (25%) are the categories of

biological processes that come after that. At least within the publicly available database of Sitka spruce nucleotides, most of the frost tolerance related genes that have been identified in other species and have homologues related to different categories of stress.

Among the genes that were downregulated during hardening in Sitka spruce, plastid and thylakoid genes were the main categories, indicating a shift of resources away from photosynthesis (Holliday et al., 2008). Proteins involved in photosynthesis and metabolic synthesis were downregulated in Siberian spruce (Kjellsen et al., 2010). In Douglas fir, translation and protein degradation were the main categories of downregulated genes (Joosen et al., 2006). Plastid (26%) and thylakoid (9%) do not make up the majority of putative frost tolerance genes with homologues in Sitka spruce (Figure 6.2). Photosynthesis-related genes do not seem to be an important category in the BPO among the identified genes (Figure 6.4).

This could be because the query sample is biased against downregulated genes. While Joosen et al. (2006) and Kjellsen et al. (2010) provide a list of downregulated genes that could be downloaded, Holliday et al. (2008) only provided a list of upregulated genes.

Interest in upregulated genes as opposed to downregulated genes is quite understandable. Genes that are upregulated during frost tolerance development seem to play a direct role in frost tolerance development, whereas down-regulated genes are involved in growth and photosynthesis (Joosen et al., 2006). Neither growth nor photosynthesis are negative traits that should be avoided.

When looking for genes or molecular markers that could be used to breed for frost tolerance, it is important to look for those that play a positive role in increasing frost tolerance, but also take into account the entirety of the plant. Breeding for the absence of growth or photosynthesis related genes is unlikely to lead to more productive plants.

5. Conclusion

There is a lack of strict definitions for frost tolerance and cold tolerance in the literature which restricted the number of publications identified in a systematic literature survey to 14, of which only four provided retrievable sequence data. Eighty-two of the 165 genes that have been identified from the four selected publications as having a role in frost tolerance development in conifers were found to have a homologue in the Sitka spruce genome. It is, therefore, expected that some of the same genes could be detected in the GWAS planned for the Sitka spruce trees phenotyped in Chapter 5. Since the samples examined are mostly QCI and QCI descendant trees, as most commercial species in the UK are, they are likely to have frost tolerance genes, as the study by Holliday et al. (2008) found that there is strong selective pressures on Sitka spruce for frost tolerance in British Columbia. Genes identified from published studies as positively correlated with frost tolerance are quite likely to be

related to the cytoplasm, involved in stress response, and have a function in catalytic activity. A notable proportion of genes reported in published studies do not appear in databases.

References

Aitken, S.N., Adams, W.T., 1996. Genetics of fall and winter cold hardiness of coastal Douglas-fir in Oregon. *Can. J. For. Res.* **26**, 1828-1837.

Altschul, S., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402.

Begum, F., Ghosh, D., Tseng, G.C., Feingold, E., 2012. Comprehensive literature review and statistical considerations for GWAS meta-analysis. *Nucleic Acids Res.* **40**, 3777-3784.

Berardini, T.Z., Mundodi, S., Reiser, L., Huala, E., Garcia-Hernandez, M., Zhang, P., Mueller, L.A., Yoon, J., Doyle, A., Lander, G., Moseyko, N., Yoo, D., Xu, I., Zoeckler, B., Montoya, M., Miller, N., Weems, D., Rhee, S.Y., 2004. Functional annotation of the *Arabidopsis* genome using controlled vocabularies. *Plant Physiol.* **135**, 745-755.

Binns, D., Dimmer, E.C., Huntley, R.P., Barrell, D.G., O'Donovan, C., Apweiler, R., 2009. The Gene Ontology - providing a functional role in proteomic studies. *Bioinformatics* **25**, 3045-3046.

Bouck, A., Vision, T., 2007. The molecular ecologist's guide to expressed sequence tags. *Mol. Ecol.* **16**, 907-924.

Carninci, P., Shibata, Y., Hayatsu, N., Sugahara, Y., Shibata, K., Itoh, M., Konno, H., Okazaki, Y., Muramatsu, M., Hayashizaki, Y., 2000. Normalization and subtraction of cap-trapper-selected cDNAs to prepare full-lenght cDNA libraries for rapid discovery of new genes. *Genome Res.* **10**, 1617-1630.

Chen, Q., Li, Y., Tan, K., Qiao, Y., Pan, S., Jiang, T., Chen, Y.-P.P., 2021. Network-based methods for gene function prediction. *Brief. Funct. Genomics* **00**, 1-9.

De La Torre, A.R., Wilhite, B., Puiu, D., St. Clair, J.B., Crepeau, M.W., Salzberg, S.L., Langley, C.H., Allen, B., Neale, D.B., 2021. Dissecting the polygenic basis of cold adaptation using genome-wide association of traits and environmental data in Douglas-fir. *Genes (Basel).* **12**, 1-28.

Eckert, A.J., Bower, A.D., Wegrzyn, J.L., Pande, B., Jermstad, K.D., Krutovsky, K. V., St. Clair, J.B., Neale, D.B., 2009. Association genetics of coastal Douglas fir (*Pseudotsuga menziesii* var. *menziesii*, *Pinaceae*). I. Cold-hardiness related traits. *Genetics* **182**, 1289-1302.

Gill, R.W., Sanseau, P., 2000. Rapid *in silico* cloning of genes using expressed sequence tags (ESTs). *Biotechnol. Annu. Rev.* **5**, 25-44.

Goto, S., Kajiya-Kanegae, H., Ishizuka, W., Kitamura, K., Ueno, S., Hisamoto, Y., Kudoh, H., Yasugi, M., Nagano, A.J., Iwata, H., 2017. Genetic mapping of local adaptation along the altitudinal gradient in

Abies sachalinensis. *Tree Genet. Genomes* **13**, 1-13.

Haddaway, N., Macura, B., Whaley, P., Pullin, A., 2018. ROSES for Systematic Review Protocols. Version 1.0. figshare. Online resource. Accessed 01/05/2021. https://doi.org/https://doi.org/10.6084/m9.figshare.5897269.v4

Holliday, J.A., Ralph, S.G., White, R., Bohlmann, J., Aitken, S.N., 2008. Global monitoring of autumn gene expression within and among phenotypically divergent populations of Sitka spruce (*Picea sitchensis*). *New Phytol.* **178**, 103-122.

Hou, L., Zhao, H., 2013. A review of post-GWAS prioritization approaches. *Front. Genet.* **4**, 1-6.

Illumina, 2021. Read length recommendations. Accessed 01/05/2021. URL: https://emea.illumina.com/science/technology/next-generation-sequencing/plan-experiments/read-length.html

Jermstad, K.D., Bassoni, D.L., Jech, K.S., Ritchie, G.A., Wheeler, N.C., Neale, D.B., 2003. Mapping of Quantitative Trait Loci controlling adaptive traits in coastal Douglas fir. III. Quantitative Trait Loci-by-environment interactions. *Genetics* **165**, 1489-1506.

Jermstad, K.D., Bassoni, D.L., Wheeler, N.C., Anekonda, T.S., Aitken, S.N., Adams, W.T., Neale, D.B., 2001. Mapping of quantitative trait loci controlling adaptive traits in coastal Douglas-fir. II. Spring and fall cold-hardiness. *Theor. Appl. Genet.* **102**, 1152-1158.

Joosen, R.V.L., Lammers, M., Balk, P.A., Bronnum, P., Konings, M.C.J.M.J.M., Perks, M., Stattin, E., van Wordragen, M.F., van der Geest, A.H.M., 2006. Correlating gene expression to physiological parameters and environmental conditions during cold acclimation of *Pinus sylvestris*, identification of molecular markers using cDNA microarrays. *Tree Physiol.* **26**, 1297-1313.

Kassambara, A., 2020. ggpubr: "ggplot2" Based Publication Ready Plots.

Kjellsen, T.D., Shiryaeva, L., Schröder, W.P., Strimbeck, G.R., 2010. Proteomics of extreme freezing tolerance in Siberian spruce (*Picea obovata*). *J. Proteomics* **73**, 965-975.

Korte, A., Farlow, A., 2013. The advantages and limitations of trait analysis with GWAS: a review. *Plant Methods* **9**, 1-9.

La Porta, N., Sablok, G., Emilliani, G., Hietala, A.M., Giovannelli, A., Fontana, P., Potenza, E., Baldi, P., 2015. Identification of low temperature stress regulated transcript sequences and gene families in Italian cypress. *Mol. Biotechnol.* **57**, 1-12.

Ma, Q., Huang, J.G., Hänninen, H., Berninger, F., 2019. Divergent trends in the risk of spring frost damage to trees in Europe with recent warming. *Glob. Chang. Biol.* **25**, 351-360.

Mimura, M., Aitken, S.N., 2007. Adaptive gradients and isolation-by-distance with postglacial migration in *Picea sitchensis*. *Heredity (Edinb).* **99**, 224-232.

Mudunuri, U., Che, A., Yi, M., Stephens, R.., 2009. bioDBnet: the biological database network.

Bioinformatics. Accessed 01/05/2021. https://biodbnet-abcc.ncifcrf.gov/db/db2dbRes.php

Nagy, L.G., Merényi, Z., Hegedüs, B., Bálint, B., 2020. Novel phylogenetic methods are needed for understanding gene function in the era of mega-scale genome sequencing. *Nucleic Acids Res.* **48**, 2209-2219.

O'Neill, G.A., Adams, W.T., Aitken, S.N., 2001. Quantitative genetics of spring and fall cold hardiness in seedlings from two Oregon populations of coastal Douglas-fir. *For. Ecol. Manage.* **149**, 305-318.

Prunier, J., Laroche, J., JEAN, B., Bousquet, J., 2011. Scanning the genome for gene SNPs related to climate adaptation and estimating selection at the molecular level in boreal black spruce. *Mol. Ecol.* **20**, 1702-1716.

R Foundation for Statistical Computing, R Core Team, 2019. R: A language and environment for statistical computing. Accessed 01/05/2021. URL http://www.r-project.org/index.html

Rodríguez-Trelles, F., Tarrío, R., Ayala, F.J., 2005. Is ectopic expression caused by deregulatory mutations or due to gene-regulation leaks with evolutionary potential? *BioEssays* **27**, 592-601.

Roy, S., Chauhan, R., Maheshwari, N., Gupta, S., Gupta, D.K., Sharma, A., 2011. *In silico* approaches in comparative genomics, structure prediction and functional characterization of secondary metabolite proteins of *Mentha* sp. *Plant Omics* **4**, 354-363.

RStudio Team, 2019. RStudio: Integrated Development for R. Accessed 01/05/2021. https://www.rstudio.com/

ThermoFisher Scientific, 2021. Qubit Fluorometric Quantification. Accessed 01/05/2021. URL https://www.thermofisher.com/uk/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/fluorometers/qubit.html

Vangestel, C., Eckert, A.J., Wegrzyn, J.L., St. Clair, J.B., Neale, D.B., 2018. Linking phenotype, genotype and environment to unravel genetic components underlying cold hardiness in coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii*). *Tree Genet. Genomes* **14**, 10.

Wachowiak, W., Balk, P.A., Savolainen, O., 2009. Search for nucleotide diversity patterns of local adaptation in dehydrins and other cold-related candidate genes in Scots pine (*Pinus sylvestris* L.). *Tree Genet. Genomes* **5**, 117-132.

Wang, F., Chen, Song, Liang, D., Qu, G.-Z., Chen, Su, Zhao, X., 2020. Transcriptomic analyses of *Pinus koraiensis* under different cold stresses. *BMC Genomics* **21**, 1-14.

Wang, J., Ma, Z., Carr, S.A., Mertins, P., Zhang, H., Zhang, Z., Chan, D.W., Ellis, M.J.C., Townsend, R.R., Smith, R.D., McDermott, J.E., Chen, X., Paulovich, A.G., Boja, E.S., Mesri, M., Kinsinger, C.R., Rodriguez, H., Rodland, K.D., Liebler, D.C., Zhang, B., 2017. Proteome profiling outperforms transcriptome profiling for coexpression based gene function prediction. *Mol. Cell. Proteomics* **16**, 121-134.

Wang, Z., Gerstein, M., Snyder, M., 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev.*

Genet. **10**, 57-63.

Wellburn, A.R., 1995. Genetic and environmental contributions to the winter hardiness of conifers. *Bot. J. Scotl.* **47**, 193-209.

Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. *J. Comput. Biol.* **7**, 203-214.

Zhao, Y., Wang, J., Chen, J., Zhang, X., Guo, M., Yu, G., 2020. A literature review of gene function prediction by modeling gene ontology. *Front. Genet.* **11**, 1-15.

Chapter 7. Thesis discussion

1. Overview

In this thesis I have examined gymnosperm frost tolerance and the techniques used to measure frost tolerance in gymnosperms, specifically focusing on the application of these methods to discern frost hardiness levels in Sitka spruce (*Picea sitchensis* [Bong.] Carr.) and their genetic basis. After a brief introduction (Chapter 1) I examined the mechanisms of frost damage and frost tolerance of gymnosperms in the literature review (Chapter 2), where I described the known changes that occur in conifers in response to frost. I also detailed the physiological processes by which gymnosperms lose and acquire frost hardiness, and the abiotic and biotic factors that influence it. There seems to be a sufficient basis from the literature to think that, while the environment affects frost tolerance, genetics also matter for frost tolerance, especially spring frost tolerance (Aitken and Adams, 1997; O'Neill et al., 2001), confirming my initial hypothesis that differences in frost tolerance are due to underlying genetic differences. Identifying genes that can confer frost tolerance would be beneficial to commercial foresters by improving yield and decreasing risk. The objective of the examination of future risks of frost damage with climate change on Sitka spruce (Chapter 3), was to estimate the effects of climate change on Sitka spruce frost damage and examine the factors breeders need to take into account to avoid future frost damage.

Despite the motivation for this research project partly being a perceived increased risk of frost damage, modelling of predicted climate change scenarios showed a reduced risk of frost damage. This confirmed the hypothesis that climate change influences frost damage, reducing the incidence of frost damage. Although such an effect was also predicted in other studies, there was a high degree of difference between species, with some species being affected positively and some negatively (Ma et al., 2019; Morin and Chuine, 2014). While other factors, such as increases due to warmer temperatures in pests like pine weevil (*Hylobius abietis* L.) which finds in Sitka spruce an alternate host (Inward et al., 2012) may worsen the long-term viability of Sitka spruce plantations in GB, the warming of temperatures and the consequent decrease in frost hardiness rate appear to be beneficial or neutral. It is also of interest to note that phenology-related frost damage has not historically been a factor in UK Sitka spruce plantations, and this research revealed that it will not become one. As many studies on frost tolerance genetics and mechanisms focus on spring phenology without studying frost hardiness on its own (Ma et al., 2019; Morin and Chuine, 2014), it is important to note that for Sitka spruce, breeding for later bud burst date will not lead to a decrease in frost damage.

In addition to the main findings, *i.e.* that frost damage in Sitka spruce will decrease with climate change, and that phenology-related frost damage is practically non-existent in GB, it was also found that

downscaled, more detailed climate models can help estimate future regional trends better, as regional differences were erased when a global model was used. The south of GB, which historically experiences fewer frosts, is predicted to have a bigger decrease than the more frost-prone north. Overall, climate change will reduce frosts, but this change will be smaller in the areas that experience the most frost.

Using a systematic mapping approach, I critically examined the methodological factors that should be considered when designing a study to measure frost tolerance (Chapter 4), observing which techniques were used in the literature, and the constraints of each method. There are three parts to the frost tolerance measurement: growing the plants, the freezing treatment, and the measurement of damage. Each of these steps has its own issues. Growing plants in the field means less control over growing conditions than in a greenhouse or growth chamber, but these growing conditions will be closer to those experienced by trees experiencing frosts in nature. Using natural frosts as treatment will give closer results to the conditions in nature, but will require more time and replication, as frosts cannot be obtained on demand. Artificial freezing treatments given in the lab, on the other hand, do not face the same time constraints, but require careful managing to ensure artificial freezing conditions are close to natural ones. Measurement techniques, which include visual assessment, electrolyte leakage, fluorometry, and differential thermal analysis, differ on what they measure, and thus obtain results that, while correlated with field observation studies, do not necessarily match them. I classified the studies depending on the technique used, factors controlled, and how well they compared to field studies, and for studies that used both field and lab studies, how the two techniques correlated. This methodological information gleaned from this chapter was used to inform the selection of the frost tolerance measurement technique. Precautions were taken to control the freezing rate, keeping it below 5 K h^{-1}, as it was seen from this review that controlling the freezing rate is very important. Knowledge obtained from hands-on experience in frost tolerance assessments was used to re-evaluate the literature.

In addition to modelling the effects of climate change, I also aimed at measuring the frost tolerance of individual genotypes and identify populations that exhibited different patterns of frost tolerance, so they could be used in a GWAS study. To achieve this goal, I investigated the susceptibility to frost damage in Sitka spruce by phenotyping using an electrolyte leakage assay with samples collected in the field, in addition to samples obtained from a commercial nursery, our industry partner, Maelor Forest Nurseries Ltd. (Chapter 5). I did not find agreement or correlation between observed incidence of frost damage in the field and a laboratory assessment using electrolyte leakage measurements of frost damage susceptibility, nor did I find distinct differences between populations. This could be because almost all samples are descendants from the same limited group of individuals that were

imported into the UK, and later used for breeding. This could mean that, due to the reduced number of alleles in the population after the importation bottleneck, the different varieties of Sitka spruce do not differ in these alleles, as they were not selectively bred for frost tolerance. As individuals genes explain as little as 1-3% of the variation (Eckert et al., 2009), finding differences between groups that share most of the same genes because they are descendants of the same original population will be complicated.

I planned to analyse the SNPs present in the samples phenotyped in Chapter 5, by using an external commercial genotyping service, in collaboration with Professor MacKay's group at the University of Oxford (Chapter 6). Due to the COVID-19 pandemic the analysis pipeline was shut down and unfortunately, I have not received data to conduct an analysis. I was thus unable to test the second initial hypothesis of this thesis, that genetic differences could be found by measuring the frost tolerance of individuals and linking them to a SNP.

In order to find whether there was a genetic basis for frost tolerance in Sitka spruce, I examined the literature on possible candidate genes for conifer frost tolerance and identified their homologue in the publicly available Sitka spruce genetic databases. I identified 4 conifer studies revealing 165 genes associated with frost tolerance. Of these, 82 possible candidate genes represented in Sitka spruce could be analysed to determine their function in relation to frost tolerance. Stress-related genes were found to be the most common category of genes identified by biological process ontology. Most genes were expressed in the cytoplasm and nucleus, and catalytic activity and protein binding were the main categories of molecular functions of the frost tolerance related genes. This desk-based analysis suggested that frost tolerance in conifers is a complex trait controlled by many genes with a wide variety of functions and roles. However, it is noteworthy that a notable proportion of genes reported in published studies do not appear in databases, preventing their inclusion in this analysis.

2. Discussion

Genome-Wide Association Studies (GWASs) can serve to study the genetic underpinnings of complex traits by associating molecular markers linked to putative causative genetic loci to a particular phenotype. While most GWASs are conducted with targeted genotyping of previously identified SNPs, association studies that utilise Whole Genome Sequencing (WGS) to identify variant SNPs are also considered GWAS. These studies are only distinguished from association studies that use SNPs by DNA sequence coverage and the ability to detect minor frequency alleles (Visscher et al., 2017). Since GWAS that use SNPs can detect allele variants present as rarely as 1 in 1000 samples, the advantage in statistical power gained from performing GWAS with WGS in comparison to SNPs is very minor.

The potential of a GWAS to identify genetic loci correlated with a trait depends on the population being studied, the joint distribution of effect size, the number of loci and allele frequency, and the distribution of the alleles in the population (Visscher et al., 2017).

Given a sample size of 100, 80% power, and type I error (α) frequency of 5×10^{-8}, only alleles present in 10% of the population with effect size (β) 1 can be detected (Visscher et al., 2017). Therefore, the sample size used in this study is insufficient to detect anything but very frequent alleles with large effects (> 0.5) on phenotype. A minimum of 1000 genotypes would be needed to detect alleles with smaller effect sizes (< 0.1). Using a large sample size is crucial in identifying markers with a small effect size (Du et al., 2018). An effect size of 0.5 would indicate that the QTL explains 50% of the variation, something that would only happen with a gene with a very large effect.

GWASs started to be used with sample sizes in the thousands in the year 2007, with seminal studies in human genotypes such as the one conducted by the Wellcome Trust on several diseases (Burton et al., 2007) and a Canadian GWAS study on type II diabetes (Sladek et al., 2007). More recently GWASs have been conducted on tree genomes. A study in Japanese cedar (*Cryptomeria japonica* [Thunb. ex L.f.] D. Don) on growth traits, wood properties and reproductive traits was conducted with 476 samples and 32,036 polymorphic SNPs, showing that using a larger number of SNPs served to identify more SNPs with significant effects (Hiraoka et al., 2018). This study identified 5 SNPs that were significantly associated with wood stiffness, 4 with growth, and 4 with reproductive traits. Another study in Japanese cedar identified 5 SNPs that were significant for different wood properties using 367 samples and a 1,032 polymorphic SNPs (Uchiyama et al., 2013). This shows that the number of samples is more important than the number of molecular markers used in the study to identify a locus. As reviewed by Du et al. (2018), low-frequency SNPs played an important role in identifying bioenergy traits in *Populus deltoides* W. Bartram ex Marshall.

Although DNA sequencing data was not obtained in the work presented in this thesis, the small sample size of 96 would be insufficient to find anything but very frequently present alleles with large effects. Few loci could be identified with this approach, and a larger sample size would certainly be beneficial in identifying more loci. A review by Visscher et al. (2017) on GWAS studies in humans concluded that complex traits are mostly coded by multiple loci, and the same happens with trees (Chen et al., 2021), with larger sample sizes leading to the discovery of more loci in human genomes (Visscher et al., 2017). Cold tolerance is a type of adaptation to the local climate, and such adaptations have a notably complex genetic architecture, as shown in an study of Loblolly pine (De La Torre et al., 2019). Climate adaptations in loblolly pine are mostly formed by the addition of genes with moderate to small effect sizes, with few genes of large effect. Genes associated with climate adaptations were located in regions of low recombination and high population differentiation, suggesting that climate

adaptations, including frost tolerance, have evolved as a result of different selection pressures acting on groups of genes rather than the effect of individual environmental variables. Thus, cold tolerance would have co-evolved with tolerance to drought and heat, optimizing overall survival rather than higher cold tolerance.

A GWAS study on the cold tolerance of Douglas fir (*Pseudotsuga menziesii* (Mirb). Franco) found 582 SNPs significantly correlated with cold tolerance, 225 of them located in coding regions, using an Illumina Infinium array containing 20k SNPs on DNA extracted out of seeds from 288 open pollinated trees (De La Torre et al., 2021). Needle frost tolerance, the trait studied in this thesis, was associated with a large (>1000) number of SNPs in the study by De la Torre et al. (2021), most of them with minor effects. A high proportion of SNPs related to cold adaptation were in non-coding regions in Douglas fir.

Identifying molecular markers for complex traits is intrinsically difficult, and could be improved by using more data (Chen et al., 2021). Genome-wide association studies in tree species are mostly limited by the availability of good quality phenotypic data. A way to overcome this problem is to use data collected from breeding programmes, as was done with data collected from a Norway spruce (*Picea abies* L. Karst) breeding programme by Chen et al. (2021), which identified 55 QTLs for commercially important properties (phenology, height, wood properties and frost tolerance), 3 of which were for frost tolerance (5 genes identified). Data from breeding programmes are highly heterogeneous; the study by Chen et al. (2021), the largest reported study for trees to date, had 4138 genotypes with associated phenology data and only 1428 genotypes associated with frost damage data (out of 5056 parental trees).

Sitka spruce has undergone an extensive breeding process, and there has been a lot of phenotypic data collected on many different Sitka spruce phenotypes. The group we were collaborating with in the University of Oxford was working on genotyping many of these trees that had been part of the breeding programme to associate many traits related to growth and wood properties to SNPs. Many complex traits are associated with each other (De La Torre et al., 2019), as frost tolerance seems to be with phenology (Chen et al., 2021), and thus leveraging already existing phenotypic data to discover unknown relationships between frost tolerance and other traits would make such GWAS more powerful. Thus, molecular markers correlated with frost tolerance could be significantly correlated with other traits, such as growth or drought tolerance, revealing the complex and interlinked nature of the regulation of frost tolerance.

3. Conclusion

While there is evidence to suggest a genetic basis for frost tolerance in Sitka spruce, as there are frost tolerance associated genes in the Sitka spruce genome, identifying genes that confer frost tolerance is far from trivial. Because of the complex nature of frost damage, tolerance, hardening and dehardening, it is hard to find a strong genetic effect, as most genes have a very small effect, and may be overshadowed by environmental effects. Genes that affect frost tolerance are present in Sitka spruce (hypothesis i) and can be identified by homology to other genes. Thus, finding genes for frost tolerance should be possible, although it requires rigorous testing for the phenotype, and to use a large and diverse enough population (hypothesis ii).

The effects of climate change on frost tolerance on Sitka spruce plantations in the UK will be positive (hypothesis iii), at least when basing these predictions just on the predicted levels of frost damage. This, however, does not mean that the need for breeding for frost tolerance will disappear.

4. Future work

Once the SNP data for the tested phenotypes is received, the data should be studied for association with the frost damage traits measured in the lab, to see whether any SNP is associated with it. If molecular markers are found, the area around the SNPs should be investigated, to find if there is any gene that is a likely candidate for the desired trait.

I recommend future researchers trying to identify frost tolerance in Sitka spruce use a bigger testing population if budget allows, as there would be a better chance of finding suitable SNPs. When measuring frost tolerance, the factors I mentioned in Chapter 4 should be taken into account, in order to obtain results that closely resemble field results. If budget allows, equipment to control the frost exposure time and a better control of freezing and thawing times would be advisable. Using temperatures below -20 °C will also allow distinguishing between trees that have a frost tolerance below -20 °C.

Although it might be tempting to genotype samples solely from field studies without phenotyping in controlled laboratory conditions, it is not possible to reliably infer the level of frost tolerance from a single natural frost damage event. In the absence of rigorous data collected from repeated cases of frost damage occurrence, reproducibility in laboratory conditions is strongly recommended. The lack of diversity in commercial Sitka spruce trees grown in the UK could be problematic when trying to identify rare alleles, as there would have been a great reduction in genetic diversity upon the introduction of Sitka spruce into the UK. Thus, it is recommended that a larger geographic pool of Sitka spruce trees from wild origins over the entire natural range, from California to Alaska, be used to compare the populations and identify possible candidates.

References

Aitken, S.N., Adams, W.T., 1997. Spring cold hardiness under strong genetic control in Oregon populations of *Pseudotsuga menziesii* var. *menziesii*. *Can. J. For. Res.* **27**, 1773-1780.

Burton, P.R., Clayton, D.G., Cardon, L.R., Craddock, N., Deloukas, P., Duncanson, A., Kwiatkowski, D.P., McCarthy, M.I., Ouwehand, W.H., Samani, N.J., Todd, J.A., Donnelly, P., Barrett, J.C., Davison, D., Easton, D., Evans, D., Leung, H.T., Marchini, J.L., Morris, A.P., Spencer, C.C.A., Tobin, M.D., Attwood, A.P., Boorman, J.P., Cant, B., Everson, U., Hussey, J.M., Jolley, J.D., Knight, A.S., Koch, K., Meech, E., Nutland, S., Prowse, C. V., Stevens, H.E., Taylor, N.C., Walters, G.R., Walker, N.M., Watkins, N.A., Winzer, T., Jones, R.W., McArdle, W.L., Ring, S.M., Strachan, D.P., Pembrey, M., Breen, G., St. Clair, D., Caesar, S., Gordon-Smith, K., Jones, L., Fraser, C., Green, E.K., Grozeva, D., Hamshere, M.L., Holmans, P.A., Jones, I.R., Kirov, G., Moskvina, V., Nikolov, I., O'Donovan, M.C., Owen, M.J., Collier, D.A., Elkin, A., Farmer, A., Williamson, R., McGuffin, P., Young, A.H., Ferrier, I.N., Ball, S.G., Balmforth, A.J., Barrett, J.H., Bishop, D.T., Iles, M.M., Maqbool, A., Yuldasheva, N., Hall, A.S., Braund, P.S., Dixon, R.J., Mangino, M., Stevens, S., Thompson, J.R., Bredin, F., Tremelling, M., Parkes, M., Drummond, H., Lees, C.W., Nimmo, E.R., Satsangi, J., Fisher, S.A., Forbes, A., Lewis, C.M., Onnie, C.M., Prescott, N.J., Sanderson, J., Mathew, C.G., Barbour, J., Mohiuddin, M.K., Todhunter, C.E., Mansfield, J.C., Ahmad, T., Cummings, F.R., Jewell, D.P., Webster, J., Brown, M.J., Lathrop, G.M., Connell, J., Dominiczak, A., Braga Marcano, C.A., Burke, B., Dobson, R., Gungadoo, J., Lee, K.L., Munroe, P.B., Newhouse, S.J., Onipinla, A., Wallace, C., Xue, M., Caulfield, M., Farrall, M., Barton, A., Bruce, I.N., Donovan, H., Eyre, S., Gilbert, P.D., Hider, S.L., Hinks, A.M., John, S.L., Potter, C., Silman, A.J., Symmons, D.P.M., Thomson, W., Worthington, J., Dunger, D.B., Widmer, B., Frayling, T.M., Freathy, R.M., Lango, H., Perry, J.R.B., Shields, B.M., Weedon, M.N., Hattersley, A.T., Hitman, G.A., Walker, M., Elliott, K.S., Groves, C.J., Lindgren, C.M., Rayner, N.W., Timpson, N.J., Zeggini, E., Newport, M., Sirugo, G., Lyons, E., Vannberg, F., Hill, A.V.S., Bradbury, L.A., Farrar, C., Pointon, J.J., Wordsworth, P., Brown, M.A., Franklyn, J.A., Heward, J.M., Simmonds, M.J., Gough, S.C.L., Seal, S., Stratton, M.R., Rahman, N., Ban, S.M., Goris, A., Sawcer, S.J., Compston, Alastair, Conway, D., Jallow, M., Rockett, K.A., Bumpstead, S.J., Chaney, A., Downes, K., Ghori, M.J.R., Gwilliam, R., Hunt, S.E., Inouye, M., Keniry, A., King, E., McGinnis, R., Potter, S., Ravindrarajah, R., Whittaker, P., Widden, C., Withers, D., Cardin, N.J., Ferreira, T., Pereira-Gale, J., Hallgrimsdóttir, I.B., Howie, B.N., Spencer, C.C.A., Su, Z., Teo, Y.Y., Vukcevic, D., Bentley, D., Compston, Alistair, 2007. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* **447**, 661–678.

Chen, Z.-Q., Zan, Y., Milesi, P., Zhou, L., Chen, J., Li, L., Cui, B., Niu, S., Westin, J., Karlsson, B., García-Gil, M.R., Lascoux, M., Wu, H.X., 2021. Leveraging breeding programs and genomic data in Norway spruce (*Picea abies* L. Karst) for GWAS analysis. *Genome Biol.* **22**, 1-30.

De La Torre, A.R., Wilhite, B., Neale, D.B., Slotte, T., 2019. Environmental Genome-Wide Association reveals climate adaptation is shaped by subtle to moderate allele frequency shifts in Loblolly Pine. *Genome Biol. Evol.* **11**, 2976–2989.

De La Torre, A.R., Wilhite, B., Puiu, D., St. Clair, J.B., Crepeau, M.W., Salzberg, S.L., Langley, C.H., Allen, B., Neale, D.B., 2021. Dissecting the polygenic basis of cold adaptation using genome-wide association of traits and environmental data in Douglas-fir. *Genes (Basel)*. **12**, 1–28.

Du, Q., Lu, W., Quan, M., Xiao, L., Song, F., Li, P., Zhou, D., Xie, J., Wang, L., Zhang, D., 2018. Genome-wide association studies to improve wood properties: Challenges and prospects. *Front. Plant Sci.* **871**, 1–10.

Eckert, A.J., Bower, A.D., Wegrzyn, J.L., Pande, B., Jermstad, K.D., Krutovsky, K. V., St. Clair, J.B., Neale, D.B., 2009. Association genetics of coastal Douglas fir (*Pseudotsuga menziesii* var. *menziesii*, *Pinaceae*). I. Cold-hardiness related traits. *Genetics* **182**, 1289-1302.

Hiraoka, Y., Fukatsu, E., Mishima, K., Hirao, T., Teshima, K.M., Tamura, M., Tsubomura, M., Iki, T., Kurita, M., Takahashi, M., Watanabe, A., 2018. Potential of genome-wide studies in unrelated plus trees of a coniferous species, *Cryptomeria japonica* (japanese cedar). *Front. Plant Sci.* **9**, 1–15.

Inward, D.J.G., Wainhouse, D., Peace, A., 2012. The effect of temperature on the development and life cycle regulation of the pine weevil *Hylobius abietis* and the potential impacts of climate change. *Agric. For. Entomol.* **14**, 348-357.

Ma, Q., Huang, J.G., Hänninen, H., Berninger, F., 2019. Divergent trends in the risk of spring frost damage to trees in Europe with recent warming. *Glob. Chang. Biol.* **25**, 351-360.

Morin, X., Chuine, I., 2014. Will tree species experience increased frost damage due to climate change because of changes in leaf phenology? *Can. J. For. Res.* **44**, 1555-1565.

O'Neill, G.A., Adams, W.T., Aitken, S.N., 2001. Quantitative genetics of spring and fall cold hardiness in seedlings from two Oregon populations of coastal Douglas-fir. *For. Ecol. Manage.* **149**, 305-318.

Sladek, R., Rocheleau, G., Rung, J., Dina, C., Shen, L., Serre, D., Boutin, P., Vincent, D., Belisle, A., Hadjadj, S., Balkau, B., Heude, B., Charpentier, G., Hudson, T.J., Montpetit, A., Pshezhetsky, A. V., Prentki, M., Posner, B.I., Balding, D.J., Meyre, D., Polychronakos, C., Froguel, P., 2007. A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature* **445**, 881–885.

Uchiyama, K., Iwata, H., Moriguchi, Y., Ujino-Ihara, T., Ueno, S., Taguchi, Y., Tsubomura, M., Mishima, K., Iki, T., Watanabe, A., Futamura, N., Shinohara, K., Tsumura, Y., 2013. Demonstration of genome-wide association studies for identifying markers for wood property and male strobili traits in *Cryptomeria japonica*. *PLoS One* **8**, 1-12.

Visscher, P.M., Wray, N.R., Zhang, Q., Sklar, P., McCarthy, M.I., Brown, M.A., Yang, J., 2017. 10 Years of GWAS discovery: biology, function, and translation. *Am. J. Hum. Genet.* **101**, 5–22.

Appendix Chapter 3

Table 3.S1. Frost damage values for forests in Great Britain, in the spring of year 2015. Easting/northing coordinates given in OSGB format.

Forest	Easting	Northing	Percentage
Brenig Llyn Bran	303380	960075	60
Black Esk	322639	595041	3
Ormidale	200266	681761	0
Three Bridges	525708	164572	0
Brownhills	403344	315890	10
Blazehill	313483	594397	90
Watermeetings	294849	613523	0
Kirtleton	328437	581413	75
Billholm	325698	599223	63
Lambdoughty	224784	592305	15
Glengennet	228496	596188	8
Bogrie	279543	587372	0
Waterhead	337860	503097	70
Marnhoul	376194	119269	25
Rashiegrain	333512	602682	8
Dykeraw	361834	608606	55
Monynut	368710	668778	0
Bryn Coch	323180	364633	3
Llandegla	321889	351416	70
Craigengillan North	246755	603071	80
Singdean	357798	601690	50
Rhyd Y Felin	308965	194337	3
Garwald Complex	322741	601241	3
Glendearg	351661	637788	0
Strone East	218158	682231	0
White Lyne	354202	578316	50
Carmacoup	278747	628866	0
Calton Forest	459768	383757	20
Cowans Law	250648	640509	15
Rhyd Wen	292003	330537	50
Mid Knock	353079	853033	5
Dollar law	317155	629645	60
Rascarrel	279884	549906	5
Maesycilyn	273400	297900	0
Riccarton	242200	634500	15
Stonedge	433200	368100	0
Hewisbridge	241500	566500	16
Lamloch and Drumjohn	257000	597674	10
Minnygryle	272200	587527	25
Milldown	382500	109700	0

Table 3.S2. Average number of days with temperatures below the threshold temperature between the onset of dehardening and the end of summer for each seed zone, according to historical data between 1960-2015.

		Index $1_{0°C}$			Index $1_{-3°C}$			Index $1_{-5°C}$		
Provenance zone	**Seed zones**	**1960-1979**	**1980-1999**	**2000-2015**	**1960-1979**	**1980-1999**	**2000-2015**	**1960-1979**	**1980-1999**	**2000-2015**
10	**101**	22.87	18.53	16.8	4.78	2.6	3.07	1.11	0.42	0.98
	102	32.82	31.94	33.42	9	7.35	9.59	3.5	2.34	3.52
	103	23.48	16.97	15.26	5.31	2.11	1.63	1.58	0.41	0.13
	104	22.82	20.51	19.93	4.6	3.01	2.74	1.23	0.59	0.36
	105	32.97	33.41	31.63	9.74	8.44	8.48	3.92	2.7	2.52
	106	27.28	27.56	27.76	6.8	5.91	6.24	2.28	1.56	1.51
	107	29.3	28.34	28.12	8.27	6.48	6.55	3.06	1.82	1.52
	108	27.65	26.42	27.89	7.17	5.9	6.93	2.44	1.51	1.68
	109	32.28	31.72	32.32	9.25	8.12	8.9	3.38	2.29	2.63
20	**201**	33.97	34.89	36.63	10.38	9.58	11.3	4.27	3.49	4
	202	30.91	31.85	32.34	8.03	7.86	8.24	2.79	2.39	2.51
	203	28.69	27.32	28.28	7.09	5.38	6.11	2.52	1.43	1.43
	204	27.92	27.53	25.94	6.72	5.77	5.42	2.39	1.56	1.3
30	**301**	26.9	25.09	27.45	6.81	6.18	7.55	2.23	1.79	2.25
	302	25.73	25.43	26.46	5.94	5.77	6.44	1.89	1.64	1.71
	303	22.54	22.6	23.71	5.2	5.62	5.34	1.61	1.94	1.3
	304	31.97	31.39	31.34	8.34	8.53	8.83	2.82	3.15	2.6
	305	20.21	19.77	18.41	4.23	4.52	3.18	1.11	1.53	0.54
40	**401**	23.99	26	24.15	4.42	5.1	4.92	1.33	1.41	1.25
	402	24.6	27.03	23.93	5.03	6.84	4.83	1.66	2.45	1.22
	403	26.1	27.95	26.23	5.71	7.49	6.24	1.88	2.75	1.39
	404	24.57	26.78	25.54	5.76	7.23	5.51	1.86	2.57	1.35
	405	22.94	26.35	23.94	4.69	6.87	5.08	1.52	2.44	1.3
	406	22.9	25.53	23.86	3.94	6.21	4.76	1.32	2.22	1.31

Table 3.S3. Average value of indices for GB for all climate models.

	CMCC-CM RCP 4.5	CMCC-CM RCP 8.5	UKCP09	UKCP18 Global	UKCP18	Historical
Emissions scenario	RCP 4.5	RCP 8.5	SRESA1B	RCP 8.5	RCP 8.5	
Scale model	Global	Global	Downscaled	Global	Downscaled	
Scale km	83	83	25	60	12	5
Index $1_{0°C}$	22.01	16.94	19.14	9.79	13.55	26.71
Index $1_{-3°C}$	7.7	5.03	5.21	1.3	2.27	6.38
Index $1_{-5°C}$	3.25	1.85	1.49	0.44	0.91	2.01
Index 2	0	0.0002	0.008	0.0007	0.003	0.02
Index 2_{min}	0	0	0.0003	0	0	0.004
Index 2_{max}	0.003	0.004	0.06	0.002	0.005	0.02
Index 3	5.78	4.93	15.62	6.39	8.74	9.19
Return time post bud burst frost* <0 °C	79	79	82	78	58	55
Return time post bud burst frost* <-3 °C	79	79	82	78	58	55
Return time post bud burst frost* <-5 °C	79	79	82	78	58	55

1. Number of frosts during growing season below threshold temperature.
2. Number of frosts after bud burst below -3 °C
3. Number of backlashes (days with minimum temperatures below the calculated frost tolerance levels) during the year.

*Return time here has a maximum limit equal to the length of the period, which differs between datasets due to data availability and file format. Our results indicate that the return time is equal to the maximum for all post bud burst frosts.

Table 3.S4. Average values of estimated bud burst values for each climate model, calculated by the GDD model.

			UKCP18 Global	UKCP18	CMCC-CM RCP 8.5	UKCP09	CMCC-CM RCP 4.5	Historical
	Emissions scenario		RCP 8.5	RCP 8.5	RCP 8.5	SRESA1B	RCP 4.5	
	Scale model		Global	Downscaled	Global	Downscaled	Global	
	Scale km		60	12	83	25	83	5
	Chill days	**Number of days**	86.43	90.7	96.87	95.69	103.08	112.97
	Estimated bud burst dates	**Julian dates**	81.2	90.88	131.55	120.37	135.86	135.73
		Date	22nd March	1st April	12th May	30th April	16th May	16th May
10	**Late estimated bud burst dates**	**Julian dates**	115.52	122.59	150.47	139.99	153.3	151.69
		Date	26th April	3rd May	30th May	20th May	2nd June	1st June
	Early estimated bud burst dates	**Julian dates**	73.19	84.69	116.42	104.15	122.11	135.73
		Date	14th March	26th March	26th April	14th April	2nd May	16th May
	Chill days	**Number of days**	92.54	99.54	115.6	101.35	127.64	124.67
	Estimated bud burst dates	**Julian dates**	90.76	93.1	134.9	120.67	136.74	134.39
		Date	1st April	3rd April	15th May	1st May	17th May	14th May
20	**Late estimated bud burst dates**	**Julian dates**	120.89	122.33	149.11	138.64	148.81	148.92
		Date	1st May	2nd May	29th May	19th May	29th May	29th May
	Early estimated bud burst dates	**Julian dates**	85.52	89.95	124.23	105.47	127.66	134.39
		Date	27th March	31st March	4th May	15th April	8th May	14th May
	Chill days	**Number of days**	85.35	86.37	90.4	86.83	94.28	97.73
	Estimated bud burst dates	**Julian dates**	63.32	74.86	122.31	107.21	128.48	127.45
		Date	4th March	16th March	2nd May	17th April	8th May	7th May
30	**Late estimated bud burst dates**	**Julian dates**	97.85	108.26	140.81	128.38	145.27	145.94
		Date	8th April	18th April	21st May	8th May	22nd May	26th May
	Early estimated bud burst dates	**Julian dates**	55.22	67.03	107.19	89.53	114.54	127.45
		Date	24th February	8th March	17th April	30th March	25th April	7th May
	Chill days	**Number of days**	85.14	85.14	88.7	85.51	91.7	90.17
	Estimated bud burst dates	**Julian dates**	59.19	66.39	120.39	105.63	126.55	122.34
		Date	28th February	7th March	30th April	16th April	7th May	2nd May
40	**Late estimated bud burst dates**	**Julian dates**	93	100.11	138.44	126.24	142.96	141.53
		Date	3rd April	10th April	18th May	7th May	23rd May	21st May
	Early estimated bud burst dates	**Julian dates**	51.43	58.27	105.64	87.68	112.91	122.34
		Date	20th February	27th February	16th April	29th March	23rd April	2nd May
	Chill days	**Number of days**	86.85	88.87	95.75	90.41	101.3	102.36
	Estimated bud burst dates	**Julian dates**	71.11	77.98	126.05	111.45	131	128.38
		Date	12th March	19th March	6th May	21st April	11th May	8th May
GB	**Late estimated bud burst dates**	**Julian dates**	104.53	110.49	143.72	131.59	146.97	145.97
		Date	15th April	20th April	24th May	12th May	27th May	26th May
	Early estimated bud burst dates	**Julian dates**	63.68	71.12	111.85	94.35	118.07	128.38
		Date	5th March	12th March	22nd April	4th April	28th April	8th May

Table 3.S5. Average number of chill days (days with mean temperatures below 5 °C) between the 1st November and bud burst, and the estimated bud burst date (given in Julian days) for each seed zone, according to historical data between 1960-2015.

		Chill days			Estimated bud burst date		
Provenance zone	**Seed zones**	**1960-1979**	**1980-1999**	**2000-2015**	**1960-1979**	**1980-1999**	**2000-2015**
10	**101**	113.93	107.04	92.01	148.57	148.38	147.81
	102	127.7	119.06	110.7	139.09	136.49	134.27
	103	97.14	93.51	88.82	140.15	139.89	132.28
	104	103.14	97.91	94.11	139.93	137.44	133.46
	105	130.39	124.5	118.45	139.35	136.82	133.67
	106	120.93	114.7	109.28	139.77	137.39	134.52
	107	113.71	104.54	98.24	133.14	131.425	128.83
	108	111.53	103.72	98.54	136.98	134.09	130.88
	109	127.65	117.21	108.86	135	132.27	130.52
20	**201**	143.16	134.73	127.4	140.22	136.36	133.64
	202	143.64	134.65	127.1	140.1	136.45	134.87
	203	118.21	107.08	99.92	131.59	130.12	128.17
	204	115.81	104.62	97.69	132.65	130.22	127.11
30	**301**	111.71	102.18	98.3	134.31	131.87	129.35
	302	114.17	105.17	99.24	132.29	128.36	125.86
	303	94.39	90.76	88.71	132.18	126.82	122.71
	304	111.52	102	96.72	134.62	130.61	128.6
	305	87.51	86.14	85.59	127.21	121.02	116.29
40	**401**	103.72	94.33	90.83	129.17	125.75	121.97
	402	95.6	89.06	87.59	127.1	122.31	117.51
	403	95.21	89.11	88.05	128.2	123.46	118.58
	404	89.79	86.44	86.27	126.47	121.66	115.62
	405	90.39	86.6	86.05	125.26	118.97	114.31
	406	94.35	88.5	86.92	126.99	121.63	117.73

Table 3.S6. Average number of backlashes (days with minimum temperatures below the level of frost hardiness) during the hardening-dehardening period (September-August) for each seed zone, according to historical data between 1960-2015.

		Index 3		
Provenance zone	**Seed zones**	**1960-1979**	**1980-1999**	**2000-2015**
10	**101**	4.56	3.3	3.3
	102	11.65	10.66	10.81
	103	5.35	3.11	2.44
	104	4.18	4.03	3.88
	105	14.11	13.11	11.61
	106	10.83	10.13	9.79
	107	12.38	11.22	9.93
	108	10.65	9.22	8.77
	109	15.59	14.04	12.44
20	**201**	19.64	18.14	17.23
	202	19.21	17.57	15.94
	203	13.23	10.95	10.21
	204	11.33	9.26	8.49
30	**301**	11.62	9.85	10.07
	302	10.54	8.83	8.24
	303	6.12	5.14	5.67
	304	10.78	9.55	8.91
	305	5.12	3.82	3.64
40	**401**	8.39	7.17	6.92
	402	8.36	7.16	5.94
	403	9.13	7.85	7.07
	404	8.31	6.74	7
	405	7.78	6.4	6.19
	406	7.01	6.04	5.38

Table 3.S7. Measurement of autocorrelation of the different indices in all climate change models and for the historical period.

Indices	Historical		UKCP09		UKCP18		UKCP18 Global		CMCC RCP 4.5		CMCC RCP 8.5	
Emissions scenario			SRESA1B		RCP 8.5		RCP 8.5		RCP 4.5		RCP 8.5	
Scale model			Downscaled		Downscaled		Global		Global		Global	
Scale km	5		25		12		60		83		83	
	Moran's I	**p value**	**Moran's I**	**p value**	**Moran's I**	**p value**	**Moran's I**	**p value**	**Moran's I**	**p value**	**Moran's I**	**p value**
Index $1_{0°C}$[1]	0.35	0.005	0.4	0.0021	0.56	0.0002	0.13	0.11	0.17	0.059	0.22	0.034
Index $1_{-3°C}$	0.32	0.008	0.34	0.0072	0.56	0.0001	0.15	0.088	0.19	0.049	0.2	0.046
Index $1_{-5°C}$	0.3	0.011	0.23	0.034	0.66	0.0001	0.39	0.0034	0.19	0.048	0.19	0.054
Index 2[2]	0.43	0.0016	0.17	0.032	0.13	0.098	-0.059	0.51	NA	NA	-0.043	0.56
Index 2_{min}	0.56	0.0001	0.03	0.19	NA	NA	NA	NA	NA	NA	NA	NA
Index 2_{max}	0.43	0.003	0.15	0.045	0.12	0.13	0.029	0.18	0.028	0.25	0.047	0.23
Index 3[3]	0.44	0.0008	0.46	0.0006	0.53	0.0001	0.16	0.079	0.39	0.003	0.42	0.0011
Chill days	0.72	0.0001	0.76	0.00	0.56	0.0005	0.31	0.0051	0.55	0.0005	0.54	0.0001
Estimated bud burst date	0.54	0.0001	0.58	0.00	0.72	0.0001	0.54	0.0003	0.53	0.0002	0.62	0.0001

1. Number of frosts during growing season below threshold temperature.
2. Number of frosts after bud burst below -3 °C.
3. Number of backlashes (days with minimum temperatures below the calculated frost tolerance levels) during the year.

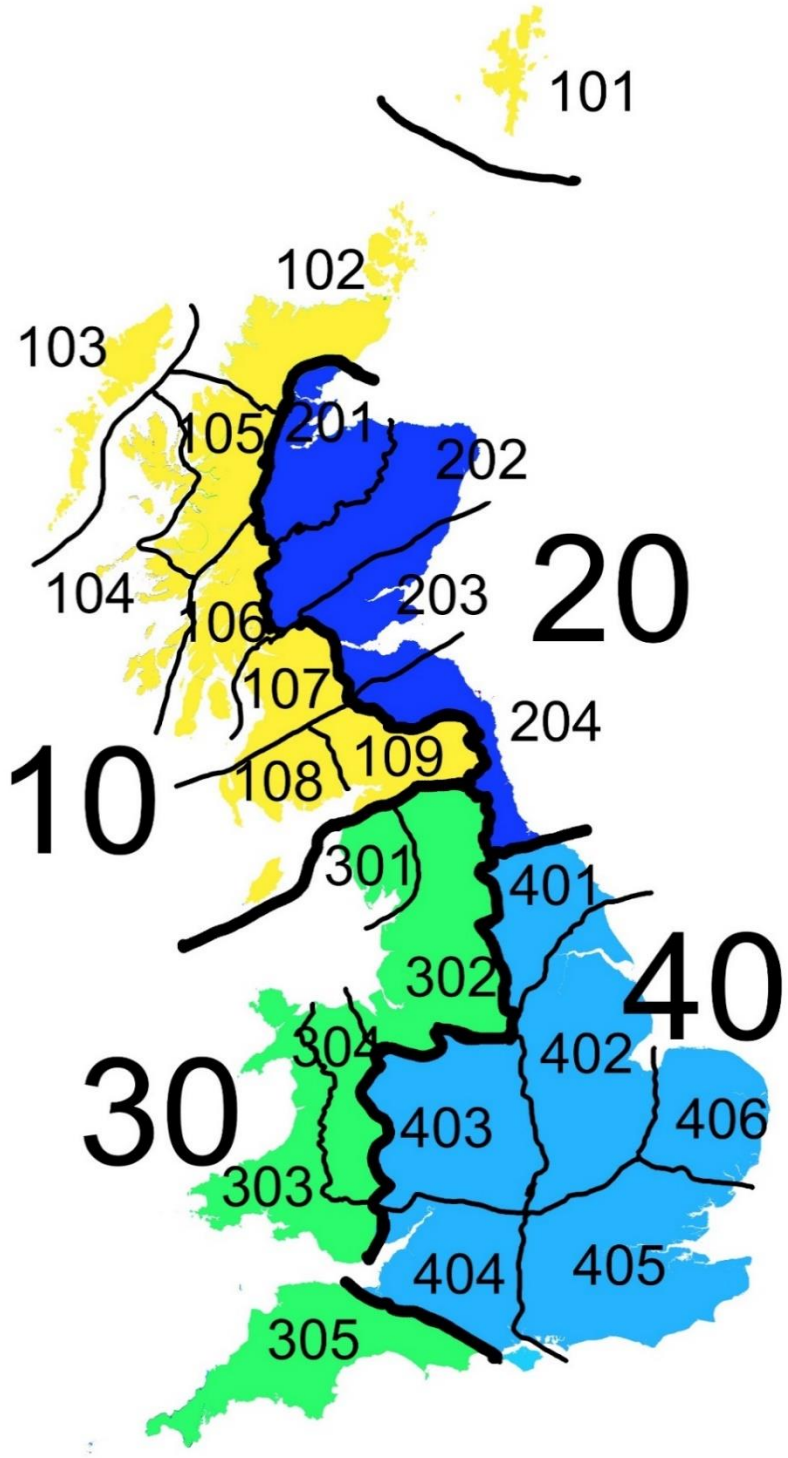

Figure 3.S1. Map of Great Britain's seed zones and provenance zones. Derived from Hubert, J and Cundall, M (2006) Choosing Provenance in Broadleaved Trees. Forestry Commission Information Note FCIN082. ©Crown Copyright. Contains Forestry Commission information licensed under the Open Government License v3.0.

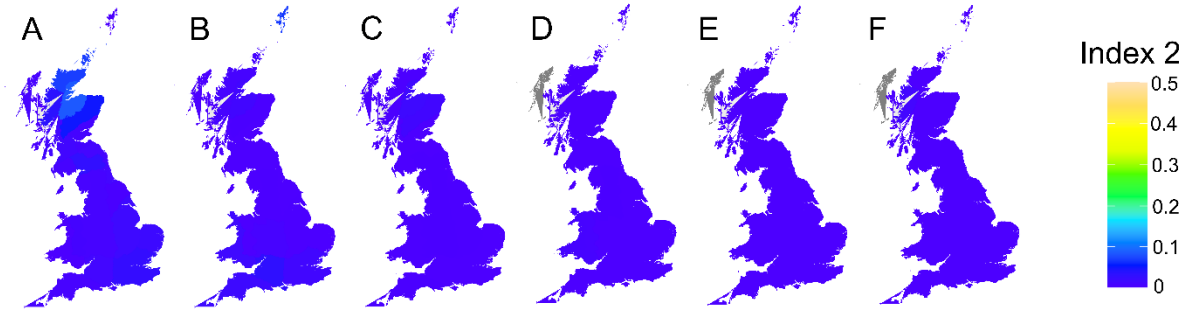

Figure 3.S2. Frequency of post bud burst date frosts < -3 °C in GB, Index 2. Panes show predictions based on different datasets. A) historical period, 1960-2012, B) UKCP09, C) UKCP18, D) UKCP18 Global model, E) CMCC-CM RCP 4.5, F) CMCC-CM RCP 8.5. Contains Forestry Commission information licensed under the Open Government License v3.0.

Appendix Chapter 4

Table 4.S1. Results of exploratory search in Web of Knowledge, done on the 20th of November 2020.

Search String	Number of hits (Web of Knowledge)	Change from previous
1. **TOPIC:** ((frost OR *freezing OR subzero OR cold*) AND (toleran* OR hard* OR resistan*)) AND (gymnosperm)	53	
2. **TOPIC:** ((frost OR *freezing OR subzero OR cold*) AND (toleran* OR hard* OR resistan*)) AND (conifer)	357	Changed search word from gymnosperm to the more common name of the most common division among gymnosperms.
3. **TOPIC:** ((frost OR *freezing OR subzero OR cold*) AND (toleran* OR hard* OR resistan*)) AND (Cedrus OR Pinus OR Cathaya OR Picea OR Pseudotsuga OR Larix OR Pseudolarix OR Tsuga OR Nothotsuga OR Keteleeria OR Abies)	1308	Changed search word from conifer to a list of the Latin names of the most common conifer species.
4. **TOPIC:** ((frost OR *freezing OR subzero OR cold*) AND (toleran* OR hard* OR resistan*)) AND (Cedrus OR Pinus OR Cathaya OR Picea OR Pseudotsuga OR Larix OR Pseudolarix OR Tsuga OR Nothotsuga OR Keteleeria OR Abies OR cedar OR celery-pine OR cypress OR fir OR juniper OR larch OR pine OR redwood OR spruce OR yew OR softwood)	1949	Added common names of the most common conifer species. Added common name for conifer wood.
5. **TOPIC:** ((frost OR *freezing OR subzero OR cold*) AND (toleran* OR hard* OR resistan*)) AND (Cycas OR Dioon OR Bowenia OR Macrozamia OR Lepidozamia OR Encephalartos OR Stangeria OR Ceratozamia OR Microcycas OR Zamia OR Ginkgo OR Welwitschia OR Gnetum OR Ephedra OR Cedrus OR Pinus OR Cathaya OR Picea OR Pseudotsuga OR Larix OR Pseudolarix OR Tsuga OR Nothotsuga OR Keteleeria OR Abies OR Araucaria OR Wollemia OR Agathis OR Phyllocladus OR Lepidothamnus OR Prumnopitys OR Sundacarpus OR Halocarpus OR Parasitaxus OR Lagarostrobos OR Manoao OR Saxegothaea OR Microcachrys OR Pherosphaera OR Acmopyle OR Dacrycarpus OR Dacrydium OR Falcatifolium OR Retrophyllum OR Nageia OR Afrocarpus OR Podocarpus OR Sciadopitys OR Cunninghamia OR Taiwania OR Athrotaxis OR Metasequoia OR Sequoia OR Sequoiadendron OR Cryptomeria OR Glyptostrobus OR Taxodium OR Papuacedrus OR Austrocedrus OR Libocedrus OR Pilgerodendron OR Widdringtonia OR Diselma OR Fitzroya OR Callitris	2168	Expanded list of Latin names to include all Latin names for gymnosperm species.

Search String	Number of hits (Web of Knowledge)	Change from previous
OR Actinostrobus OR Neocallitropsis OR Thujopsis OR Thuja OR Fokienia OR Chamaecyparis OR Callitropsis OR Cupressus OR Juniperus OR Xanthocyparis OR Calocedrus OR Tetraclinis OR Platycladus OR Microbiota OR Austrotaxus OR Pseudotaxus OR Taxus OR Cephalotaxus OR Amentotaxus OR Torreya OR cedar OR celery-pine OR cypress OR fir OR juniper OR larch OR pine OR redwood OR spruce OR yew OR conifer OR softwood)		
6. **TOPIC:** ((frost OR *freezing OR subzero OR cold*) AND (toleran* OR hard* OR resistan*)) AND (Cycas OR Dioon OR Bowenia OR Macrozamia OR Lepidozamia OR Encephalartos OR Stangeria OR Ceratozamia OR Microcycas OR Zamia OR Ginkgo OR Welwitschia OR Gnetum OR Ephedra OR Cedrus OR Pinus OR Cathaya OR Picea OR Pseudotsuga OR Larix OR Pseudolarix OR Tsuga OR Nothotsuga OR Keteleeria OR Abies OR Araucaria OR Wollemia OR Agathis OR Phyllocladus OR Lepidothamnus OR Prumnopitys OR Sundacarpus OR Halocarpus OR Parasitaxus OR Lagarostrobos OR Manoao OR Saxegothaea OR Microcachrys OR Pherosphaera OR Acmopyle OR Dacrycarpus OR Dacrydium OR Falcatifolium OR Retrophyllum OR Nageia OR Afrocarpus OR Podocarpus OR Sciadopitys OR Cunninghamia OR Taiwania OR Athrotaxis OR Metasequoia OR Sequoia OR Sequoiadendron OR Cryptomeria OR Glyptostrobus OR Taxodium OR Papuacedrus OR Austrocedrus OR Libocedrus OR Pilgerodendron OR Widdringtonia OR Diselma OR Fitzroya OR Callitris OR Actinostrobus OR Neocallitropsis OR Thujopsis OR Thuja OR Fokienia OR Chamaecyparis OR Callitropsis OR Cupressus OR Juniperus OR Xanthocyparis OR Calocedrus OR Tetraclinis OR Platycladus OR Microbiota OR Austrotaxus OR Pseudotaxus OR Taxus OR Cephalotaxus OR Amentotaxus OR TORreya OR cedar OR celery-pine OR cypress OR fir OR juniper OR larch OR pine OR redwood OR spruce OR yew OR conifer OR softwood) AND (test* OR technique* OR measure* OR treat* OR trait OR analys*)	1483	Added search word for techniques, as it was found that search was not specific enough.

Table 4.S2. List of studies included in this systematic map.

Authors	Article Title	Publication Year
Abrahamsson, S; Nilsson, JE; Wu, H; Gil, MRG; Andersson, B	Inheritance of height growth and autumn cold hardiness based on two generations of full-sib and half-sib families of *Pinus sylvestris*	2012
Adams, GT; Perkins, TD	Assessing cold tolerance in *Picea* using chlorophyll fluorescence	1993
Aho, ML	Autumn frost hardening of one-year-old *Pinus-sylvestris* (l) seedlings - effect of origin and parent trees	1994
Aitken, SN; Adams, WT	Genetics of fall and winter cold hardiness of coastal Douglas-fir in Oregon	1996
Aitken, SN; Adams, WT	Spring cold hardiness under strong genetic control in Oregon populations of *Pseudotsuga menziesii* var. *Menziesii*	1997
Aitken, SN; Adams, WT; Schermann, N; Fuchigami, LH	Family variation for fall cold hardiness in two Washington populations of coastal Douglas-fir (*Pseudotsuga menziesii* var *menziesii* (Mirb) Franco)	1996
Aldrete, A; Mexal, JG; Burr, KE	Seedling cold hardiness, bud set, and bud break in nine provenances of *Pinus greggii* Engelm.	2008
Amundson, RG; Kohut, RJ; Laurence, JA	Mineral-nutrition, carbohydrate content and cold tolerance of foliage of potted red spruce exposed to ozone and simulated acidic precipitation treatments	1990
Amundson, RG; Kohut, RJ; Laurence, JA; Fellows, S; Colavito, LJ	Moderate water-stress alters carbohydrate content and cold tolerance of red spruce foliage	1993
Andersson, B	Aftereffects of maternal environment on autumn frost hardiness in *Pinus sylvestris* seedlings in relation to cultivation techniques	1994
Andersson, B	Autumn Frost Hardiness of *Pinus sylvestris* Offspring from Seed Orchard Grafts of Different Ages	1992
Andersson, B	Effect of Maternal Soil Treatment on First Year Growth and Autumn Frost Hardiness of *Pinus sylvestris* L. Full-sib Families	1989
Andersson, B; Fedorkov, A	Longitudinal differences in Scots pine frost hardiness	2004

Authors	Article Title	Publication Year
Anekonda, TS; Adams, WT; Aitken, SN	Influence of second flushing on genetic assessment of cold hardiness in coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco)	1998
Anekonda, TS; Adams, WT; Aitken, SN; Neale, DB; Jermstad, KD; Wheeler, NC	Genetics of cold hardiness in a cloned full-sib family of coastal Douglas-fir	2000
Aronsson, A.	Influence of photo- and thermoperiod on the initial stages of frost hardening and dehardening of phytotron-grown seedlings of Scots Pine (*Pinus sylvestris*) and Norway Spruce (*Picea abies* (L.) Karst.).	1975
Aronsson, A.	Frost hardiness in Scots pine (*Pinus sylvestris* L.) II. Hardiness during winter and spring in young trees of different mineral nutrient status.	1980
Bachofen, C; Wohlgemuth, T; Ghazoul, J; Moser, B	Cold temperature extremes during spring do not limit the range shift of Mediterranean pines into regions with intermittent frost	2016
Baldi, P; Pedron, L; Hietala, AM; La Porta, N	Cold tolerance in cypress (*Cupressus sempervirens* L.): a physiological and molecular study	2011
Balduman, LM; Aitken, SN; Harmon, M; Adams, WT	Genetic variation in cold hardiness of Douglas-fir in relation to parent tree environment	1999
Bannister, P; Lord, JM	Comparative winter frost resistance of plant species from southern Africa, Australia, New Zealand, and South America grown in a common environment (Dunedin, New Zealand)	2006
Bansal, S; Harrington, CA; St Clair, JB	Tolerance to multiple climate stressors: a case study of Douglas-fir drought and cold hardiness	2016
Bansal, S; St Clair, JB; Harrington, CA; Gould, PJ	Impact of climate change on cold hardiness of Douglas-fir (*Pseudotsuga menziesii*): environmental and genetic considerations	2015
Barney, D. L.; Bauer, M.; Jensen, J.	Survival, frost susceptibility, growth, and disease resistance of corkbark and subalpine fir grown for landscape and Christmas trees.	2013

Authors	Article Title	Publication Year
Bauer, H.; Nagele, M.; Comploj, M.; Galler, V.; Mair, M.; Unterpertinger, E.	Photosynthesis in cold acclimated leaves of plants with various degrees of freezing tolerance.	1994
Bauer, H; nagele, M; Comploj, M; Galler, V; Mair, M; unterpertinger, E	Photosynthesis after freezing stress in plants with various degrees of freezing tolerance	1992
Beck, EH; Heim, R; Hansen, J	Plant resistance to cold stress: Mechanisms and environmental signals triggering frost hardening and dehardening	2004
Benowicz, A; L'Hirondelle, S; El-Kassaby, YA	Patterns of genetic variation in mountain hemlock (*Tsuga mertensiana* (Bong.) Carr.) With respect to height growth and frost hardiness	2001
Benowicz, A; Stoehr, M; Hamann, A; Yanchuk, AD	Estimation of the F2 generation segregation variance and relationships among growth, frost damage, and bud break in coastal Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) wide-crosses	2020
Bervaes, J. C. A. M.; Kylin, A.	Long and short term development of frost hardiness in *Pinus silvestris*, and heavy particle adenosine triphosphatase.	1972
Bigras, FJ; Bertrand, A	Responses of *Picea mariana* to elevated CO2 concentration during growth, cold hardening and dehardening: phenology, cold tolerance, photosynthesis and growth	2006
Bigras, FJ; D'Aoust, AL	Hardening and dehardening of shoots and roots of containerized black spruce and white spruce seedlings under short and long days	1992
Bigras, FJ; D'Aoust, AL	Influence of photoperiod on shoot and root frost tolerance and bud phenology of white spruce seedlings (*Picea glauca*)	1993
Bigras, FJ; Gonzalez, A; D'Aoust, AL; Hebert, C	Frost hardiness, bud phenology and growth of containerized *Picea mariana* seedlings grown at three nitrogen levels and three temperature regimes	1996

Authors	Article Title	Publication Year
Bigras, FJ; Hebert, C	Freezing temperatures and exposure times during bud break and shoot elongation influence survival and growth of containerized black spruce (*Picea mariana*) seedlings	1996
Bigras, FJ; Margolis, HA	Shoot and root sensitivity of containerized black spruce, white spruce and jack pine seedlings to late fall freezing	1997
Binder, WD; Fielder, P	Chlorophyll fluorescence as an indicator of frost hardiness in white spruce seedlings from different latitudes	1996
Binnie, SC; Grossnickle, SC; Roberts, DR	Fall acclimation patterns of interior spruce seedlings and their relationship to changes in vegetative storage proteins	1994
Blodner, C; Skroppa, T; Johnsen, O; Polle, A	Freezing tolerance in two Norway spruce (*Picea abies* [L.] Karst.) Progenies was physiologically correlated with drought tolerance	2005
Bower, AD; Aitken, SN	Geographic and seasonal variation in cold hardiness of whitebark pine	2006
Boyce, RL	Patterns of foliar injury to red spruce on whiteface mountain, New York, during a high-injury winter	1995
Buchner, O; Neuner, G	A low-temperature freezing system to study the effects of temperatures to-70 degrees C on trees in situ	2009
Buchner, O; Neuner, G	Winter frost resistance of *Pinus cembra* measured in situ at the alpine timberline as affected by temperature conditions	2011
Burr, K. E.; Tinus, R. W.	Effect of the timing of cold storage on cold hardiness and root growth potential of Douglas-fir.	1988
Burr, K. E.; Tinus, R. W.; Wallner, S. J.; King, R. M.	Relationships among cold hardiness, root growth potential and bud dormancy in three conifers.	1989
Burr, K. E.; Tinus, R. W.; Wallner, S. J.; King, R. M.	Comparison of 3 cold hardiness tests for conifer seedlings	1990
Burr, K. E.; Wallner, S. J.; Tinus, R. W.	Ethylene and ethane evolution during cold-acclimation and deacclimation of ponderosa pine	1991

Authors	Article Title	Publication Year
Burr, K. E.; Wallner, S. J.; Tinus, R. W.	Heat tolerance, cold-hardiness, and bud dormancy relationships in seedlings of selected conifers	1993
Calamassi, R; Paoletti, E; Strati, S	Frost hardening and resistance in three Aleppo pine (*Pinus halepensis* Mill.) Provenances	2001
Calkins, J. B.; Swanson, B. T.	Plant cold acclimation, hardiness, and winter injury in response to bare soil and groundcover-based nursery field management systems.	1998
Calme, S; Margolis, HA; Bigras, FJ	Influence of cultural-practices on the relationship between frost tolerance and water-content of containerized black spruce, white spruce, and jack pine-seedlings	1993
Cannell, M. G. R.; Sheppard, L. J.; Smith, R. I.; Murray, M. B.	Autumn frost damage on young *Picea sitchensis* 2. Shoot frost hardening, and the probability of frost damage in Scotland.	1985
Cannell, M. G. R.; Tabbush, P. M.; Deans, J. D.; Hollingsworth, M. K.; Sheppard, L. J.; Philipson, J. J.; Murray, M. B.	Sitka spruce and Douglas fir seedlings in the nursery and in cold storage: root growth potential, carbohydrate content, dormancy, frost hardiness and mitotic index.	1990
Cape, JN; Leith, ID; Fowler, D; Murray, MB; Sheppard, LJ; Eamus, D; Wilson, RHF	Sulfate and ammonium in mist impair the frost hardening of red spruce seedlings	1991
Carles, S; Lamhamedi, MS; Stowe, DC; Bernier, PY; Veilleux, L; Margolis, HA	Relationships between frost hardiness, root growth potential, and photosynthesis of nursery-grown white spruce seedlings	2011
Carles, S; Lamhamedi, MS; Stowe, DC; Margolis, HA; Bernier, PY; Veilleux, L; Fecteau, B	Frost tolerance of two-year-old *Picea glauca* seedlings grown under different irrigation regimes in a forest nursery	2008
Carles, S; Lamhamedi, MS; Stowe, DC; Veilleux, L; Margolis, HA	An operational method for estimating cold tolerance thresholds of white spruce seedlings in forest nurseries	2012
Cavieres, LA; Rada, F; Azocar, A; Garcia-Nunez, C; Cabrera, HM	Gas exchange and low temperature resistance in two tropical high mountain tree species from the Venezuelan Andes	2000

Authors	Article Title	Publication Year
Chang, C. Y.; Unda, F.; Zubilewich, A.; Mansfield, S. D.; Ensminger, I.	Sensitivity of cold acclimation to elevated autumn temperature in field-grown *Pinus strobus* seedlings.	2015
Charra-Vaskou, K; Charrier, G; Wortemann, R; Beikircher, B; Cochard, H; Ameglio, T; Mayr, S	Drought and frost resistance of trees: a comparison of four species at different sites and altitudes	2012
Chen, Y. P.; Zhang, J.; Yang, Z. J.; Yu, F.; Li, Y. Q.	Relationship between nitric oxide accumulation, anti-oxidative system and freezing tolerance in the leaves of *Sabina* during cold adaptation.	2012
Cherry, ML; El-Kassaby, YA	Growth, morphology, and cold hardiness of *Chamaecyparis nootkatensis* seedlings originating from an abbreviated reproductive cycle	2002
Christersson, L.	The influence of photoperiod and temperature on the development of frost hardiness in seedlings of *Pinus silvestris* and *Picea abies*.	1978
Christersson, L.	Frost hardiness development in rapid- and slow-growing Norway Spruce seedlings.	1975
Climent, J; Silva, FCE; Chambel, MR; Pardos, M; Almeida, MH	Freezing injury in primary and secondary needles of Mediterranean pine species of contrasting ecological niches	2009
Coleman, MD; Hinckley, TM; Mcnaughton, G; Smit, BA	Root cold hardiness and native distribution of sub-alpine conifers	1992
Colombo, S. J.	Frost hardening spruce container stock for overwintering in Ontario.	1997
Colombo, S. J.	Bud dormancy status, frost hardiness, shoot moisture content, and readiness of black spruce container seedlings for frozen storage.	1990
Colombo, S. J.; Gellert, S.	Frost hardiness testing: an Ontario update.	2002
Colombo, S. J.; Glerum, C.; Webb, D. P.	Winter hardening in first-year black spruce (*Picea mariana)* seedlings.	1989
Colombo, S. J.; Raitanen, E. M.	Frost hardening in first-year eastern larch (*Larix laricina*) container seedlings.	1993

Authors	Article Title	Publication Year
Colombo, SJ	Timing of cold temperature exposure affects root and shoot frost hardiness of *Picea mariana* container seedlings	1994
Colombo, SJ; Glerum, C; Webb, DP	Daylength, temperature and fertilization effects on desiccation resistance, cold hardiness and root growth potential of *Picea mariana* seedlings	2003
Colombo, SJ; Raitanen, EM	Frost hardening in white cedar container seedlings exposed to intermittent short days and cold temperatures	1991
Colombo, SJ; Zhao, SY; Blumwald, E	Frost hardiness gradients in shoots and roots of *Picea mariana* seedlings	1995
Connolly, BM; Orrock, JL	Exogenous application of methyl jasmonate alters *Pinus resinosa* seedling response to simulated frost	2018
Corcuera, L; Gil-Pelegrin, E; Notivol, E	Intraspecific Variation in *Pinus pinaster* PSII Photochemical Efficiency in Response to Winter Stress and Freezing Temperatures	2011
Coursolle, C; Bigras, FJ; Margolis, HA; Hebert, C	Dehardening and second-year growth of white spruce provenances in response to duration of long-night treatments	1997
Dalen, LS; Johnsen, O; Lonneborg, A; Yaish, MW	Freezing tolerance in Norway spruce, the potential role of pathogenesis-related proteins	2015
Dalen, LS; Johnsen, O; Ogner, G	CO(2) enrichment and development of freezing tolerance in Norway spruce	2001
Danusevicius, D; Jonsson, A; Eriksson, G	Variation among open-pollinated families of *Picea abies* (L.) Karst-in response to simulated frost desiccation	1999
Davradou, M; Hawkins, BJ	Effects of plantation location and population on the seasonal freezing tolerance of yellow-cedar (*Chamaecyparis nootkatensis*) stecklings	1998
De Jesus-Re, G; Vargas-Hernandez, JJ; Cruz-Huerta, N; Lopez-Upton, J	Populations of *Pinus radiata* D. Don differ in low-temperature tolerance and growth rate	2020

Authors	Article Title	Publication Year
De Waal, L; Mitchell, RG; Hodge, GR; Chirwa, PW	The use of field and artificial freezing studies to assess frost tolerance in natural populations of *Pinus oocarpa*	2018
Deha, DH; Thornton, FC; Waite, CE; Ingle, MA	Ambient cloud deposition reduces cold tolerance of red spruce seedlings	1991
Dirr, M. A.; Lindstrom, O. M., Jr.; Lewandowski, R.; Vehr, M. J.	Cold hardiness estimates of woody taxa from cultivated and wild collections.	1993
Driessche, R. Van den	Prediction of cold hardiness in Douglas Fir seedlings by index of injury and conductivity methods.	1976
Driessche, R. Van den	Prediction of frost hardiness in Douglas Fir seedlings by measuring electrical impedance in stems at different frequencies.	1973
Dubuc, Y; Dubuc, J; Bigras, FJ	An inexpensive portable freezer for in situ freezing in the field	2002
Dueck, TA; Dorel, FG; Terhorst, R; Van De Reerden, LJ	Effects of ammonia, ammonium-sulfate and sulfur-dioxide on the frost sensitivity of scots pine (*Pinus sylvestris* l)	1990
Duncan, P. D.; White, T. L.; Hodge, G. R.	First-year freeze hardiness of pure species and hybrid taxa of *Pinus elliottii* (Engelmann) and *Pinus caribaea* (Morelet).	1996
Eamus, D	Assimilation and stomatal conductance responses of red spruce to midwinter frosts and the constituent ions of acid mist	1993
Ericsson, T.; Andersson, B.	Can field mortality of lodgepole pine (*Pinus contorta* var. *Latifolia*) families be predicted based on early freezing test results?	1997
Eysteinsson, T; Karlman, L; Fries, A; Martinsson, O; Skulason, B	Variation in spring and autumn frost tolerance among provenances of Russian larches (*Larix* Mill.)	2009
Fan, SH; Grossnickle, SC	Clonal variation in gas exchange and freezing tolerance development of interior spruce (*Picea glauca* (Moench) Voss x *P. engelmannii* Parry ex Engelm.) During autumn acclimation	1999
Feild, TS; Brodribb, T	Stem water transport and freeze-thaw xylem embolism in conifers and angiosperms in a Tasmanian treeline heath	2001

Authors	Article Title	Publication Year
Fernandez, M; Royo, A; Gil, L; Pardos, JA	Effects of temperature on growth and stress hardening development of phytotron-grown seedlings of Aleppo pine (*Pinus halepensis* Mill.)	2003
Floistad, IS	Effects of excessive nutrient supply and short day treatment on autumn frost hardiness and time of bud break in *Picea abies* seedlings	2002
Floistad, IS; Granhus, A	Bud break and spring frost hardiness in *Picea abies* seedlings in response to photoperiod and temperature treatments	2010
Floistad, IS; Kohmann, K	Effects of thawing procedure on frost hardiness, carbohydrate content and timing of bud break in *Picea abies*	2001
Floistad, IS; Kohmann, K	Influence of nutrient supply on spring frost hardiness and time of bud break in Norway spruce (*Picea abies* (L.) Karst.) Seedlings	2004
Folk, R. S.; Grossnickle, S. C.; Major, J. E.; Arnott, J. T.	Influence of nursery culture on western red cedar. II. Freezing tolerance of fall-planted seedlings and morphological development of fall- and spring-planted seedlings.	1994
Gillies, SL; Binder, WD	The effect of sub-zero temperatures in the light and dark on cold-hardened, dehardened and newly hushed white spruce (*Picea glauca* [Moench] Voss) seedlings	1997
Glerum, C.	Annual trends in frost hardiness and electrical impedance for seven coniferous species.	1973
Gomory, D; Foffova, E; Kmet, J; Longauer, R; Romsakova, I	Norway spruce (*Picea abies* [l.] Karst.) Provenance variation in autumn cold hardiness: adaptation or acclimation?	2010
Gooch, NJ; Nzokou, P; Cregg, BM	Effect of Indoor Exposure on the Cold Hardiness and Physiology of Containerized Christmas Trees	2009
Greer, D. H.; Stanley, C. J.; Warrington, I. J.	Photoperiod control of the initial phase of frost hardiness development in *Pinus radiata*.	1989
Greer, DH; Robinson, LA; Hall, AJ; Klages, K; Donnison, H	Frost hardening of *Pinus radiata* seedlings: effects of temperature on relative growth rate, carbon balance and carbohydrate concentration	2000

Authors	Article Title	Publication Year
Grossnickle, SC	Relationship between freezing tolerance and shoot water relations of western red cedar	1992
Grossnickle, SC; Russell, JH	Yellow-cedar and western redcedar ecophysiological response to fall, winter and early spring temperature conditions	2006
Guak, S; Olsyzk, DM; Fuchigami, LH; Tingey, DT	Effects of elevated CO(2) and temperature on cold hardiness and spring bud burst and growth in Douglas-fir (*Pseudotsuga menziesii*)	1998
Hadley, JL; Amundson, RG; Laurence, JA; Kohut, RJ	Physiological-response to controlled freezing of attached red spruce branches	1993
Halman, JM; Schaberg, PG; Hawley, GJ; Eagar, C	Calcium addition at the Hubbard Brook Experimental Forest increases sugar storage, antioxidant activity and cold tolerance in native red spruce (*Picea rubens*)	2008
Hannerz, M.; Westin, J.	Growth cessation and autumn-frost hardiness in one-year-old *Picea abies* progenies from seed orchards and natural stands.	2000
Hannerz, M; Westin, J	Autumn frost hardiness in Norway spruce plus tree progeny and trees of the local and transferred provenances in central Sweden	2005
Hansen, J. M.	Effects of nutritional factors on frost hardening in *Larix leptolepis* (Sieb & Zucc.) Gord.	1992
Hattemer, H. H.; Konig, A.	Geographic variation of early growth and frost resistance in Douglas-Fir.	1975
Hawkins, B. J.; Stoehr, M.	Growth, phenology, and cold hardiness of 32 Douglas-fir full-sib families.	2009
Hawkins, BJ	Photoperiod and night frost influence the frost hardiness of *Chamaecyparis nootkatensis* clones	1993
Hawkins, BJ; davradou, M; pier, D; shortt, R	Frost hardiness and winter photosynthesis of *Thuja plicata* and *Pseudotsuga menziesii* seedlings grown at 3 rates of nitrogen and phosphorus supply	1995

Authors	Article Title	Publication Year
Hawkins, BJ; Henry, G; Whittington, J	Frost hardiness of *Thuja plicata* and *Pseudotsuga menziesii* seedlings when nutrient supply varies with season	1996
Hawkins, BJ; McDonald, SE	Photoperiod influences dehardening of *Chamaecyparis nootkatensis* seedlings	1993
Hawkins, BJ; Stoehr, M	Growth, phenology, and cold hardiness of 32 Douglas-fir full-sib families	2009
Hawkins, BJ; Sweet, GB; Greer, DH; Bergin, DO	Genetic-variation in the frost hardiness of *Podocarpus totara*	1991
Hawkins, CDB; Shewan, KB	Frost hardiness, height, and dormancy of 15 short-day, nursery-treated interior spruce seed lots	2000
Hawley, GJ; Schaberg, PG; Eagar, C; Borer, CH	Calcium addition at the Hubbard Brook Experimental Forest reduced winter injury to red spruce in a high-injury year	2006
Haynes, CL; Lindstrom, OM; Dirr, MA	Cooling and warming effects on cold hardiness estimations of 3 woody ornamental taxa	1992
Heiskanen, J.; Sutinen, S.; Hyvönen, J.	Effects of light exposure in freezing temperatures on winter damage to foliage of Norway spruce container seedlings in mid and late winter: pilot experiments in open field.	2015
Hellergren, J.	Frost hardiness development in *Pinus silvestris* seedlings in response to fertilization.	1981
Hodge, GR; Dvorak, WS; Tighe, ME	Comparisons between laboratory and field results of frost tolerance of pines from the southern USA and Mesoamerica planted as exotics	2012
Hodge, GR; Weir, RJ	Freezing stress tolerance of hardy and tender families of loblolly-pine	1993
Hofmann, M; Jager, M; Bruelheide, H	Relationship between frost hardiness of adults and seedlings of different tree species	2014
Holopainen, J. K.	Growth and visible responses of Scots pine (*Pinus sylvestris* L.) Seedlings to simulated summer frost.	1988
Hurme, P; Repo, T; Savolainen, O; Paakkonen, T	Climatic adaptation of bud set and frost hardiness in Scots pine (*Pinus sylvestris*)	1997

Authors	Article Title	Publication Year
Ishizuka, W; Ono, K; Hara, T; Goto, S	Influence of low- and high-elevation plant genomes on the regulation of autumn cold acclimation in *Abies sachalinensis*	2015
Jacobson, JS; Heller, LI; L'Hirondelle, SJ; Lassoie, JP	Phenology and Cold Tolerance of *Picea rubens* Sarg. Seedlings Exposed to Sulfuric and Nitric Acid Mist	1992
Jalkanen, RE; Redfern, DB; Sheppard, LJ	Nutrient deficits increase frost hardiness in Sitka spruce (*Picea sitchensis*) needles	1998
Johnsen, O	Freeze-testing Young *Picea abies* Plants A Methodological Study	1989
Johnsen, O	Phenotypic Changes in Progenies of Northern Clones of *Picea abies* (L.) Karst. Grown in a Southern Seed Orchard I. Frost Hardiness in a Phytotron Experiment	1989
Johnsen, O; Skreppa, T	Provenances and families show different patterns of relationship between bud set and frost hardiness in *Picea abies*	2000
Johnsen, O; Skroppa, T; Haug, G; Apeland, I; Ostreng, G	Sexual reproduction in a greenhouse and reduced autumn frost hardiness of *Picea abies* progenies	1995
Johnsen, O; Skroppa, T; Junttila, O; Daehlen, OG	Influence of the female flowering environment on autumn frost-hardiness of *Picea abies* progenies	1996
Jones, GE; Cregg, BM	Budbreak and winter injury in exotic firs	2006
Jonsson, A.; Eriksson, G.; Dormling, I.; Ifver, J.	Studies on frost hardiness of *Pinus contorta* Dougl. Seedlings grown in climate chambers.	1981
Jonsson, AM; Ingerslev, M; Raulund-Rasmussen, K	Frost sensitivity and nutrient status in a fertilized Norway spruce stand in Denmark	2004
Joosen, RVL; Lammers, M; Balk, PA; Bronnum, P; Konings, MCJM; Perks, M; Stattin, E; Van Wordragen, MF; van der Geest, AHM	Correlating gene expression to physiological parameters and environmental conditions during cold acclimation of *Pinus sylvestris*, identification of molecular markers using cDNA microarrays	2006
Joyce, D. G.	Adaptive variation in cold hardiness of eastern larch, *Larix laricina*, in northern Ontario.	1988

Authors	Article Title	Publication Year
Kandemir, GE; Kaya, Z; Temel, F; Onde, S	Genetic Variation in Cold Hardiness and Phenology Between and Within Turkish Red Pine (*Pinus brutia* Ten.) Populations: Implications for Seed Transfer	2010
Kathke, S; Bruelheide, H	Differences in frost hardiness of two Norway spruce morphotypes growing at Mt. Brocken, Germany	2011
Kontinen, K; Luoranen, J; Rikala, R	Growth and frost hardening of *Picea abies* seedlings after various night length treatments	2007
Korhonen, A; Lehto, T; Repo, T	Frost hardiness of mycorrhizal and non-mycorrhizal Scots pine under two fertilization treatments	2015
Larsen, JB; Wellendorf, H	Early Test in *Picea abies* Full Sibs by Applying Gas Exchange, Frost Resistance and Growth Measurements	1990
Lazarus, BE; Schaberg, PG; Hawley, GJ; deha1, DH	Landscape-scale spatial patterns of winter injury to red spruce foliage in a year of heavy region-wide injury	2006
Lehtinen, MT; Pulkkinen, P	Effects of Scots pine paternal genotypes of two contiguous seed orchards on the bud set and frost hardening of first-year progeny	2017
Leinonen, I; Repo, T; Hanninen, H	Changing environmental effects on frost hardiness of Scots pine during dehardening	1997
Levkoev, E; Mehtatalo, L; Luostarinen, K; Pulkkinen, P; Zhigunov, A; Peltola, H	Development of height growth and frost hardiness for one-year-old Norway spruce seedlings in greenhouse conditions in response to elevated temperature and atmospheric CO2 concentration	2018
L'Hirondelle, SJ; Binder, WD	Temperature stress tolerance of conifer seedlings after exposure to UV-B radiation	2005
L'Hirondelle, SJ; Simpson, DG; Binder, WD	Overwinter storability of conifer planting stock: operational testing of fall frost hardiness	2006
Lindgren, K; Hallgren, JE	Cold-acclimation of *Pinus contorta* and *Pinus sylvestris* assessed by chlorophyll fluorescence	1993

Authors	Article Title	Publication Year
Lindgren, K; Nilsson, JE	Cold Acclimation of *Pinus sylvestris* and *Pinus contorta* Provenances as Measured by Freezing Tolerance of Detached Needles	1992
Lindstrom, A; Stattin, E; Grans, D; Wallin, E	Storability measures of Norway spruce and Scots pine seedlings and assessment of post-storage vitality by measuring shoot electrolyte leakage	2014
Lu, PX; Colombo, SJ; Sinclair, RW	Cold hardiness of interspecific hybrids between *Pinus strobus* and *P. wallichiana* measured by post-freezing needle electrolyte leakage	2007
Lu, PX; Joyce, DG; Sinclair, RW	Geographic variation in cold hardiness among eastern white pine (*Pinus strobus* L.) Provenances in Ontario	2003
Lucas, P. W.; Cottam, D. A.; Sheppard, L. J.; Francis, B. J.	Growth responses and delayed winter hardening in Sitka spruce following summer exposure to ozone.	1988
Luis, VC; Taschler, D; Hacker, J; Jimenez, MS; Wieser, G; Neuner, G	Ice nucleation and frost resistance of *Pinus canariensis* seedlings bearing needles in three different developmental states	2007
Lund, AE; Livingston, WH	Freezing cycles enhance winter injury in *Picea rubens*	1999
Luoranen, J; Konttinen, K; Rikala, R	Frost Hardening and Risk of a Second Flush in Norway Spruce Seedlings after an Early-Season Short-Day Treatment	2009
Luoranen, J; Lahti, M; Rikala, R	Frost hardiness of nutrient-loaded two-year-old *Picea abies* seedlings in autumn and at the end of freezer storage	2008
Luoranen, J; Riikonen, J; Rikala, R; Sutinen, S	Frost hardiness, carbohydrates and bud morphology of *Picea abies* seedlings after different lengths of freezer storage	2012
Mabaso, F; Ham, H; Nel, A	Frost tolerance of various *Pinus* pure species and hybrids	2019
Malmqvist, C; Wallertz, K; Lindstrom, A	Storability and freezing tolerance of Douglas fir and Norway spruce seedlings grown in mid-Sweden	2017
Man, RZ; Colombo, S; Lu, PX; Dang, QL	Effects of winter warming on cold hardiness and spring budbreak of four boreal conifers	2016
Man, RZ; Lu, PX; Dang, QL	Cold hardiness of white spruce, black spruce, jack pine, and lodgepole pine needles during dehardening	2017

Authors	Article Title	Publication Year
Man, RZ; Lu, PX; Dang, QL	Cold tolerance of black spruce, white spruce, jack pine, and lodgepole pine seedlings at different stages of spring dehardening	2020
Margolis, H. A.; Vézina, L. P.	Atmospheric CO2 enrichment and the development of frost hardiness in containerized black spruce seedlings.	1990
Martin, M; Gavazov, K; Korner, C; Hattenschwiler, S; Rixen, C	Reduced early growing season freezing resistance in alpine treeline plants under elevated atmospheric CO2	2010
Martinez Meier, A.; Mondino, V.; Gallo, L.	Evaluation of damage by late frosts in provenances trials of Douglas-fir introduced in Argentinian North Patagonia.	2005
Martz, F; Sutinen, ML; Kivineemi, S; Palta, JP	Changes in freezing tolerance, plasma membrane H+-ATPase activity and fatty acid composition in *Pinus resinosa* needles during cold acclimation and de-acclimation	2006
McKay, HM; Mason, WL	Physiological indicators of tolerance to cold-storage in Sitka spruce and Douglas-fir seedlings	1991
Mellerowicz, EJ; Coleman, WK; Riding, RT; Little, CHA	Periodicity of cambial activity in *Abies balsamea*. 1. Effects of temperature and photoperiod on cambial dormancy and frost hardiness	1992
Meng, P; Bai, XF; Li, HD; Song, XD; Zhang, XL	Cold hardiness estimation of *Pinus densiflora* var. *Zhangwuensis* based on changes in ionic leakage, chlorophyll fluorescence and other physiological activities under cold stress	2015
Menzies, M. I.; Burdon, R. D.; Holden, D. G.; Warrington, I. J.	Family variation and potential for genetic gain in frost resistance of *Pinus radiata*.	1987
Mexal, J. G.; Timmis, R.; Morris, W. G.	Cold-hardiness of containerized loblolly pine seedlings. Its effect on field survival and growth.	1979
Murray, M. B.; Cape, J. N.; Fowler, D.	Quantification of frost damage in plant tissues by rates of electrolyte leakage.	1989

Authors	Article Title	Publication Year
Neuner, G; Beikircher, B	Critically reduced frost resistance of *Picea abies* during sprouting could be linked to cytological changes	2010
Neuner, G; Huber, B; Plangger, A; Pohlin, JM; Walde, J	Low temperatures at higher elevations require plants to exhibit increased freezing resistance throughout the summer months	2020
Nicoll, BC; Redfern, DB; mckay, HM	Autumn frost damage: Clonal variation in Sitka spruce	1996
Nielsen, CCN; Rasmussen, HN	Frost hardening and dehardening in *Abies procera* and other conifers under differing temperature regimes and warm-spell treatments	2009
Nilsson, J. E.; Andersson, B.	Performance in freezing tests and field experiments of full-sib families of *Pinus sylvestris* L.	1987
Nilsson, JE	Seasonal changes in phenological traits and cold hardiness of F1-populations from plus-trees of *Pinus sylvestris* and *Pinus contorta* of various geographical origins	2001
Nilsson, JE	Variation in Early Winter Hardening within Families of *Pinus sylvestris* (L.) From Northern Sweden	1990
Nilsson, JE; Eriksson, G	Freeze Testing and Field Mortality of *Pinus sylvestris* (L.) In Northern Sweden	1986
Nilsson, JE; Walfridsson, EA	Autumn Freeze Testing of One-year Reciprocal Families of *Pinus sylvestris* (L.)	1990
Nzokou, P; Nikiema, P	The influence of three plant growth regulators on susceptibility to cold injury following warm winter spells in fraser fir [*Abies fraseri* (Pursh) Poir] and Colorado blue spruce (*Picea pungens*)	2008
Odlum, KD; Blake, TJ	A comparison of analytical approaches for assessing freezing damage in black spruce using electrolyte leakage methods	1996
Odlum, KD; Blake, TJ; Kim, YT; Glerum, C	Influence of photoperiod and temperature on frost hardiness and free amino-acid-concentrations in black spruce seedlings	1993

Authors	Article Title	Publication Year
Ogren, E	Relationship between temperature, respiratory loss of sugar and premature dehardening in dormant Scots pine seedlings	1997
Ögren, E.	Effects of climatic warming on cold hardiness of some northern woody plants assessed from simulation experiments.	2001
Ogren, E; Nilsson, T; Sundblad, LG	Relationship between respiratory depletion of sugars and loss of cold hardiness in coniferous seedlings over-wintering at raised temperatures: Indications of different sensitivities of spruce and pine	1997
O'Neill, GA; Adams, WT; Aitken, SN	Quantitative genetics of spring and fall cold hardiness in seedlings from two Oregon populations of coastal Douglas-fir	2001
O'Neill, GA; Aitken, SN; Adams, WT	Genetic selection for cold hardiness in coastal Douglas-fir seedlings and saplings	2000
Pardos, M; Climent, J; Almeida, H; Calama, R	The role of developmental stage in frost tolerance of *Pinus pinea* L. Seedlings and saplings	2014
Persson, T; Andersson, B; Ericsson, T	Relationship between Autumn Cold Hardiness and Field Performance in northern *Pinus sylvestris*	2010
Prada, E; Alia, R; Climent, J; Diaz, R	Seasonal cold hardiness in maritime pine assessed by different methods	2014
Prada, E; Climent, J; Alia, R; Diaz, R	Life-history correlations with seasonal cold hardiness in maritime pine	2016
Puertolas, J; Gil, L; Pardos, JA	Effects of nitrogen fertilization and temperature on frost hardiness of Aleppo pine (*Pinus halepensis* Mill.) Seedlings assessed by chlorophyll fluorescence	2005
Pulkkinen, P.	Frost hardiness development and lignification of young Norway spruce seedlings of southern and northern Finnish origin.	1993
Puttonen, P; Arnott, JT	Influence of photoperiod and temperature on growth, gas-exchange, and cold-hardiness of yellow cypress stecklings	1994

Authors	Article Title	Publication Year
Raisanen, M; Repo, T; Lehto, T	Cold acclimation was partially impaired in boron deficient Norway spruce seedlings	2007
Raisanen, M; Repo, T; Lehto, T	Cold Acclimation of Norway Spruce Roots and Shoots after Boron Fertilization	2009
Raisanen, M; Repo, T; Lehto, T	Effect of thawing time, cooling rate and boron nutrition on freezing point of the primordial shoot in Norway spruce buds	2006
Raisanen, M; Repo, T; Rikala, R; Lehto, T	Does ice crystal formation in buds explain growth disturbances in boron-deficient Norway spruce?	2006
Ramirez-Cuevas, Y; Rodriguez-Trejo, DA	Frost resistance in *Pinus hartwegii* subjected to different potassium treatments	2010
Rehfeldt, G. E.	Cold acclimation in populations of *Pinus contorta* from the northern Rocky Mountains.	1980
Reinikainen, J.; Huttunen, S.	The level of injury and needle ultrastructure of acid rain-irrigated pine and spruce seedlings after low temperature treatment.	1989
Repo, T; Hanninen, H; Kellomaki, S	The effects of long-term elevation of air temperature and CO2 on the frost hardiness of Scots pine	1996
Repo, T; Zhang, G; Ryyppo, A; Rikala, R	The electrical impedance spectroscopy of Scots pine (*Pinus sylvestris* L.) Shoots in relation to cold acclimation	2000
Repo, T; Zhang, G; Ryyppo, A; Vapaavuori, E; Sutinen, S	Effects of freeze-thaw injury on parameters of distributed electrical circuits of stems and needles of scots pine-seedlings at different stages of acclimation	1994
Riikonen, J; Kontunen-Soppela, S; Ossipov, V; Tervahauta, A; Tuomainen, M; Oksanen, E; Vapaavuori, E; Heinonen, J; Kivimaenpaa, M	Needle metabolome, freezing tolerance and gas exchange in Norway spruce seedlings exposed to elevated temperature and ozone concentration	2012
Riikonen, J; Luoranen, J	An Assessment of Storability of Norway Spruce Container Seedlings in Freezer Storage as Affected by Short-Day Treatment	2020

Authors	Article Title	Publication Year
Rikala, R; Repo, T	Frost Resistance and Frost Damage in *Pinus sylvestris* Seedlings during Shoot Elongation	1987
Rixen, C; Dawes, MA; Wipf, S; Hagedorn, F	Evidence of enhanced freezing damage in treeline plants during six years of CO2 enrichment and soil warming	2012
Rose, R; Haase, D	Chlorophyll fluorescence and variations in tissue cold hardiness in response to freezing stress in Douglas-fir seedlings	2002
Rostad, H; Granhus, A; Floistad, IS; Morgenlie, S	Early summer frost hardiness in *Picea abies* seedlings in response to photoperiod treatment	2006
Ryyppö, A.; Sutinen, S.; Mäenpää, M.; Vapaavuori, E.; Repo, T.	Frost damage and recovery of Scots pine seedlings at the end of the growing season.	1997
Ryyppo, A; Repo, T; Vapaavuori, E	Development of freezing tolerance in roots and shoots of Scots pine seedlings at nonfreezing temperatures	1998
Sakai, A.; Malla, S. B.	Winter hardiness of tree species at high altitudes in the East Himalaya, Nepal.	1981
Sakai, A.; Wardle, P.	Freezing resistance of New Zealand trees and shrubs.	1978
Sakai, A.; Weiser, C. J.	Freezing resistance of trees in North America with reference to tree regions.	1973
Sarvaš, M.	Changes in cold hardiness of silver fir and larch bare-rooted seedlings during autumn and spring.	2004
Schaberg, P. G.; Strimbeck, G. R.; Hawley, G. J.; deha1, D. H.; Shane, J. B.; Murakami, P. F.; Perkins, T. D.; Donnelly, J. R.; Wong, B. L.	Cold tolerance and photosystem function in a montane red spruce population: physiological relationships with foliar carbohydrates.	2000
Schaberg, PG; deha1, DH; Hawley, GJ; Murakami, PF; Strimbeck, GR; mcnulty, SG	Effects of chronic N fertilization on foliar membranes, cold tolerance, and carbon storage in montane red spruce.	2002
Schaberg, PG; Hennon, PE; D'Amore, DV; Hawley, GJ	Influence of simulated snow cover on the cold tolerance and freezing injury of yellow-cedar seedlings	2008
Schaberg, PG; Hennon, PE; D'Amore, DV; Hawley, GJ; Borer, CH	Seasonal differences in freezing tolerance of yellow-cedar and western hemlock trees at a site affected by yellow-cedar decline	2005

Authors	Article Title	Publication Year
Schuch, U. K.; Duryea, M. L.; Fuchigami, L. H.	Frost hardiness as acquired by Douglas-fir seedlings in three Pacific Northwest nurseries.	1989
Sebastian-Azcona, J; Hacke, UG; Hamann, A	Adaptations of white spruce to climate: strong intraspecific differences in cold hardiness linked to survival	2018
Sheel Bansal; Harrington, C. A.; St. Clair, J. B.	Tolerance to multiple climate stressors: a case study of Douglas-fir drought and cold hardiness.	2016
Sheppard, L. J.; Smith, R. I.; Cannell, M. G. R.	Frost hardiness of *Picea rubens* growing in spruce decline regions of the Appalachians.	1989
Sheppard, LJ	Soil dressing with sulphur: does it reduce frost hardiness in spruce seedlings?	1997
Sheppard, LJ; Cape, JN; Leith, ID	Influence of acidic mist on frost hardiness and nutrient concentrations in red spruce seedlings. 1. Exposure of the foliage and the rooting environment	1993
Sheppard, LJ; Crossley, A; Harvey, FJ; Wilson, D; Cape, JN	Field application of acid mist to a single clone of Sitka spruce: Effects on foliar nutrition and frost hardiness	1997
Sheppard, LJ; Franssen, I; Cape, JN	Frost hardiness of Norway spruce treated with acid mist evaluation of the electrolyte leakage rate technique	1995
Sheppard, LJ; Leith, ID; Cape, JN	Effects of acid mist on mature grafts of Sitka spruce .1. Frost hardiness and foliar nutrient concentrations	1994
Sheppard, LJ; Rosengren, U; Emmett, BA	Do nitrogen additions change the sensitivity of detached shoots from Sitka and Norway spruce to freezing temperatures? Evidence from three field manipulation studies	2003
Simpson, D. G.	Frost hardiness, root growth capacity, and field performance relationships in interior spruce, lodgepole pine, Douglas-fir, and western hemlock seedlings.	1990
Simpson, D. G.	Seasonal and geographic origin effects on cold-hardiness of white spruce buds, foliage, and stems	1994

Authors	Article Title	Publication Year
Skroppa, T	Within-population Variation in Autumn Frost Hardiness and its Relationship to Bud-set and Height Growth in *Picea abies*	1991
Smiley, E. T.; Shirazi, A. M.	Fall fertilization and cold hardiness in landscape trees.	2003
St Clair, JB	Genetic variation in fall cold hardiness in coastal Douglas-fir in western Oregon and Washington	2006
Stevenson, JF; Hawkins, BJ; Woods, JH	Spring and fall cold hardiness in wild and selected seed sources of coastal Douglas-fir	1999
Strimbeck, GR; Kjellsen, TD	First frost: Effects of single and repeated freezing events on acclimation in *Picea abies* and other boreal and temperate conifers	2010
Strimbeck, GR; Schaberg, PG; Deha, DH; Shane, JB; Hawley, GJ	Midwinter dehardening of montane red spruce during a natural thaw	1995
Stushnoff, C; Tinus, RW; Esensee, VD	Analysis of freezing in buds of Douglas-fir seedlings by simultaneous detection of ultrasonic emissions and differential thermal-analysis	1992
Sundblad, LG; Andersson, B	No difference in frost hardiness between high and low-altitude *Pinus sylvestris* (l) offspring	1995
Sundblad, LG; Andersson, M; Geladi, P; Salomonson, A; Sjostrom, M	Fast, nondestructive measurement of frost hardiness in conifer seedlings by VIS plus NIR spectroscopy	2001
Sundblad, LG; Sjostrom, M; Malmberg, G; Oquist, G	Prediction of frost hardiness in seedlings of scots pine (*Pinus sylvestris*) using multivariate-analysis of chlorophyll-a fluorescence and luminescence kinetics	1990
Sutinen, ML; Palta, JP; Reich, PB	Seasonal differences in freezing stress resistance of needles of *Pinus nigra* and *Pinus resinosa* - evaluation of the electrolyte leakage method	1992
Sutinen, ML; Raitio, H; Nivala, V; Ollikainen, R; Ritari, A	Effects of emissions from copper-nickel smelters on the frost hardiness of *Pinus sylvestris* needles in the subarctic region	1996

Authors	Article Title	Publication Year
Tan, WX	Impacts of nursery cultural treatments on stress tolerance in 1+0 container white spruce (*Picea glauca* [Moench] Voss) seedlings for summer-planting	2007
Taulavuori, K; Taulavuori, E; Niinimaa, A; Laine, K	Frost resistance and PH of cell effusate in needles of artificially deacclimated Scots pine (*Pinus sylvestris*)	1996
Teklemariam, TA; Blake, TJ	Phenylalanine ammonia-lyase-induced freezing tolerance in jack pine (*Pinus banksiana*) seedlings treated with low, ambient levels of ultraviolet-B radiation	2004
Thomas, BR; Lester, DT	An examination of regional, provenance, and family variation in cold hardiness of *Pinus monticola*	1992
Timmis, R.	Critical frost temperatures for Douglas-Fir cone buds.	1977
Timmis, R.; Worrall, J.	Translocation of dehardening and bud-break promoters in climatically 'split' Douglas Fir.	1974
Tinus, RW; Bourque, JE; Wallner, SJ	Estimation of cold hardiness of Douglas-fir and Engelmann spruce seedlings by differential thermal-analysis of buds	1985
Toivonen, A; Rikala, R; Repo, T; Smolander, H	Autumn Colouration of First Year Pinus sylvestris Seedlings during Frost Hardening	1991
Valkonen, M. J.; Hänninen, H.; Pelkonen, P.; Repo, T.	Frost hardiness of Scots pine seedlings during dormancy.	1990
Van den Driessche, R	Measurement of frost-hardiness in 3 douglas fir at 3 nurseries by an electrical method	1970
Vangestel, C; Eckert, AJ; Wegrzyn, JL; St Clair, JB; Neale, DB	Linking phenotype, genotype and environment to unravel genetic components underlying cold hardiness in coastal Douglas-fir (*Pseudotsuga menziesii* var. *Menziesii*)	2018
Vann, DR; Strimbeck, GR; Johnson, AH	Effects of ambient levels of airborne chemicals on freezing resistance of red spruce foliage	1992

Authors	Article Title	Publication Year
Velasco, MH	Treatments for induction of cold hardiness in *Picea abies* (L.) Karst. And *Pinus sylvestris* L. Seedlings pre-cultivated under light-emitting diodes - impact of photoperiod and temperature including energy consumption and seedling quality after cold storage	2020
Viveros-Viveros, H; Saenz-Romero, C; Vargas-Hernandez, JJ; Lopez-Upton, J; Ramirez-Valverde, G; Santacruz-Varela, A	Altitudinal genetic variation in *Pinus hartwegii* Lindl. I: Height growth, shoot phenology, and frost damage in seedlings	2009
Waite, CE; Deha, DH; Rebbeck, J; Schier, GA; Johnson, AH	The influence of elevated ozone on freezing tolerance of red spruce seedlings	1994
Warrington, I. J.; Jackson, A. K. H.	Injury to radiata pine as influenced by freezing and thawing rate, and low temperature duration.	1981
Westin, J.; Sundblad, L. G.; Strand, M.; Hällgren, J. E.	Phenotypic differences between natural and selected populations of *Picea abies*. I. Frost hardiness.	2000
Wheeler, NC; Jermstad, KD; Krutovsky, K; Aitken, SN; Howe, GT; Krakowski, J; Neale, DB	Mapping of quantitative trait loci controlling adaptive traits in coastal Douglas-fir. IV. Cold-hardiness QTL verification and candidate gene mapping	2005
Wiemken, V; Kossatz, L; Ineichen, K	Frost hardiness of Norway spruce grown under elevated atmospheric CO2 and increased nitrogen fertilizing	1996
Wulff, A; Sheppard, L; Leith, I	Evaluation of electrolyte leakage, chlorophyll fluorescence and ultrastructural techniques for detecting effects of acid mist on frost hardiness of sitka spruce shoots	1994
Zapata-Valenzuela, JA; Ogut, F; Kegley, A; Cumbie, P; Isik, F; Li, BL; mckeand, SE	Seedling Evaluation of Atlantic Coastal and Piedmont Sources of *Pinus taeda* L. And Their Hybrids for Cold Hardiness	2015
Zhang, G; Li, YQ; Dong, SH	Assessing frost hardiness of *Pinus bungeana* shoots and needles by electrical impedance spectroscopy with and without freezing tests	2010

Authors	Article Title	Publication Year
Zhang, G; Sun, BB; Li, Z; Di, B; Xiang, DY; Meng, Y	Effects of soil temperature on bud break, shoot and needle growth, and frost hardiness in *Pinus sylvestris* var. *Mongolica* saplings during dehardening	2017
Zhao, SY; Colombo, SJ; Blumwald, E	The induction of freezing tolerance in jack pine-seedlings - the role of root plasma-membrane H+-ATPase and redox activities	1995
Zhao, XP; Hui, B; Hu, LX; Cheng, QZ; Via, BK; Nadel, R; Starkey, T; Enebak, S	Potential of near infrared spectroscopy to monitor variations in soluble sugars in Loblolly pine seedlings after cold acclimation	2017