Biological defences of Sitka spruce against the weevil *Hylobius abietis*: investigation of genetic variability and molecular basis to facilitate resistance breeding

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A thesis submitted for the degree of

Doctor of Philosophy

TRINITY 2024

Declaration

I declare that this thesis was composed by myself and that the work contained herein is my own except:

- In Chapter 2 where the data collection was completed by both myself and Oliver Spacey
- In Chapter 3 where the field trial was planted by myself, Professor John Mackay and Laura Guillardín-Calvo
- In Chapter 4 where, the damage area data collection was completed by myself, William Hoaen, George MacKay and James Letcher. The sequence quality control and alignment was completed by Tin Hang Hung, who is part of the ForesTree group at the University of Oxford.

All information from external resources has been fully referenced within. The work has not been submitted for any degree or professional qualification except as specified.

Impact of COVID-19 statement

This DPhil was undertaken during the COVID-19 pandemic which severely disrupted my progress at the start of the project. The experiment in Chapter 1 had to be delayed by 3 months due to not being allowed to return to University. Seeds required for the provenance experiment were also delayed due to the lack of staff in postal facilities and increased processing time through customs meaning that they had to be planted a year late. This meant that when the germination and survival of seedlings were impacted by a fungus, we did not have any time to resow them, so the size of the experiment had to be reduced. The reduced working capacity of the department, lack of in person supervision, delayed access to growth facilities, and severe impact on my mental health meant disruption to the DPhil was significant. I estimate that it caused over 4 months of delays to the project which could not be recouped.

Barley Rose Collier Harris, Trinity term 2024

Word count: 38,000 excluding references and appendices

Acknowledgements

This thesis is the culmination of 4 years of hard work, and I could not have done it or got through those years without so many amazing people. Firstly, thank you to the people who got me on the path – Caroline Dean and Jo Hepworth. You taught me how to love Plant Science.

My supervisor John MacKay, thank you for seeing the potential in me and helping me grow. Your patience, support, and guidance have been invaluable. Sorry, I almost killed you with the tree planting. The ForesTree Biology Group for being there to inspire, help, and keep me laughing when no one else understood that trees grow REALLY slowly. Plus, George, Will and Ollie, your help over the summers was invaluable. Special thanks to Henry for all your help as I learnt to code, Heather and Hayley for being wonderful friends and showing me life and science post PhD and Laura for all the time spent in the office, lab and cold forested hillsides thank you for keeping me going.

Thank you to my second supervisor Roger Moore, and the rest of the Ento team at Forest Research (especially Molly, who got roped into coming to the field). I had such an incredible 3 months at NRS. Also, a huge thanks to my industrial sponsors Dave and Helen, from Forestart, for your hospitality and backing, you really went above and beyond!

Thanks to the people in Oxford who made it feel like home, Channel 4's The Bubble, KCBC and my DTP Cohort. Zoe and Jai, thank you for making my time in HBAC the most fun imaginable. Andrew and Caitlin, thank you for always being there to talk when I needed you. Alice, thank you for everything; from the first hour of the first day in that lift, we've been in this together, and you've been wonderful; I can't wait to see where you go next!

To all my friends who asked how it was going, kept me smiling, listened to me talk about how many trees I had killed that week, and never questioned if I could do this – thank you. The Landmark Trust Team, my oldest Norfolk friends, and so many more. Thanks to the most perfect pet a girl could dream of and my late-night study companion – Rowena.

Huge thank you to my family, especially Mum, Nanny, Grandad, and Maisha. I love you all. This has stopped me from coming home more than I would have liked, but your phone calls and visits meant everything. You kept me going through every tough revision session from school, exam stress at uni, and now this. Without you, it wouldn't have been possible.

And finally, thank you to James. You have helped me more than I can ever explain. Your love, support, and faith in me have been unwavering. You've helped with every experiment and read every chapter. You are the home I can't wait to go back to at the end of every day. Thank you for everything. P.S. Sorry, you're not first author; it just doesn't work like that.

General Abstract

Climate change is expected to expand the range and shorten the life cycle of many insect species, increasing pressure on crop species from numerous pests, including *Hylobius abietis* (L.) (the large pine weevil). *H. abietis* poses a significant threat to European forestry, particularly affecting *Picea sitchensis* (Bong.) Carriére (Sitka spruce), the most widely planted commercial conifer in the UK. Mature weevils feed on conifer bark, which can girdle saplings, causing high mortality, with untreated sapling death rates exceeding 50%. Current protection relies heavily on treating saplings with pesticides for several years until they are large enough to withstand attack. These pesticides are costly and environmentally harmful and are slated for imminent withdrawal. Many known conifer herbivory defences exhibit substantial additive genetic variation and function dose-dependently, suggesting their potential for resistance breeding programs. Significant additive variation in resistance to *H. abietis* has been observed in several conifer species but has not been previously tested in *P. sitchensis*. This research aimed to investigate the genetic variability of the biological defences of the exotic conifer *P. sitchensis* against the weevil *H. abietis and* try to unravel its molecular basis to facilitate resistance breeding.

Using a greenhouse trial, we assessed whether genetic variation in resistance to *H. abietis* exists in the UK breeding population of *P. sitchensis*. Contrary to our hypothesis, family-level variation within this population did not significantly impact the damage area caused by *H. abietis*. This finding could result from genetic uniformity within the UK population, mainly from a single source, Haida Gwaii. Therefore, the current UK breeding population offers limited potential to support the development of a resistance breeding program, indicating a need to introduce new germplasm. In a field trial, significant variation in resistance to *H. abietis* was detected in *P. sitchensis* from different provenances across the native range, confirming that natural resistance varies across populations. This finding is promising for pest management, suggesting that incorporating new germplasm could facilitate a resistance breeding program. Additionally, known resistance to a different pest, *Pissodes strobi*, in *P. sitchensis*, appears to confer resistance to *H. abietis*, as provenances with higher resistance to *P. strobi* exhibited significantly lower mortality from *H. abietis*.

Understanding the molecular response of plants to herbivory attacks can help to identify resistance mechanisms. Previous studies have shown that many genes are induced by biotic stress in conifers, some of which are linked to increased resistance. We ran an RNA sequencing study to assess the transcriptional response in *P. sitchensis* to *H. abietis* exposure. We observed a large localised transcriptional response in the bark with significant involvement of jasmonate signalling and a smaller systemic transcriptional response in the needles, indicating a potential systemic defence

response. The differentially expressed genes (DEGs) in the needles suggested hormonal crosstalk between auxin and jasmonate in systemic defence responses. Subsequently, we compared the transcriptional response of saplings exposed to and potentially primed by previous attack and naïve trees after a period of *H. abietis* exposure. We found a small but biologically insightful set of DEGs in both bark and needles, including genes involved in defence priming. However, no significant difference in damage area was observed, indicating that while defences may be primed, the effect may not be strong enough to reduce damage from *H. abietis* in the conditions used here.

We built a knowledge base on the *H.abietis* and *P. sitchensis* system, which has direct applications in this system but could also apply to other conifers. Observing significant variation in *H. abietis* damage across *P. sitchensis's* native range but not within the UK breeding population underscores the need to study broader genetic diversity for resistance breeding. The observation that resistance to another pest conferred resistance to *H. abietis* allows the use of known biomarkers for testing resistance and suggests the importance of testing this in other species:pest systems. We developed two resources for future resistance breeding: more resistant provenances and a set of differentially expressed genes for further study.

Table of Contents

Declaration 2

Impact of COVID-19 statement 2

Acknowledgements 3

General Abstract 5

Table of Contents 7

List of Figures 11

List of Tables 13

Chapter 1: General Introduction 15

- Project background 15
- Sitka spruce in the UK 17
- Hylobius abietis 18
  - Life Cycle 18
  - Factors affecting the rate of feeding 18
  - The effects of climate change 19
- Current UK Management of Hylobius abietis 20
- Breeding conifers for insect resistance 23
  - Case Study: Sitka Spruce with resistance against white pine weevil 25
  - Case Study: White spruce with resistance to Spruce budworm 27
- Experimental methods for phenotyping resistance to *Hylobius abietis* 29
- Potential for genetic resistance to *Hylobius abietis* studies in conifers 30
  - Picea abies (Norway spruce) 30
  - Pinus radiata (Monterey pine) 30
  - Pinus pinaster (Maritime pine) 31
- Conifer defences against insect attack 31
  - Constitutive defences 31
  - Induced defences 32
- Rational and objectives 34
- References 36

Chapter 2: The genetic variability of resistance to *Hylobius abietis* in *Picea sitchensis* from the UK Breeding population in a controlled environment 46

- Abstract 46
- Introduction 46
- Materials 49
  - Insects 49

Plant material 49
Experimental set up 50
Damage measurements 51
Meta data 52
Tissue samples 52
Power analysis 52
Statistical analysis 53
Results 54
Response variables – number of scars and area of damage 54
Tree characteristics 56
Weevil characteristics 58
Effect of experimenter 59
Effect of Family 60
Results of models 61
Discussion 65
Impact of covariates 65
Impact of family 66
Implications for Integrated Pest Management 67
References 69
Chapter 3: The genetic variability of resistance to *Hylobius abietis* in *Picea sitchensis* from across its native range in a field environment. 74
Abstract 74
Introduction 74
Materials 77
Plant material 77
Field site specification 79
Experimental set up 80
Damage measurements 80
Tissue samples 82
Statistical analysis 82
Results 84
Provenance Material 84
Level of *H. abietis* damage over time 84
Level of *H. abietis* damage at two individual time points 85
Tree mortality 86
Geographical Variables 90

P. strobi resistant and susceptible full-sib crosses 92
Discussion 95
Level of *H. abietis* damage 95
Tree Mortality 96
Impacts and future directions 98
References 99
Chapter 4: Molecular response to attack by *Hylobius abietis* and the effects of priming in *Picea sitchensis*. 104
Abstract 104
Introduction 104
Methods 107
Insects 107
Plant material 108
H. abietis exposure experiment 108
Sampling for RNA sequencing 109
RNA isolation and sequencing 110
RNA sequence analysis 110
Results 112
Damage response 112
RNA sequencing and read mapping 113
Differential expression across the dataset 114
Constitutive differences between Needles and Bark 116
Effect of Treatment on Gene Expression 118
Gene Ontology Analysis 122
Discussion 128
Response of *P. sitchensis* to *H. abietis* attack 128
Uncovering priming 132
Future directions 134
References 135
Appendices 140
Appendix 1 140
Appendix 2 142
Appendix 3 144
Appendix 4 147
Appendix 5 149
Appendix 6 152

Chapter 5: General Discussion and Conclusions 157
No significant variation in resistance to *H. abietis* was detected in *P. sitchensis* from the UK Breeding population in a controlled environment 157
Significant variation in resistance to *H. abietis* in *P. sitchensis* from different provenances was detected in a field trial 158
Resistance to *Pissodes strobi* confers resistance to *H. abietis* 159
Large localised and smaller systemic transcriptional response to attack by H. abietis 159
Small but biologically insightful transcriptomic changes in primed trees 160
Knowledge base and resources developed 161
Future research directions 161
Impacts and potential applications of the research 162
References 164

List of Figures

Chapter 1

Figure 1 Control options for Hylobius abietis as part of the current integrated management plan 23

Chapter 2

Figure 2 Temperature and humidity over a 24-hour period 50

Figure 3 The articulated box around the stem of a sapling 51

Figure 4 Temporal variation in metrics of damage assessment. 55

Figure 5 The height (cm) against Diameter at base (mm) of each sapling 56

Figure 6 Variation across different diameters against the metrics of damage assessment. 57

Figure 7 Frequency distribution of saplings across different but burst stages. 58

Figure 8 Variation across weights of weevil pairs against the metrics of damage assessment. 59

Figure 9 Frequency distribution of number of saplings with different amounts of damage, separated out by experimenter and by day. 60

Figure 10 Variation across families against the metrics of damage assessment. 61

Chapter 3

Figure 11 Map showing the native range of *P. sitchensis* and the locations of the 18 provenances selected for the field trial. 77

Figure 12 Damage experienced by saplings during field trial. 81

Figure 13 Number of saplings in each damage class at each time point by provenance. 84

Figure 14 Mortality of provenances. 87

Figure 15 Geographical distribution of mortality and resistance. 88

Figure 16 Assumptions test of models 88

Figure 17 Mortality against height for each provenance 89

Figure 18 Mortality of the two pooled groups of seven 'resistant' provenances and 11 'susceptible' provenances. 90

Figure 19 Height(cm) at the start of experiment against latitude, longitude, and elevation. 91

Figure 20 Number of saplings in each damage class at each time point by cross 92

Figure 21 Mortality of crosses 94

Figure 22 Mortality against height for each cross demonstrating the Cross-By-Height interaction. 94

Chapter 4

Figure 23 Experimental plan showing three treatments 107

Figure 24 Experimental set-up 109

Figure 25 Boxplot showing area of bark consumed in the second exposure period for W and W2 113

Figure 26 Principal component analysis of the regularised log transformation of the gene expression in the technical replicates 114

Figure 27 Principal component analysis of the regularised log transformed gene expression data for the 72 samples 115

Figure 28 Hierarchical cluster analysis of the 500 genes with most significant variation in abundance 116

Figure 29 Over-representation analysis of GO terms for biological processes between bark and needles 118

Figure 30 Principal component analysis of the regularised log transformation of the gene expression data for the 36 bark samples. 120

Figure 31 Number of DEGs between each treatment at each timepoint in each tissue. 121

Figure 32 Venn diagrams showing how many DEGs are shared between different gene lists. 122

Figure 33 Over-representation analysis of GO terms for biological processes within DEG lists from bark samples between W1 and Cont treatment at each TP 123

List of Tables

Chapter 2

Table 1 Full list of all 13 full sib families used in the experiment 49

Table 2 The required sample size to be able to detect each effect size 53

Table 3 The mean and standard deviation for each of the dependent variables per day 55

Table 4 The results of the three main models 62

Chapter 3

Table 5 Information about the 18 Provenances selected. 78

Table 6 Information on the 11 full-sib crosses used for exploratory analysis in the field trial. 79

Table 7 Results of likelihood ratio tests using CLMMs on damage score by provenance data by day 85

Table 8 Results of likelihood ratio tests using CLMMs on damage score by provenance data on the final time point 86

Table 9 Results of likelihood ratio tests using glmers on mortality by provenance 86

Table 10 Results of likelihood ratio tests using lmers on collection Latitude, longitude and elevation on the height at the start of the experiment. 91

Table 11 Results of likelihood ratio tests using Cumulative link mixed models (CLMMs) on damage score by cross 93

Chapter 4

Table 12 Results of likelihood ratio tests using lmers. On damage area by treatment between W2 and W1 112

Table 13 RNA Sequencing quality 114

Table 14 DEGs in Bark between W2 and W1 samples 126

Chapter 1: General Introduction

Project background

Hylobius abietis (L.) (the large pine weevil) is a major pest to UK and European forestry. Mature weevils feed on the bark of conifers and often cause death of saplings. This has a large economic impact as without treatment, over 50% of seedlings will die (I. Willoughby, Moore, and Nesbet 2017). Currently, the most common method of protection is the regular application of pesticides to the seedlings until they are deemed to no longer be at risk. These pesticides are both expensive and damaging to the environment and are due to be withdrawn from the market imminently. As the global temperature continues to increase due to climate change, it is likely that the range of this pest will change, and its life cycle will shorten (Inward, Wainhouse, and Peace 2012; David Wainhouse, Inward, and Morgan 2014). In the UK *Picea sitchensis* (Bong.) Carriére (Sitka spruce) is the third largest crop by land area and contributes £1bn to the economy each year (Forest Research 2020). *P. sitchensis* is particularly badly affected by *H. abietis*. Since Sitka spruce has no known resistance to the large pine weevil, resistance breeding is not currently possible. This project will address this problem.

The project developed a new area of research which comes with several large challenges. The initial challenge is that tree breeding in the UK focuses on growth and form traits rather than resistance and *H. abietis* resistance has never been looked for in Sitka spruce. Therefore, this project will have to create a range of new resources and frameworks and may face new unknown challenges. In addition, the long generation time and draft state of the genome of Sitka Spruce will make the experiments slow to set up and the data difficult to process. However, the use of accelerated growth conditions and collaboration with existing projects internationally may help to overcome some of these difficulties.

This chapter will cover the general background of the project. It will review the current literature available about:

1. *H. abietis* and *P. sitchensis* as our study system
2. Different ways of managing insect attack in conifers, both by breeding for resistance and using silviculture.
3. The studies that have been done looking at resistance to *H. abietis,* summarising both their key results and methods
4. Constitutive and induced defences to attack in conifers

At the very end of this chapter, I set out the rationale of the project, stating the questions which need to be answered and laying out the main objectives.

Sitka spruce in the UK

Sitka spruce is a very important commercial crop for the UK. It accounts for 60% of the volume of softwoods harvested annually (J. Moore 2011) and contributes over £1bn towards the total £2.5 billion gross value added to the UK economy annually by all forestry and primary wood processing (Forest Research 2020). Sitka spruce is a popular crop in forestry as it grows vigorously even in poor soils and has good wood properties. Its natural range is the west coast rainforest in North America from southern Alaska to northern California (Peterson and Martin 1997) and it is a popular non-native crop in Europe, especially in the British Isles. It was first introduced to the UK in 1831 by David Douglas however, the main seed imports to Britain came post 1922 from Haida Gwaii (formerly know as Queen Charlotte Islands) (MacDonald and Al 1957). Positive trial results led to increased planting, meaning that between 1920 and 1980, the composition of conifers planted changed from 11% to 68% Sitka spruce and has remained at such high levels ever since.

In order to improve the crop, the selection of superior Sitka spruce known as 'plus trees' began in 1963, based on growth and form traits (Fletcher and Faulkner 1972). As a result, around 1800 individuals from Haida Gwaii were selected as plus trees (Lee and Connolly 2010). Seeds were collected from these trees after wind-pollination and grown in common garden experiments; the performance of the daughter trees in the same location makes it possible to calculate the genetic merit of the parent or its breeding value. The Sitka spruce breeding population was formed by a subsequent selection of the best of those plus trees based on breeding values for three top economic traits: growth rate, stem form and wood density. The first 240 trees were selected using mean performances weighted across sites and estimates for family heritability (Lee 2001) a further 120 trees were selected more recently using breeding values based on mixed model analysis (Lee and Connolly 2010). These trees make up the Sitka spruce General Breeding Population for the UK. As these trees form a population which introduced to the UK from one small area in the natural range and have been subsequently bred for specific growth traits they are unlikely to reflect the full genetic diversity of the species.

The most common UK silvicultural practice for Sitka spruce is to plant saplings on a clear fell site when they are 20-40cm in height (approximately 2 years of age) at an initial density of 2 x 2 m to get commercially acceptable yields with rotation lengths of around 35 years (J. Moore 2011). The current government target is to increase tree canopy and woodland cover from 14.5% to 16.5% of the total land area in England by 2050 (HM Government 2023) to support both environmental and ecological goals; this will involve increasing both conservation planting and commercial forestry.

Hylobius abietis

The large pine weevil, *Hylobius abietis* (L.), causes large losses in forestry across its entire European range from Norway down to Spain. If control measures are not used, *H. abietis* adults eat the bark of young saplings, girdling the trees and causing high levels of mortality, especially in areas that use clearcutting which augments the population (Långström and Day 2004; Day et al. 2004).

Life Cycle

H. abietis adults migrate by flight in spring and are attracted to cut stumps by the volatiles the stumps emit, including monoterpenes α-pinene and ethanol (Nordlander 1987; Nordenhem and Eidmann 1991). The adults then breed in the freshly cut stumps and in summer each female lays 25+ eggs in the surrounding soil and bark of the roots which provides food for the larvae (Nordlander, Nordenhem, and Bylund 1997). An egg laid in early summer in the UK climate typically reaches the larval stage by late autumn and the adult stage by the following late spring (Scott and King 1974). However, this process can take up to 4 years if the egg is laid later in the season or if the climate is colder (Day et al. 2004). When the adults emerge, they walk to find nearby living trees; they feed on the bark of surrounding conifer trees and their flight muscles quickly develop. Some adults remain at their emergence site, and some migrate by flight in spring, potentially several kilometres, and are attracted to the volatiles released by fresh clear-cuts (Tan et al. 2011). Weevil densities are highest for the two years following clear-cutting and infestations are more concentrated in areas after clearcutting, which is the most common silvicultural practice for conifer plantations throughout Europe (Nilsson et al. 2010). Adults *H. abietis* can live for up to 4 years (Eidmann 1979).

Factors affecting the rate of feeding

H. abietis feeds on the roots and bark of the branches in the crowns of the trees as well as the bark of both the newly planted conifer seedlings and adult conifers surrounding the clear-cut (Örlander et al. 2000). However, only feeding on the bark of the stems of the newly planted conifer seedlings is of commercial concern. This is because high levels of feeding on the small stems of the trees result in a large proportion of the bark being removed, and often, the wounds coalesce together, and the stem is girdled (a complete ring of bark is removed around the entire circumference of the stem). Girdling causes the tree to be unable to transport water and nutrients from its roots to shoots, and therefore, the saplings die.

An individual *H. abietis* will eat around 20 mm^2 of bark per day but this figure varies greatly depending on several factors. *H. abietis* adults are around 12 mm long, larger adults have an increased rate of feeding (D. Wainhouse, Boswell, and Ashburner 2004) and the females consume significantly more bark per day than the males (Bylund, Nordlander, and Nordenhem 2004).

Silvicultural practices and geographical location also affect *H. abietis* bark consumption. Mounding and scarification are both silvicultural practices which involve disturbing the soil before planting, these practices both lead to lower levels of attack and mortality, saplings planted in mineral soil are also less attacked than those in humus (Nordlander et al. 2011) and feeding rate is reduced in bark with a lower nitrogen content (D. Wainhouse, Boswell, and Ashburner 2004). The debarked area, has been shown to be lower on saplings with a larger stem diameter (Nordlander et al. 2011), as these tend to have thicker bark and thus a greater resin duct density which negatively correlates with the amount of feeding by *H. abietis* (D. Wainhouse et al. 2005). In branches of adult trees, area debarked peaks at a diameter of 11 mm and is lower for both smaller and larger branches, however as this is a proportion rather than raw area these two results are not comparable (Örlander et al. 2000). Local climate also affects the feeding rate of *H. abietis* as they will feed more in warmer more stable conditions, but they avoid feeding during the mid-day sun (Bylund, Nordlander, and Nordenhem 2004).

Although *H. abietis* can eat the bark of broad-leaf trees, they prefer conifers and will avoid broad leaves in the presence of a conifer (Manlove, Styles, and Leather 1997). Different species of conifers get varying levels of damage. In field studies, saplings of *Pseudotsuga menziesii* (Mirb.) Franco (Douglas Fir) and *P. sitchensis* receive far more damage than *P. abies* or *P. Sylvestris* (Wallertz, Nordenhem, and Nordlander 2014). These authors reported that 60% of *P. menziesii* and *P. sitchensis* saplings were severely damaged or killed when no other controls were applied compared to 49% and 27% in *P. abies* or *P. Sylvestris,* respectively.

The effects of climate change

The IPCC's fourth assessment report 2007 states that "It is very likely that all land regions will warm in the 21st century." Within the natural range of *H. abietis,* it predicts an increase in average temperature of 2.3°C to 5.3°C along with shorter snow periods and increases in annual and extreme precipitation (Christensen et al. 2007). This change in climate is likely to have numerous effects on the plant pest interactions, including that of *H. abietis* and its hosts. Barredo et al collected data in 2015 looking at the distribution of *H. abietis* and integrated both host and pest species distribution data and species distribution modelling using current and predicted climate data to try to assess the potential distribution of the large pine weevil under both current and future climates (Barredo et al. 2015). Their study predicted that in 2080 the potential suitable habitat of *H. abietis* would decrease by 23-27% as the distribution moved towards more northern and higher elevation regions. However, the model only had limited access to pest distribution data from the Global Biodiversity Information Facility (GBIF 2014) which didn't include any data for the warmer or cooler extremes of their true range. The current range already of both *H. abietis* and its hosts extends as far north as north-

western Finland and Sweden (Zas et al. 2014; Nilssen 1984) but could extend further. However, it is unlikely there will be much range retraction in the south as *H. abietis* currently survive in the warm climates of Spain (López-Villamor et al. 2019), they develop healthily at temperatures of at least 25°C (Inward, Wainhouse, and Peace 2012) and during the warmest parts of the day they are able to avoid the heat by burrowing into the cooler ground. Population distribution may change with a higher density of *H. abietis* occurring in higher latitudes (Nordlander et al. 2017) and at higher elevations (Chinellato et al. 2014) which currently have a very low insect density.

The warming climate will also affect the life cycle of *H. abietis*. In the warmer parts of its range, the life cycle of *H. abietis* from egg to egg takes one or two years but in cooler areas this can extend to over four years (Lekander et al. 1985). The warmer climate is likely, therefore, to speed up the life cycle as more larval development will occur during warmer winters, which is supported by a constantly high abundance of food. Studies in lab conditions demonstrate that higher temperatures both speed up development and reduce the likelihood of prepupal dormancy (Inward, Wainhouse, and Peace 2012; David Wainhouse, Inward, and Morgan 2014) meaning that it is more common to have one generation per year (be uni-voltine). This would mean that *H. abietis* population is likely to increase as temperatures increase. Higher temperatures were also shown to increase size, fecundity and egg size, which led to larger larvae with decreased mortality. Thus, the warming climate may extend the geographical range of *H. abietis* and increase *H. abietis* density, especially in more elevated areas and northern latitudes. Taken together, these changes could increase damage levels and economic losses to forestry by *H. abietis,* especially in areas where damage levels are currently low.

Current UK Management of Hylobius abietis

Hylobius abietis is one of the most serious pests to conifer saplings in the UK. If protective measures are not taken, on average 50% of transplanted seedlings will die each year; and in some cases, mortality can be up to 100% (I. Willoughby, Moore, and Nesbet 2017). The saplings are at risk for the first three years after they are planted with the highest risk being in the first two years before the stem diameter gets large enough to reduce the likelihood of girdling (Morgan 1999). To help manage pests and reduce pesticide usage more broadly in forestry, a decision key has been devised (I. Willoughby 2004) , which can also be applied to *H. abietis*.

1. Identify the problem and evaluate its potential and likely consequences.
2. Consider control options
    a. Take no action (ill-advised with *H. abietis*),

    b. Avoid the problem by changing silviculture or tree species
    c. Take remedial action (last resort)
3. Consider which remedial action is suitable (non-chemical is preferable but may not always be practical)
4. Consider which chemical methods are appropriate.

This framework was used to set up an integrated management plan for *H. abietis*. When considering control options taking no action is likely to lead to large losses. The problem could be mostly avoided by changing silvicultural practices, but this is not always practical.

The use of continuous cover forestry (CCF) techniques rather than clear-felling has been shown to reduce damage done by *H. abietis* to almost negligible levels (B. Mason et al. 2003) however, the conversion of sites to a CCF management style is very time-consuming (Kerr et al. 2010) and adoption rates are often low. In the UK only 5% of the area which could be used for CCF currently is being managed using this technique (W. L. Mason 2015; Malcolm, Mason, and Clarke 2001).

H. abietis can survive at a high population density for up to two generations (5 years) in stumps after clear-felling has occurred; after this time, they will begin to migrate away. Therefore, if the site is left to lie fallow for 4-5 years after clear-felling, the risk of damage is much reduced (R. Moore 2004). This option causes revenue loss, can increase flood and nitrate release risk and the strategy will not work if there are neighbouring sites which attract the weevils to the area instead.

There are other techniques which can be used in combination with these to avoid the problem. These include using brambles as an alternative food source to divert *H. abietis*, having open areas of mineral soil directly around the saplings as *H. abietis* tend to avoid walking over exposed mineral soil, and using vigorous seedlings with good root systems and larger diameter stems which are more likely to be able to withstand the initial attack (Morgan 1999; David Wainhouse and Brough 2007).

If the problem cannot be avoided there are also numerous remedial actions which can be taken both chemical and non-chemical. There are three major non-chemical methods that have been tested in the management of *H. abietis* in the UK: physical protection, mulching and de-stumping and biological control using nematodes. Physical protection in the form of full tree shelters are effective in the UK but considered too expensive (I. Willoughby, Moore, and Nesbet 2017) and other forms of physical protection are only effective if *H. abietis* populations are already very low (Moore et al, unpublished) and the ground has already scarified. Mulching or de-stumping can significantly reduce *H. abietis* populations, if done quickly after felling, before the emergence. However, the population

will not be eliminated, and de-stumping can increase water acidification, cause soil carbon losses and reduce the nutrient status of the site (Moffat, Nisbet, and Nicoll 2011).

The use of entomopathogenic nematodes (EPN) as a bio pesticide has shown substantial success. Currently only one species: *Steinernema carpocapsae,* is used commercially. When applied to stumps, the infective juveniles enter the larvae or pupa of *H. abietis*, introducing bacteria which break down the host tissue to create a nutrient-rich food for the nematodes. This reduces emergence by 20-70% for up to two years, reducing damage inflicted in the treated area curbing population (Williams et al. 2013; Torr, Wilson, and Heritage 2005; Brixey, Moore, and Milner 2006). EPN are safe for plants and vertebrates, do not affect non-target Coleoptera (Dillon et al. 2012) and are able to penetrate into the stumps below soil level which pesticides often can't reach (Torr, Wilson, and Heritage 2005). However, up to two-thirds of conifer sites in the UK are not able to use EPN as they are too steep or remote (I. Willoughby, Moore, and Nesbet 2017).

Although non-chemical remedial action is preferable it is not always practical so to allow for forest regeneration, young plants are generally treated with insecticides to avoid excessive *H. abietis* losses. There are three main time points in which saplings may be sprayed – pre-planting, immediately post-planting or later post-planting as a top up if the *H. abietis* population is particularly high. There are three insecticides which are commonly used in the UK. Alpha-cypermethrin and cypermethrin are both non systemic pyrethroid insecticides which kill insects by stopping nerve impulses (MacBean 2012). Alpha-cypermethrin is a pre-planting spray used in a nursery setting so the insecticide is absorbed into the bark pre-planting reducing contamination risk to the environment and providing 3-7 months of high-level protection from *H. abietis* (I. H. Willoughby et al. 2020). If required cypermethrin is then used as a post-planting top up to provide an additional 1-3 months of protection; however, it is currently being phased out across Europe over its potential environmental impacts as it does have to be applied wet and therefore can contaminate water courses where it is very toxic to the aquatic environment (European Commission 2012). The most used insecticide in the UK to control *H abietis* is Acetamiprid, a synthetic neonicotinoid insecticide which blocks postsynaptic nicotinic receptors in the central nervous system killing the insect (MacBean 2012). Acetamiprid can either be applied pre-planting in the nursery or post planting as a top up and studies have shown it works as well as or better than alpha-cypermethrin (I. H. Willoughby et al. 2020). Acetamiprid is ~500 times less toxic to aquatic life than Alpha-cypermethrin and cypermethrin.

The Forestry Stewardship Council (FSC) classifies all three as 'highly hazardous pesticides' (HHPs) (Forestry Stewardship Council 2019). The environmental and social risk assessment for and Acetamiprid requires estate of 5000+ acers to provide "financial or in kind contributions to research

into chemical and non-chemical alternatives" (Forestry Stewardship Council 2020) (such as this PhD) but there is no obligation to follow these ERSAs. Acetamiprid as the least hazardous insecticide has been the main pesticide used in the UK for the last 3 years. However, the Health and Safety Executive has withdrawn the use of Acetamiprid as a plant protection product, it is no longer on sale and will not be able to be applied from July 2024 (Purdy 2023). Private growers are delaying intended restocks for the winter of 2024/25 but this is not possible for larger restocks, which could result in high levels of losses and then to uneven crop establishment as dead plants have to be replaced. The lack of currently available pesticides and the heavy losses if they are not used mean other avenues need to be explored.

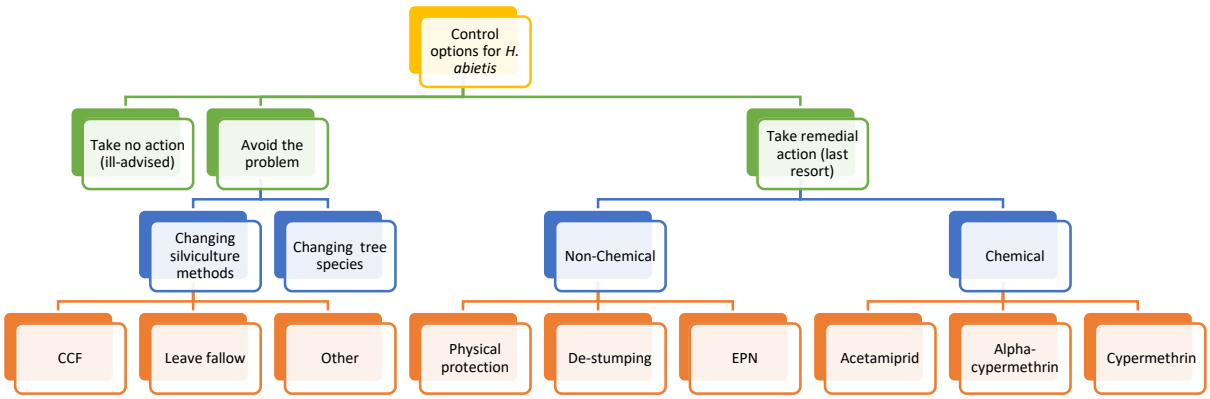


Figure 1 Control options for Hylobius abietis as part of the current integrated management plan (I. Willoughby, Moore, and Nesbet 2017)

Breeding conifers for insect resistance

Being long-lived and sessile trees have evolved to cope with insect attacks; however, with the effects of both climate change and international trade, the threat to trees from pests is increasing (Roques 2010). This increasing threat reduces the already small margins earned in commercial forestry and has a much larger and wider economic impact (Lovett et al. 2016; Aukema et al. 2011). Management of insect pests has traditionally focused on eradication or containment and, once an insect is prevalent in an area, on mitigating its effects. This often requires costly, time-consuming, and environmentally damaging applications of pesticides or physical barriers but can give fast and effective results in the short term. More recently there has been a surge in interest in breeding trees for resistance (Woodcock et al. 2018; Telford et al. 2015; Sniezko and Koch 2017).

Telford et al (2015) define resistance as "the ability of an individual host tree to use genetically encoded mechanisms to defend against or withstand attack by an invading organism, with an associated and measurable increase in fitness compared with hosts who do not employ these

mechanisms". Resistance can be qualitative and, therefore, complete resistance may be achieved and will be often controlled by one gene, whereas quantitative or partial resistance is controlled by many genes. Induced or constitutive resistance can also be referred to in terms of tolerance where the tree is attacked but is able to survive and/or is not impacted (Woodcock et al. 2018). Resistance is often under varying levels of environmental and genetic control, with interaction, meaning that a resistant tree in one area may not necessarily be resistant in a different environment, which may present different growth conditions or pest population pressures; therefore, understanding these levels of control is hugely important.

There are several notable benefits to resistance breeding. Firstly, by using trees with increased resistance there is a reduction in cost of other pest controls such as pesticides. Secondly, large-scale, long-term deployment will have the potential to decrease the insect population. Thirdly, resistance can contribute to an integrated management system (Leather 1996). Breeding resistant trees is a difficult task especially as most tree breeding currently only selects for growth and wood traits. Several reviews discuss the basic steps involved in developing and using resistant trees (Woodcock et al. 2018; Sniezko and Koch 2017) which can be summarised as follows:

(1) Selection of plant host pest system,
(2) Researching natural resistance in the population, through looking at the natural population and that of related species as well as rigorous trials checking for heritable resistance,
(3) Developing a population of resistant trees, using selective breeding. This could be biomarker-assisted if markers have been discovered but an understanding of the resistance mechanism is not required (Smalley and Guries 1993)
(4) Propagation of resistant material at a large scale using natural processes and seedling and clonal seed orchards
(5) Planting of resistant trees to maximise benefits and minimise risk of resistance being overcome
(6) Sustained monitoring to see if resistance is being overcome and to help guide future studies

There is an interest in resistance breeding in trees, with research and breeding programs identifying genetic variation in resistance but very few have led to the operational planting of resistant material. Of 274 activities documented by the FAO as "activities are now documented on breeding for insect and disease resistance in forest trees" but only 24 were on insect pests in conifers. Of those 24, most had detected variation in resistance at either the seedling or provenance trial level but only 3 are 'breeding programmes with deployed resistant material' (FAO 2015). These are resistance to the green spruce aphid in Sitka spruce in Denmark (Harding, Roulund, and Wellendorf 2003) and

resistance to the white pine weevil in both Sitka spruce and white spruce in Canada (René Alfaro et al. 2004; King et al. 2011). This is partially to do with the slow generation times of trees, and so new methods such as breeding without breeding will hopefully speed up this process (El-Kassaby et al. 2011), though this will be difficult in conifers as they often have large complex genomes.

Conifers have mechanisms to minimise and prevent damage caused by insect herbivory; these are a mix of constitutive, induced, chemical and physical defences (Franceschi et al. 2005; Ali and Agrawal 2012). Some of these traits demonstrate additive genetic variation (López-Goldar et al. 2019; X. Moreira, Zas, and Sampedro 2013) and directly affect resistance (Phillips and Croteau 1999). Different subpopulations may also vary in several traits which could affect insect herbivory including release of volatiles, size or vigour, and nutrient content (Björklund, Nordlander, and Bylund 2005). Although few programmes have been successful, there is potential for many to be developed. The success of breeding resistant lines of Sitka Spruce against the white pine weevil (René Alfaro, King, and vanAkker 2013) and the potential to breed white spruce resistant to spruce budworm are of particular note (Parent et al. 2020), as is the discovery of additive genetic variation in susceptibility/resistance to *H. abietis* in several different species (Zas et al. 2005; 2008; Puentes et al. 2018).

Case Study: Sitka Spruce with resistance against white pine weevil

1: The Problem

Sitka spruce is a popular crop in forestry as it grows vigorously even in poor soils and has good wood properties. Its natural range is as part of the coastal rainforest in North America from southern Alaska to northern California (Peterson and Martin 1997) as well as being popular in Europe as a non-native crop. However, its use in reforestation in North America has been in steep decline since the 1980s due to an increase in white pine weevil, *Pissodes strobi* Peck (Coleoptera: Curculionidae) infestation. It is estimated that a serious infestation over 30–40 years decreases stand volume by 30–40% (Heppner and Turner 2006).

The *P. strobi* adults overwinter in the ground at the base of the trees and emerge in spring. They then crawl to the terminal leader where they mate and lay eggs in the bark. In the summer, the larvae hatch and mine down eating the phloem and making pupal chambers in the xylem which girdles and therefore kills the previous year's leader (Silver 1968). As the terminal leader is killed, lateral branches begin to grow upward and fork the main stem, making it unsellable (René Alfaro 1989).

2: The Solution

Initially there were only three ways to control *P. strobi*. Clipping and disposal of infested leaders was labour intensive and had to be done annually for many years and had variable success. Spraying with

insecticides (initially DDT) was also expensive, impractical over large areas and polluted the area. The only viable strategy was avoidance, i.e. stop use of *P. sitchensis* (Hall 1994). *P. strobi* require a set temperature to develop so risk zones were created based on temperature (McMullen 1976). These Zones then dictated what proportion of Sitka spruce could be grown. However, the discovery of genetic resistance meant that this strategy could be superseded.

The first evidence of genetic resistance to in Sitka spruce to *P. strobi* was seen in the 1940s when only two trees remained untacked in a heavily infested plantation (René Alfaro 1989; Silver 1968). A provenance test was set up in 1974 in the Salmon Valley (Canada) to test for growth traits. However, measurements of natural weevil attacks over 15 years demonstrated that some provenances appeared to be more resistant than others (René Alfaro and Ying 1990). This was a very positive result and the British Columbia (BC) ministry of forests increased investment in the Sitka spruce weevil resistance program allowing the development of many new trials. Three areas in BC were identified as the source of resistant genotypes: Haney, the Big Qualicum River, which both have high incidence of *P. strobi* and the Skeena River which is an area of natural hybridization between Sitka and white spruce (Ying 1991; King, Alfaro, and Cartwright 2004). Many of the early trials had to be ended as natural weevil populations were too slow to build up or too variable. In later trials, the natural weevil population was augmented with the addition of caught weevils to each tree to standardise the testing (René Alfaro, King, and vanAkker 2013). The detection of resistant trees and provenances made it possible to begin to look at the underlying mechanisms of resistance – namely increased density of resin canals and sclereid or stone cells which both repel the attack of *P. strobi* (King et al. 2011). The augmented trials also produced reliable results much faster, within 4 years of adding the weevils, allowing faster selection of resistant trees to create an F1 population. The F1 population allowed heritability estimates to be calculated which showed that resistance was highly heritable (Individual and family heritability were 0.5 and 0.9 respectively) (Xoaquín Moreira, Alfaro, and King 2012). The progeny of resistant trees received fewer attacks and had higher levels of constitutive defences. Several families showed very high levels of resistance, and one clone, 898, has been intensely studied as it is both resistant and grows tall and straight. There are now several different categories of seed available, category B seed is from 'weevil resistant' provenances and category A seed which is seed from seed orchards using weevil resistant parents. Since the deployment of this seed, the attack rate has been more than halved (King, Alfaro, and Cartwright 2004) and is predicted to eventually result in a reduction of over 80% (King and Alfaro 2009), meaning that Sitka spruce can now be planted even in high hazard zones. However, the amounts of Sitka planted, and the deployment of resistant seed is still closely monitored.

It took over 20 years for this to become possible with a huge amount of funding and scientific effort, which is clearly not possible for each host-pest system. Since this pioneering case study there have been huge advances in sequencing and biotechnology which should allow for faster resistance breeding and selection. Nevertheless, we have yet to see the effects of these technological advances as this is still one of the most successful examples of tree breeding for resistance and the only example of successfully breeding and implementing conifer resistance to insect attack.

Case Study: White spruce with resistance to Spruce budworm

1 The Problem

Spruce budworm (SBW) *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae) is one of the most damaging pests facing coniferous forests in North America (Blais 1983; Hardy, Lafond, and Hamel 1983). SBW cause severe defoliation by feeding on the foliage of their hosts, and larvae mostly feed on new growth. This reduces growth and reduces the chance of tree survival.

SBW attacks both natural stands and plantations of several conifers including *Abies balsamea* and *Picea glauca* to which it causes economic devastation as well as *Picea rubens* and *Picea mariana* which it effects to a lesser extent (Hennigar et al. 2008). SBW has an outbreak approximately every 30 years (Jardon, Morin, and Dutilleul 2003). The last outbreak started in the 1970s and over 55 million hectares of forest in eastern Canada were affected (Blais 1983), there is an also a current outbreak which, since 2006, has led to the defoliation of over 8.1 million hectares in Quebec (Ministère des Forêts 2018). These outbreaks cause huge damage with losses ranging from 3-68 m^3/ha of wood (Gray and MacKinnon 2006) causing billions of dollars of losses to the local economy (Chang et al. 2012).

Outbreaks used to be responded to with the widespread use of now banned chemical insecticides and instead now rely on the costly aerial application of the biological insecticide Bacillus thuringiensis (Bt) and the insect growth regulator Mimic R. The Bt gene (cry1Ab) was successfully overexpressed in white spruce and some lines were lethal to SBW (Lachance et al. 2007) but because transgenic trees are not permitted commercially in Canada this strategy cannot be used. Currently, early intervention strategies are providing a promising possibility; however, they are also time-consuming and require constant monitoring (MacLean 2019). Research suggests that White Spruce has evolved some resistance to SBW (Mageroy et al. 2015; Lamara et al. 2018) which has been reinforced by quantitative genetics analysis (Méndez-Espinoza et al. 2018) and transcriptional data (Parent et al. 2020).

2 The Solution

At present there is no application of resistance breeding in white spruce as a defence to SBW. However, many of the main steps towards successful selection of resistant trees have already been achieved. The first evidence that there may be naturally occurring resistance to SBW in *P. glauca* came from a plantation in Southern Quebec where susceptible heavily defoliated trees were found growing next to much more lightly defoliated trees (Bauce and Kumbaşli 2007; Daoust et al. 2010). When the chemical compositions of the resistant and susceptible plants were examined and found to have varying levels of many different components including sugars (Clancy 1992), phenolic compounds (Delvas et al. 2011), monoterpenes and tannins (Daoust et al. 2010). The resistant ones were also found to have a higher constitutive, and induced (Parent et al. 2018), level of accumulation of two hydroxyacetophenones, piceol and pungenol, but not their respective glucosides (Mageroy et al. 2015; Delvas et al. 2011). These hydroxyacetophenones have been shown to reduce development in female SBW, slow larval development and increase larval mortality (Delvas et al. 2011; Parent et al. 2017) and SBW is seen to have longer intermeal intervals on resistant trees even in no choice experiments (Despland et al. 2011). The biosynthetic pathway was elucidated and showed that the hydroxyacetophenones build up as inactive glucosides which are then transformed into their active state due to the cleavage of the glucoside bond (Mageroy, Jancsik, et al. 2017). Transcriptome profiling of 24,000 genes in resistant and susceptible trees showed that *Pgβglu-1* was the most differentially expressed and that it accumulated in the foliage (Mageroy et al. 2015). *In vitro* assays demonstrated that *Pgβglu-1* was capable of cleaving the glucoside bond in picein and pungenin to make the hydroxyacetophenones (Mageroy et al. 2015) and transgenic overexpression in white spruce did the same *in planta* (Mageroy, Lachance, et al. 2017). This defence strategy was found to be highly heritable by both heritability estimates from sib analysis (Méndez-Espinoza et al. 2018) and from a kinship matrix (Parent et al. 2017). Studies also looked to see if there were any metabolic or growth trade-offs to accumulating hydroxyacetophenones and *Pgβglu-1* transcrips in white spruce of a range of ages, but no clear trade-offs were found (Lamara et al. 2018; Méndez-Espinoza et al. 2018).

Resistance breeding should be possible for white spruce as a defence against SBW, as a heritable marker has been identified that is correlated with increased resistance. However, there are still gaps in the understanding as nothing is known about how *Pgβglu-1* is regulated or compartmentalised. In addition, monogenic resistance is easily overcome due to the strong selection pressure it elicits. Therefore, more resistance genes should be found before a resistance program is started. Transcriptome analysis and association studies have already suggested that a range of other genes are also involved and this should be further looked into (Lamara et al. 2018; Parent et al. 2020).

Experimental methods for phenotyping resistance to *Hylobius abietis*

When aiming to phenotype resistance against *H. abietis*, damage level is often used as a proxy for susceptibility which is the inverse of resistance. There are various experimental techniques which have been used in past experiments, which were considered in the present project.

One approach is using choice experiments, which can be in the greenhouse or field. Choice experiments, especially those on field sites, create a more representative of the real-world system where the weevils would have the option to feed on many different seedlings. It is hard to distinguish if a sapling has recorded low levels of damage because the weevils are repelled from it or attracted to a different sapling. Alternatively, experiments can be run as non-choice experiments where the weevil only has one food source, these are run in the lab or greenhouse. Comparisons between levels of damage done by *H. abietis* on seven species showed mostly consistent results between the field trial and lab experiment, e.i. *P. sitchensis* and *P. menziesii* were the most damaged (Wallertz, Nordenhem, and Nordlander 2014). In the past many lab experiments have been done using cut stem twigs often referred to as cafeteria bioassays (D Wainhouse et al. 2005) however, cut branches are unable to respond to weevil attack and deploy induced defences and when compared, patterns of weevil feeding on twigs differed from those on living trees (Zas et al. 2019).

There are 3 main ways that studies have measured damage area. The first method is the one used in Chapter 2 as well as in several previous studies (Bylund, Nordlander, and Nordenhem 2004; Leather, Ahmed, and Hogan 1994; Doležal, Kleinová, and Davídková 2021) . The insects are allowed to damage the area which is then wrapped in a see-through material and the areas of damage are measured directly either by being are drawn round, measured with transparent millimetre paper or imaged. The benefit of this method is it should yield a quantitative result rather than an estimation and it also allows for the number of scars to be measured, however it is very time intensive. Subjective assessments can be used level of damage is scored on a scale (Zas et al. 2014; 2006; 2008; Puentes et al. 2018). This method is much faster, so it allows for many more trees to be assessed in a single day, and is commonly used in field trials (including ours in Chapter 3) rather than greenhouse experiments. However, it can lead to ordinal data which can be more complex to analyse and doesn't allow for quantitative assessments of different levels of damage. The final way is by comparing the area of scars to areas of a known size (Zas et al. 2019; Puentes et al. 2021; Nordlander et al. 2011). This method was used in Chapter 4. It allows for quantitative results and is fast but could be subjective.

Potential for genetic resistance to *Hylobius abietis* studies in conifers

Due to *H. abietis* having a large range and being a generalist herbivore, which causes widespread damage across Europe, genetic variation in resistance has been studied in several species, particularly in spruces (*Picea spp.*) and pines (Pinus *spp.*).

Picea abies (Norway spruce)

Significant genetic variation in resistance to the *H. abietis* between different half-sib families was seen in *P. abies* (Zas et al. 2017). As there were low individual heritability estimates (h_i^2 consistently below 0.09) and moderate family heritability estimates (h_f^2 0.53 to 0.81), which suggests that breeding using forward selection for higher resistance is unrealistic but positive backward selection of only the more resistant families could lead to substantial genetic gains. A different study also looking at half-sib families of Norway spruce also found a genetic component to variation between families in both plant growth and resistance with family heritability estimates for resistance ranging from 0.1 to 0.31 (Puentes et al. 2018). This study also showed that saplings derived from stomatic embryogenesis (SE) were significantly less damaged than those from seed, showing potential to increase resistance by both selection and SE. There is also evidence from a study using several different clones of *P. abies* to examine genotypic variation in defence inducibility and treatment with methyl jasmonate (MeJA), which chemically induces resistance mechanisms (Puentes et al. 2021). The authors showed that clones reacted differently and that clones with a higher level of constitutive resistance had the smallest reduction in area debarked if treated with MeJA. This inverse relationship has been seen in other conifers but does not hold up at a forest population level (Howe et al. 2020).

Pinus radiata (Monterey pine)

Pinus radiata is a non-native species within the *H. abietis* range. Zas et al (2008) looked at the effect of genetic variation on the susceptibility of half-sib *Pinus radiata* seedlings to *H. abietis* damage and found highly significant differences between families for all the damage and susceptibility traits. They reported additive genetic coefficients of variation between 13.3% and 34.3%, moderate individual heritability estimates (h_i^2 0.16), and high family heritability estimates (h_f^2 up to 0.78), which would suggest that breeding for resistance is possible (Zas et al. 2008). However, two further experiments disagree with these results – both used branches of *P. radiata* from different half-sib families in a 'cafeteria style' experiment in petri dishes; they showed no significant variation between half-sib families with or without MeJA application (X. Moreira, Zas, and Sampedro 2013; Zas, Moreira, and Sampedro 2011).

Pinus pinaster (Maritime pine)

Pinus pinaster is native species within *H. abietis'* range. Differences among families of *P. pinaster* were highly significant for several damage traits inversely related to resistance, with high estimated family heritability (0.6-0.8) but only moderate individual heritability (0.1-0.2), so it is likely notable gains could be made through family selection (Zas et al. 2005). Evidence of genetic variation for tolerance to *H. abietis* in *P. pinaster* was also seen in 'cafeteria style' bioassays to look at tolerance and induced resistance to *H. abietis* and exhaustive field trials. The damage x family interaction was significant for tree survival after insect damage (Zas, Moreira, and Sampedro 2011) showing the relationship between damage level and survival differs between families suggesting varied tolerance levels.

Conifer defences against insect attack

As sessile organisms, plants have evolved a range of both constitutive (expressed as part of normal development) and inducible (activated after attack commences) defences to pests and pathogens (Krokene 2015; Wilkinson et al. 2019). Conifers have very long generation times and must defend themselves against many attacks during their lifetime, which are often from shorter-lived and, therefore, more rapidly evolving biotic stressors. As a result, they rely on a broad range of defence strategies, many of which are generalist.

Constitutive defences

Constitutive defences are pre-formed, so they may be active prior to attack and are often under genetic control. They include mechanical and chemical defences, which act as a first line of protection and offer a broad range of resistance to several pests and pathogens. The periderm contains many mechanical defences including tough lignified stone cells which deter tunnelling (Whitehill, Henderson, Schuetz, et al. 2016) or suberized cell walls create a hydrophobic barrier which prevents access to several pathogens, and limits access to other pests which are only able to enter through openings in the periderm for gaseous exchange (Franceschi et al. 2005). Calcium oxalate crystals also provide protection against herbivory, especially in non-Pinaceae species, where, alongside fibre rows they act as a barrier to bark-boring insects (Hudgins, Krekling, and Franceschi 2003). The cortex also forms an important barrier against pests and pathogens, especially during the early stages of growth. Like the periderm it contains stone cells and calcium oxalate crystals as well as constitutive resin ducts and phenolics within the Polyphenolic Parenchyma (PP) cells (Franceschi et al. 2005).

Oleoresin cell ducts can vary constitutively in number or size, and the increased flow of oleoresin from more or bigger resin ducts increases resistance against bark-boring pests, including to *P. strobi*

and *Ips spp*, by encasing them and therefore restricting their movement (Kane and Kolb 2010; Whitehill et al. 2019). The composition of the oleoresin can also vary constitutively between trees with different monoterpene and diterpenoid profiles (Robert et al. 2010). Terpenes can both be repellents and toxic to pests (Boncan et al. 2020). Oleoresin ducts also act as constitutive defences in the needles against defoliators (Björkman, Larsson, and Bommarco 1997). The PP cells synthesise and store a range of phenolic compounds which are both antifeedants and antifungal (Franceschi et al. 2005).

Variation in levels of constitutive defences have been shown to be linked with varied levels of resistance (Kane and Kolb 2010; O'Neill et al. 2002; Robert et al. 2010; Whitehill, Henderson, Schuetz, et al. 2016; Whitehill, Henderson, Strong, et al. 2016). As resources are limited in plants, if more resources are allocated to high levels of constitutive defence there is a trade-off with all other plant functions including growth (Herms and Mattson 1992) and reproduction (Herms and Mattson 1991). In *Pinus pinaster* a negative correlation was seen between basal increment area and resin duct density (Vázquez-González et al. 2020). There for it may be beneficial to plants to only activate some defences when they are exposed to a pest or pathogen.

Induced defences

Plants may respond to attack by up-regulating some defences, which are referred to as inducible. This means some costly defences are only activated when required and have a lower growth trade-off for the plant than constitutive defences. Many induced defences are associated with increased resistance, such as the development of more PP cells and swelling of existing PP cells (Franceschi et al. 2005; Krokene 2015), increased terpenes levels and numbers of traumatic resin ducts (Miller et al. 2005), increases in oleoresin concentration (Zas et al., 2011) and upregulation of defence-related genes (Kovalchuk et al., 2015). There has been evidence to demonstrate that these responses are detectable in other undamaged parts of the plant and are therefore systemic (Green and Ryan 1972). Many signalling pathways are activated in these induced responses including jasmonate (Li et al. 2002) and the ethylene signalling (Ralph et al. 2007).

Some induction stimuli can lead to acquired resistance (AR), which causes enhanced resistance to pests and pathogens (Wilkinson et al. 2019). AR can involve two non-exclusive mechanisms the prolonged upregulation of inducible defences, but this is costly to the plant, and defence priming. An example of the prolonged upregulation of inducible defences is the increase in traumatic resin ducts which causes the accumulation of oleoresins in response to herbivory in *P. sitchensis* (Miller et al. 2005). Alternatively, some stimuli, including exogenous methyl jasmonate application, have been shown to prime plants, so that during a subsequent attack the plant is able to respond faster and

more strongly with no, or relatively small growth trade-offs (Buswell et al. 2018; Fedderwitz et al. 2016; Lundborg et al. 2016; Puentes et al. 2021; Sampedro, Moreira, and Zas 2011).

Rational and objectives

With a warming climate and sharply rising human population, competition for natural resources is increasing dramatically. Commercial forestry is vital to provide sustainable natural resources for building and development. Increased global trade and climate change both enhance pest pressure on forestry plantations, and resistance breeding could offer an environmentally friendly solution. The most planted commercial tree species in the UK, *Picea sitchensis*, suffers from relatively few pests, but *Hylobius abietis* can cause very high levels of mortality and is a major problem for the industry. The major pesticides for *H. abietis* are all being phased out. There is a growing interest in resistance breeding to expand the options for *H. abietis* integrated pest management, but its potential has not been investigated in *P. sitchensis*. The overall aim of this research was to investigate the genetic variability of the biological defences of *P. sitchensis* against the weevil *H. abietis,* and try to unravel its molecular basis to facilitate resistance breeding. The project had four objectives:

1) **To determine if variation in resistance to *H. abietis* exists in *P. sitchensis***

   This objective had two components:

   a) Initially, we wanted to study the UK breeding population of *P. sitchensis* (Chapter 2). This population is from a narrow geographic origin and has been bread for two generations for growth and form traits. We wanted to see if these conditions had any effect on variation in resistance.
   b) Subsequently, we wanted to assess populations of *P. sitchensis* from a wider area across its native range to see if there would be more variation in resistance of interest (Chapter 3). Given the two species did not co-evolve, this variation would likely be the result of natural selection on generalist resistance traits, not species-specific.

2) **To determine if resistance to *Pissodes strobi* confers resistance to *H. abietis***
   By comparing mortality caused by *H. abietis* to levels of known resistance to *P. strobi* in both natural populations (termed provenance material) to detect population effects, and full-sib crosses between individuals of know *P. strobi* resistance (Chapter 3).
3) **To assess the transcriptional response to H. abietis exposure**
   An RNA sequencing study will compare the transcript abundance between control and attacked saplings, as a means to rapidly gain insight into the molecular response to weevil attack. The study will also examine both bark and needle tissue to see if the response is localised or systemic (Chapter 4)
4) **To determine if prior *H. abietis* exposure primes *P. sitchensis* defences**

This involved a comparison of saplings that have been previously attacked and naïve trees when they experience controlled exposure to *H. abietis* (Chapter 4). This study assessed both the damage level and transcript abundance to determine if resistance has increased and detect changes in defence activity that could signify priming.

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Chapter 2: The genetic variability of resistance to *Hylobius abietis* in *Picea sitchensis* from the UK Breeding population in a controlled environment.

Abstract

Hylobius abietis is a bark eating insect pest, if protection measures are not taken it girdles the stems of saplings leading levels of mortality unacceptable in commercial plantations. The existing pesticides used against *H. abietis* are being phased out so new options must be explored as part of integrated pest management. *Picea sitchensis* accounts for 50% of commercial conifers planted in Great Britain and is thought to originate from a single source. We compared damage level to controlled exposure to *H. abietis* from 13 families within the UK breeding population to assess the level of genetic variability in resistance against *H. abietis*. We saw no significant impact of family on damage level however there was a significant impact of weevil weight with larger *H. abietis* consuming more bark. These observations suggest the current breeding population has a low potential for the development of a resistance breeding program and could potentially benefit from the integration of new germplasm, from a broader range of sources, to increase variation in resistance traits and facilitate resistance selection.

Introduction

Hylobius abietis is one of the most severe pests to saplings in Europe. If protective measures are not taken, on average, 50% of transplanted seedlings die each year, and in some cases, mortality can be up to 100% (Willoughby et al., 2017). Damage is estimated to cost around 120 million euros annually (Lalík et al., 2021). The saplings are at risk for the first three years after they are planted out with the highest risk being in the first two years, before the stem diameter gets large enough to reduce the likelihood of them being girdled (Morgan, 1999). Although non-chemical remedial action to pests is preferable to chemical, it is not always practical or possible to allow for forest regeneration, and young plants must be treated with insecticides to avoid excessive losses to *H. abietis*. There are three insecticides that can currently be used in the UK: Alpha-cypermethrin, cypermethrin and Acetamiprid. Alpha-cypermethrin and cypermethrin were phased out in favour of Acetamiprid, which is ~500 times less toxic (I. H. Willoughby et al. 2020), and Acetamiprid will be fully withdrawn from the market in July 2024 (Purdy, 2023). The Forestry Stewardship Council (FSC) classifies all three of them as 'highly hazardous pesticides' (Forestry Stewardship Council, 2019), discouraging their use. The lack of currently available pesticides and the heavy losses mean that novel avenues must be

explored in addition to the options available within integrated pest management. This study looks to see if there is any genetic variability in the resistance level to *H. abietis* in the UK breeding population of the most widely planted conifer in the British Isles, *P. sitchensis*.

Integrated pest management (IPM) is recommended for dealing with *H. abietis*. Alongside pesticides, silvicultural techniques (Fallow ground, manipulation of ground vegetation and continuous cover forestry), which regulate weevil pressure and other forms of direct remedial action, have also been explored. Including entomopathogenic nematodes (Williams et al., 2013), mulching and de-stumping and alternative physical protections such as wax, polysaccharide and sand (Galko et al., 2022; Hardy et al., 2020; I. H. Willoughby et al., 2022). Resistance breeding has not currently been investigated as an additional component of IPM for *H. abietis* in *P. sitchensis* despite it being the object of research in other conifer species across Europe.

Conifers have many defences to provide resistance to herbivory, including resins, volatile metabolites and stone cells (Alfaro et al., 2013; Delvas et al., 2011; Miller et al., 2005). Often these defences have large additive genetic variation (Westbrook et al., 2015) and work in a dose dependent manner (Phillips & Croteau, 1999) so they could be exploited for resistance breeding. Significant additive variation in resistance to *H. abietis* has been detected in conifer species which are commonly used in mainland Europe. In *Picea abies*, significant genetic variation in resistance was consistent across two trials and family heritability estimates were high (0.58-0.81) for survival, which could allow for backwards selection of best parents (Zas et al., 2017); however, family heritability of debarked area was much lower ranging from 0.04 to 0.42. Similar family heritability estimates were seen in native *Pinus pinaster* at around 0.8 for the qualitative 'wound' score (Zas et al., 2005) and there was a strong significant effect of the family (Zas et al., 2006). In exotic *Pinus radiata*, family heritability was 0.78 for the 'wound' score (Zas et al., 2008). These studies were all done as field trials. However, in no-choice experiments on cut sections stem in *P. radiata* (Moreira et al., 2013) and on individual saplings in *P. pinaster* (Sampedro et al., 2011), no significant difference was seen between families in damage levels. In a further study, no significant differences between the family were seen in tolerance (defined here as a family × damage interaction on pine fitness traits) to Hylobius attack in *P. radiata* but were in *P. pinaster* (Zas et al., 2011). These results, though sometimes contradictory, are promising but highlight the difficulties of comparing studies with different designs.

Not much of the recent work done on resistance breeding in trees has led to operational planting. Of 274 activities documented by the FAO as "activities are now documented on breeding for insect and disease resistance in forest trees" only three are on conifers and have led to 'breeding programmes with deployed resistant material' (FAO, 2015). These are resistance to the green spruce aphid in *P.*

sitchensis in Denmark (Harding et al., 2003) and resistance to the white pine weevil in both *P. sitchensis* and white spruce in Canada (Alfaro et al., 2004; King et al., 2011).

From, the 1960's *P. sitchensis* became a major species of interest (Forest Research, 1998). To create a breeding population, 2800 plus trees were selected from *P. sitchensis* stands on a range of sites across the UK based on growth and form traits (Fletcher, 1992). From these, 360 of the best plus trees were subsequently selected based on breeding values for three top economic traits: growth rate, stem form and wood density (Lee, 2001; Lee & Connolly, 2010) which make up the current UK breeding population. These trees are all thought to originate from a single provenance – Haida Gwaii (formerly, Queen Charlotte Islands) (Lee et al., 2013).

H. abietis is a sever pest across Europe however in the UK it poses a particularly large problem. *P. sitchensis* accounts for 60% of the volume of softwoods harvested annually in the UK (J. Moore, 2011) and studies show that *P. sitchensis* is more attractive and more heavily attacked than other conifer species (Leather et al., 1999; Wallertz et al., 2014). In addition, *H. abietis* populations in the UK are four times higher than other areas of Europe in meaning the pressure the pressure faced by seedlings is also higher (Per Comm R. Moore 2024). Even with over 60 years of breeding to improve growth traits in *P. sitchensis* in the UK and the successful resistance programs in *P. sitchensis* for green spruce aphid and white pine weevil in North-America, which both show high levels of additive variation, we have been unable to find any other studies which investigate the genetic variability, or consider using selection and breeding of *P. sitchensis* to *H. abietis*.

Before commencing the study, the working hypothesis was:

"Naturally-occurring resistance to *H. abietis* is genetically variable within the UK Sitka spruce breeding population".

The objective of this study was to examine levels of resistance to *H. abietis* specifically within the current UK population of improved *P. sitchensis*. To do this, we conducted a no choice damage experiment, where the weevils were placed on each tree and the area of damage was directly measured. Resistance can then be considered as the inverse of damage area. We then analysed the differences in damage area between families and individuals.

Materials

Insects

Adult *Hylobius abietis* were collected from the new North Highland Forest District site (South Dalchork OS Map grid 24215), Scotland, UK. Twenty-five *P. sitchensis* billets were cut and placed at the location on Friday 28/8/20. The billets were then assessed and *H. abietis* collected twice a week for a total of 4 weeks in total 1039 weevils were collected. The weevils were posted to Oxford where they were stored at room temperature inside plastic containers. They were fed and cleaned every two weeks on fresh *P. sitchensis* branches collected from adult trees at Bagley wood, Oxfordshire, United Kingdom. In total, 520 *H. abietis* were used in the experiment; if any died, they were removed and replaced with a different individual from the stock.

Plant material

The experiment used 20 saplings of *P. sitchensis* per family from 13 different full-sib families from two different nurseries (seven from Maelor and six from Forestry England). They were extracted from the nursery beds and delivered as bare-root saplings.

Maelor (anonymised)	Forestry England
MFN10	PF92TE
MFN4	PF93TE
MFN3	PF103TE
MFN9	PF109TE
MFN2	PF114TE
MFN12	PF115TE
MFN1	

Table 1 Full list of all 13 full sib families used in the experiment and their nurseries.

Saplings were transferred to 3L square pots and with 2.9 L of M2 pot and bedding, medium nutrient, professional growing media (Levington advance, UK) and placed in the greenhouse to acclimatise for a week. The conditions in the greenhouse were 16-hour light:8-hour dark cycle at 30-20°C. Trees were watered 3 times per week. Temperature and humidity were also measured (Figure 2). One week before the experiment, lower branches and needles were removed with scissors so that the

cages used for the *H. abietis* damage experiment could be applied easily on the saplings.

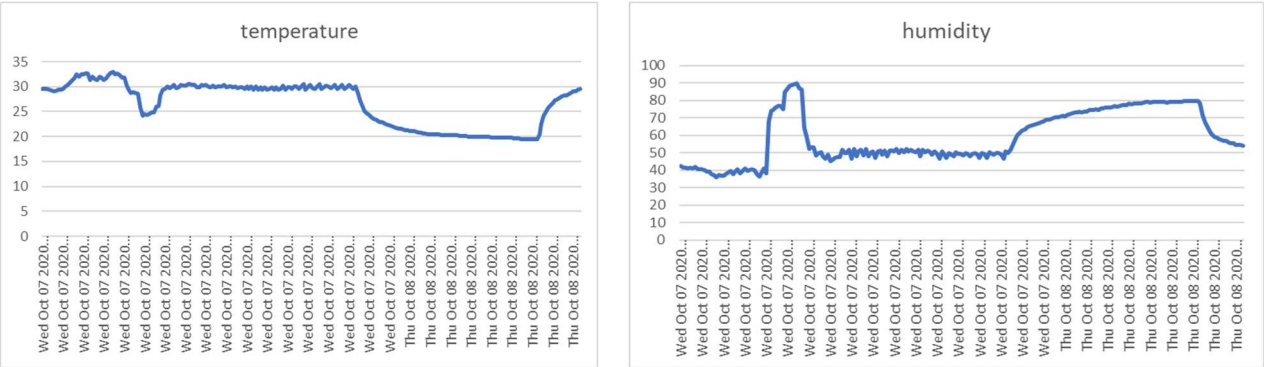


Figure 2 Temperature and humidity over a 24 hour period 7/10/2020 - 8/10/2020. Peak in humidity and dip in temperature as trees watered in the morning then staying constant at 30°C and 50% humidity for reminder of 16 hour light cycle. Over 8 hour dark cycle temperature drops to 20°C and humidity rises to 80%.

The experiment was set out in a complete randomised block design with ten blocks and two factors, family(13) and experimenter(2). There were 26 saplings in each block, two replicates from each family, each experimenter measured one tree from each family within each block. The randomisation was completed by Edgar II (Brown, 2010). The experiment was performed twice 29/09/2020 to 1/10/2020 and 6/10/2020 to 8/10/2020, blocks one to five were in the first repetition and six to ten were in the second.

Experimental set up

An experiment to assess levels of damage across the 13 different families was set up by exposing the saplings to adult *H. abietis* within small cages (Figure 3). The cages were wooden articulated boxes with a clear viewing window. The boxes measured 8x8x5cm. The boxes had a 2cm diameter hole drilled at either end so they could slip over the stem, and the gap was plugged with a sponge.



Figure 3 Images showing the articulated box around the stem of a sapling open (L) and closed (R)

Two weevils (1 male and 1 female) were placed into each cage; the cage was attached to the stem near the bottom of the tree for 3 days and the weevils were allowed to feed. Every 24 hours the weevils were briefly removed for damage measurements. Damage area was considered to be the inverse of resistance. If the weevils had escaped or died this was noted and they were replaced.

Damage measurements

The cage was removed and see-through cellophane was wrapped around the stem, and the debarked areas were traced using a felt tip pen. The whole area of damage was measured regardless of depth, though damage was mostly down to the xylem. The cellophane was then removed and photographed with a Samsung Galaxy A10 against a white background. The cellophane had a 1 cm bar, marked in the picture for calibration. Each image was then analysed using FIJI (Schindelin et al., 2012). A macro was created worked out the number of scars, the total area of damage and the average area of each scar.

The total of damage area was measured on days 1, 2, 3 (at 24h intervals) and the area of damage per day was also calculated. For day 1, this was the total area recorded on day 1. For days 2 and 3, this was calculated by removing the total area of damage measured on the previous day. If the area per day was calculated to be less than 0 this was assumed to be due to experimenter error and the area was set to 0.

Meta data

Various measurements were taken. Stem Diameter just above the collar was measured with electronic digital callipers (LDS1021, Linear tools) to the nearest 0.1mm. Sapling height was measured with a tape measure to the nearest cm from the soil to the tip of the needles on the tallest shoot when they were held up straight. The developmental stage of the apical bud of the sapling was recoded, from fully closed and dormant to complete budburst with active growth as described (Dhont et al., 2010). Weevils were weighed to the nearest 0.001g using a professional digital mini-scale (TL series). Sex of weevil was detected by inspecting the underside of the weevil, the males have a slight depression on their last sternite. (Wilson et al., 1966)

Tissue samples

Tissue samples were then taken for potential further analysis; these were taken from the freshest needles at the tip of the seedlings, placed into Eppendorf tubes in cool bags with ice blocks and placed in a -80 °C Freezer within 12 hours. These have not been used in this study but could be used in later work.

Power analysis

A power analysis was done using the pwr.f2.test function from the pwr package in R (Champely et al., 2020) before the experiment started to come up with the experimental size. This assumed data would be analysed using a general linear model.

Where the numerator degrees of freedom (u) was 14 as there were 13 families as categorical variables, and two continuous variables: tree diameter and weevil weight (1+1+(13-1)), significance level was 0.05 (Type 1 error probability) and power was 0.8 (1 - type 2 error probability). Cohen's f^2 was calculated as effect size/1-effect size. There was no previous data looking at effect size of family in P. sitchensis so by convention when doing sample size estimates 0.02 is considered small, 0.15 is considered medium and 0.35 is considered large.The denominator degrees of freedom (v) were then used to calculate the total sample size (n). As v=n-u+1 (

Table 2).

Effect size	Sample size
0.02	910

0.15	116
0.35	47

Table 2 The required sample size to be able to detect each effect size with a significance level of 0.05 and a power of 0.8. assuming a generalised linear model with 14 degrees of freedom.

We used 260 samples, so an effect size of 0.069 should be detectable with a power of 80% and a significance level of 0.05. Post hoc power analysis was then repeated after the experiment was done using the actual effect sizes with the package simr (Green & MacLeod, 2016).

Statistical analysis

All coding was done in R (R Core Team, 2023). Graphics were made using the ggplot2 package (Wickham et al., 2023)

Models were made using lmer and glmer functions from the lme4 package (Bates et al., 2023). The fixed effects were family, total weight of weevil pair and diameter at the base. The random effects were experimenter, tree ID and block. The blocks were numbered 1 to 10 with 1-5 being the block in the first experiment and 5-10 being the blocks in the second. All random variables are explicitly nested using unique name combinations. Models were validated by visually inspecting the residuals against the fitted values, using the DHARMa package (Hartig & Lohse, 2022) to simulate and plot scaled residuals as well as to check for within-group deviation from uniformity and the Levene-test was used for homogeneity of variance across treatments.

Model selection was done using the lmerTest package (Kuznetsova et al., 2020). A mixed-effects model comparison using the likelihood ratio test (Chisq) between the models using the anova function from the car package (Fox et al., 2023). To do this, the models had to be refitted to maximum likelihood (ML) estimation instead of restricted maximum likelihood (REML) to obtain appropriate degrees of freedom for the test statistics.

Post hoc testing was done using the emmeans package (Lenth et al., 2024) for differences between each family mean through multiple comparisons using the Tukey adjustment and to get an estimate mean for each family.

Results

Several response variables were calculated from our data. First, we look at variation as a function of sapling and weevil characteristics, and of experimenter. Second, we implement statistical models to examine the effect of family, weight of weevil, days of exposure to the weevil, and tree diameter at base.

Response variables – number of scars and area of damage

The cumulative area of damage was determined daily and was observed to increase over time as did the standard deviation. The mean cumulative area of damage increased from 131 mm^2 (S.D. = 63.7) on day 1 to 394 mm^2 (S.D. = 177), showing that the data became more spread out each day. The large spread of data could be due to difference between the experimenters' measurements (as discussed later) as well as the large between subject variation seen in Figure 4.

The area of damage per day was calculated from the daily cumulative damage and stayed reasonably consistent. The lowest damage was on day 2 where mean damage area was 126 mm^2 (S.D. = 75.0) and the highest damage day was day 3 at 139 mm^2 (S.D. = 94.8) but this pattern varied between individual trees (Figure 4). The data show that the average area consumed by the weevils remained reasonably constant at around 60 mm^2 per weevil per day. However, there are large standard deviations caused by large between subject variation.

The number of feeding scars was also recorded and increased over the three days from 3.5 on day 1 to 7.92 on day 3. However, the increase between day 1 and two was much greater than that between day 2 and 3. This disparity is due to the small area of bark available for feeding on day 3 causing some of the scars to begin to coalesce as the weevils ate more of the remaining bark; therefore, for some individuals the number of scars decreased (Figure 4).

Overall, there was a weak positive correlation between number of scars and damage area as indicated by Peason's correlation coefficient of 0.32. However, this pattern is non consistent over days, with Peason's correlation coefficient varying from 0.39 on day 1, to 0.12 on day 2 and -0.24 on day 3. This observation showed that as the area of damage increases over time, the scars coalesce so by day 3, the more damaged trees have a lower number of scars. The metrics that will be used to create models are cumulative area on day 3 as this represents the total of all the data collected (which was log-transformed in the models to correct for heteroscedasticity seen in the residual plots), number of feeding scars on day 2, and area of damage per day.

	Cumulative Area (mm^2)		*Number of Feeding Scars*		*Area of damage per day (mm^2)*	
	Mean	σ	Mean	σ	Mean	σ
Day 1	131	63.9	3.50	1.84	131	63.9
Day 2	256	115	6.07	2.64	126	75.0
Day 3	394	177	7.92	3.43	139	94.8

Table 3 The mean and standard deviation for each of the dependent variables per day

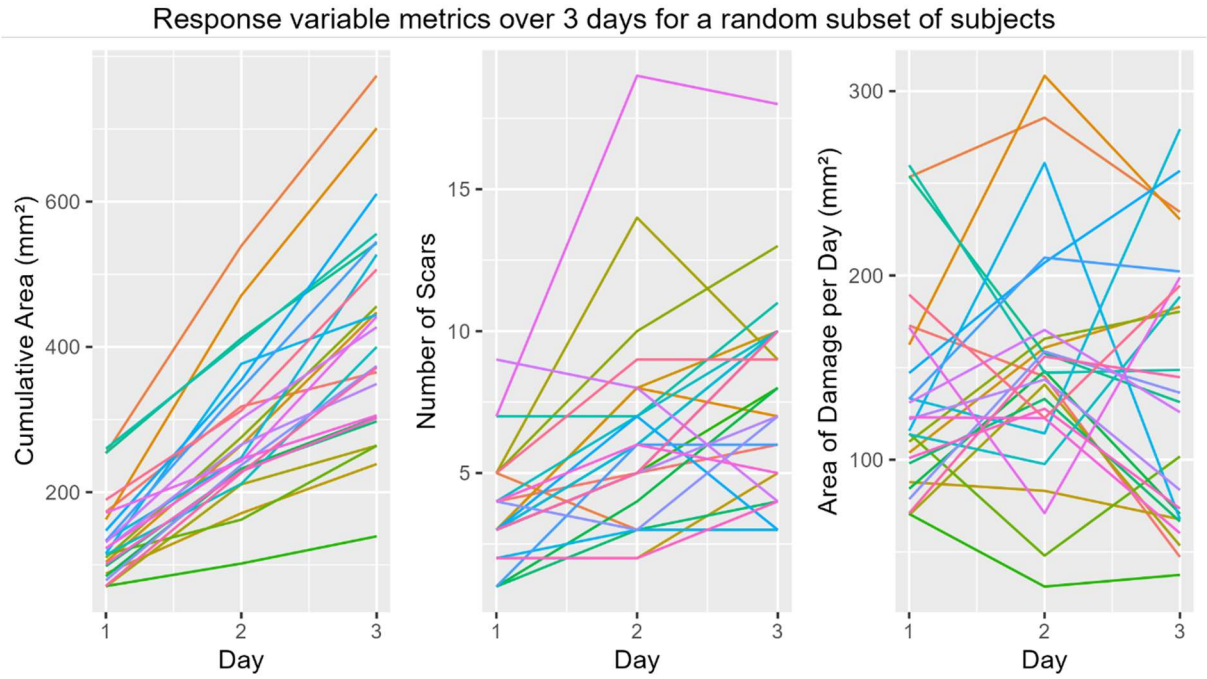


Figure 4 Temporal variation in metrics of damage assessment. Individual sapling data are shown for random subset of 26 of the 260 individuals to illustrate the between subject variation and the overall patterns of the three response variables against time.

Tree characteristics

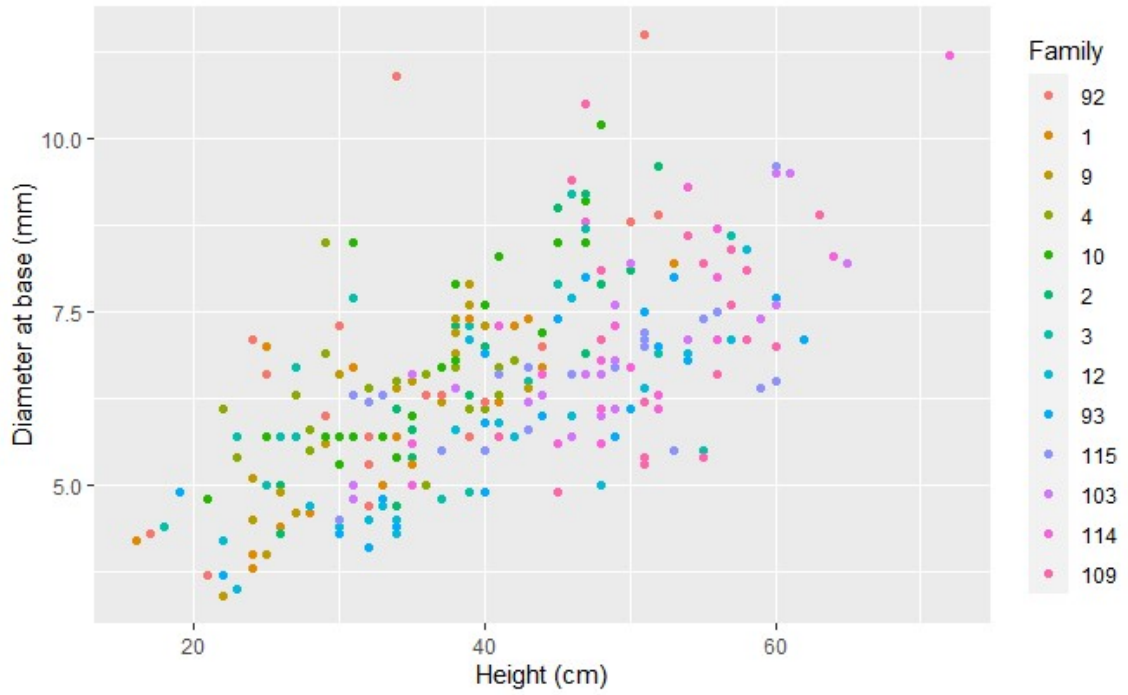


Figure 5 The height (cm) against Diameter at base (mm) of each individual showing a strong correlation between the two measurements. Colour represents the different families.

The height of saplings and diameter at base were measured on each individual before the start of the experiment, and the data were normally distributed overall and within families, but each family had a different mean. The saplings grown by Forestry England were generally larger than those grown at Maelor.

The height and diameter at the base are highly correlated (Figure 5) with a Peason's correlation coefficient of 0.63. Therefore, only one of them will be used when creating the model to avoid collinearity. Diameter will be used rather than height as the weevils were all given access to the same length of sapling, but these could have been different diameters.

There is no correlation between diameter and the log of cumulative damage area on day 3 or area of damage per day. There is a weak positive Peason's correlation coefficient (0.28) between diameter at base and number of scars on day 2 suggesting that larger trees have more scars. Diameter at the site of the articulated cage was not measured so we cannot be sure if larger diameter at base means larger area of bark available, but it is a logical assumption.

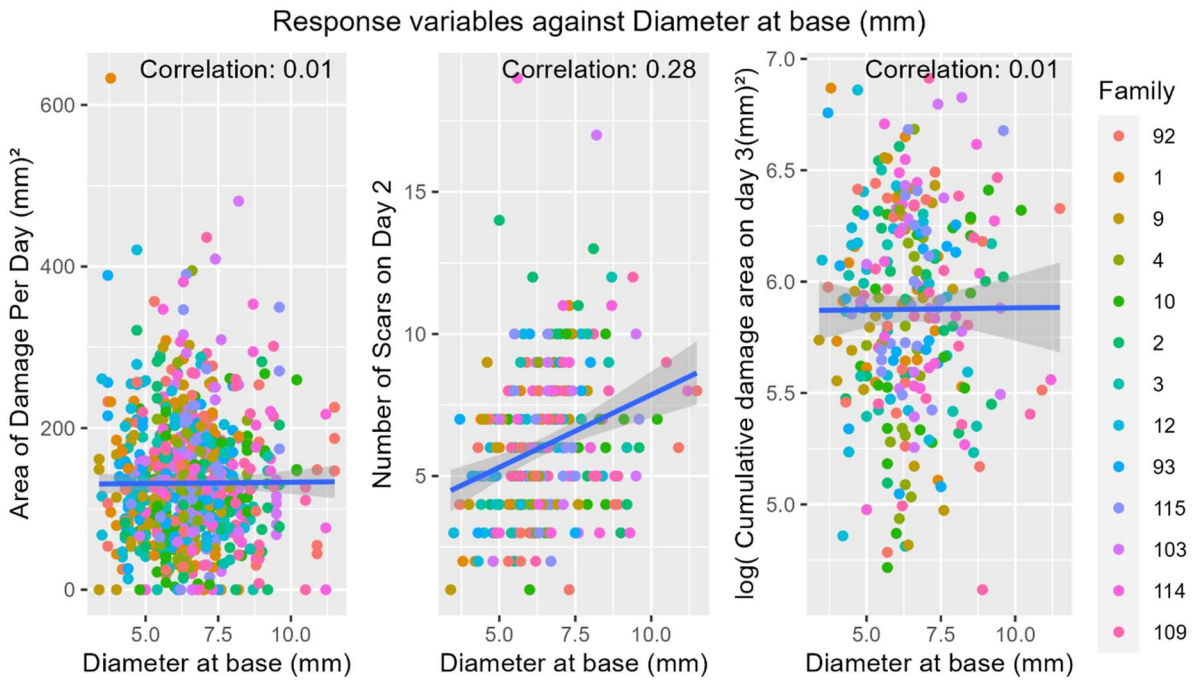


Figure 6 Variation across different diameters against the metrics of damage assessment. Pearson's rank correlation coefficient is shown in the top corner and the line of best fit is shown in blue. Families are represented by different colours

Visual inspection shows no differences in the three response variables across all the different bud burst stages. However, there is variation between families in different stages of bud burst.

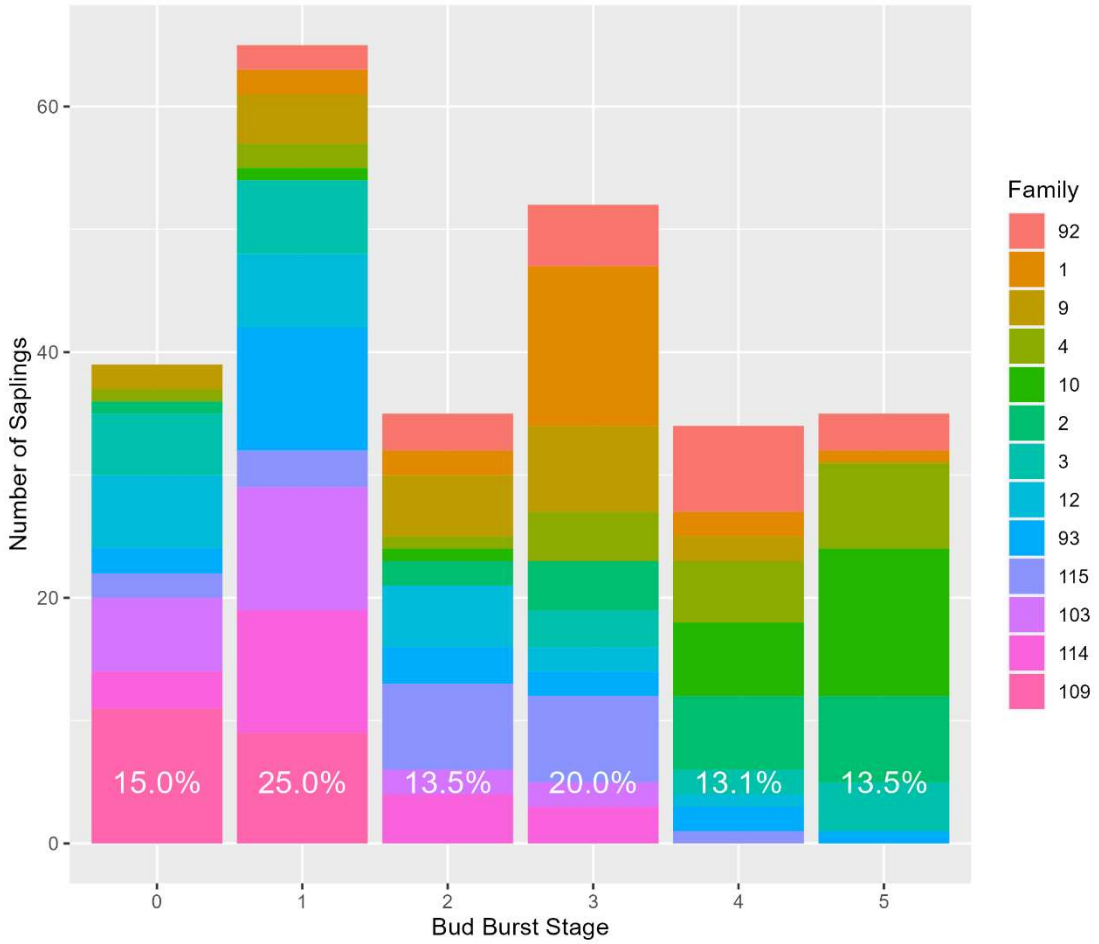


Figure 7 Frequency distribution of saplings across different bud burst stages. % of saplings in that stage shown in white Different colours represent different families.

Weevil characteristics

Weevils were sexed and weighted before the start of the experiment. Both genders had a normal distribution, but males were significantly lighter than females, with a mean of 0.091 g for males versus 0.107 g for females. A Welch two sample t-test gave a P value of p-value = 2.525e-11. A similar weight difference between sexes has been reported in other work (Inward et al., 2012).

A pair of weevils with one female and one male were placed on each sapling, so the weight of pairs was analysed. There is effectively no corelation between weight of weevil pair and number of scars, but there is a weak corelation between weight of weevil pair and area of damage per day and log of cumulative damage area on day 3 (Peason's correlation coefficient of 0.19 and 0.27 respectively). These data show that larger weevils may consume a larger area of bark but that they do not feed in more different places.

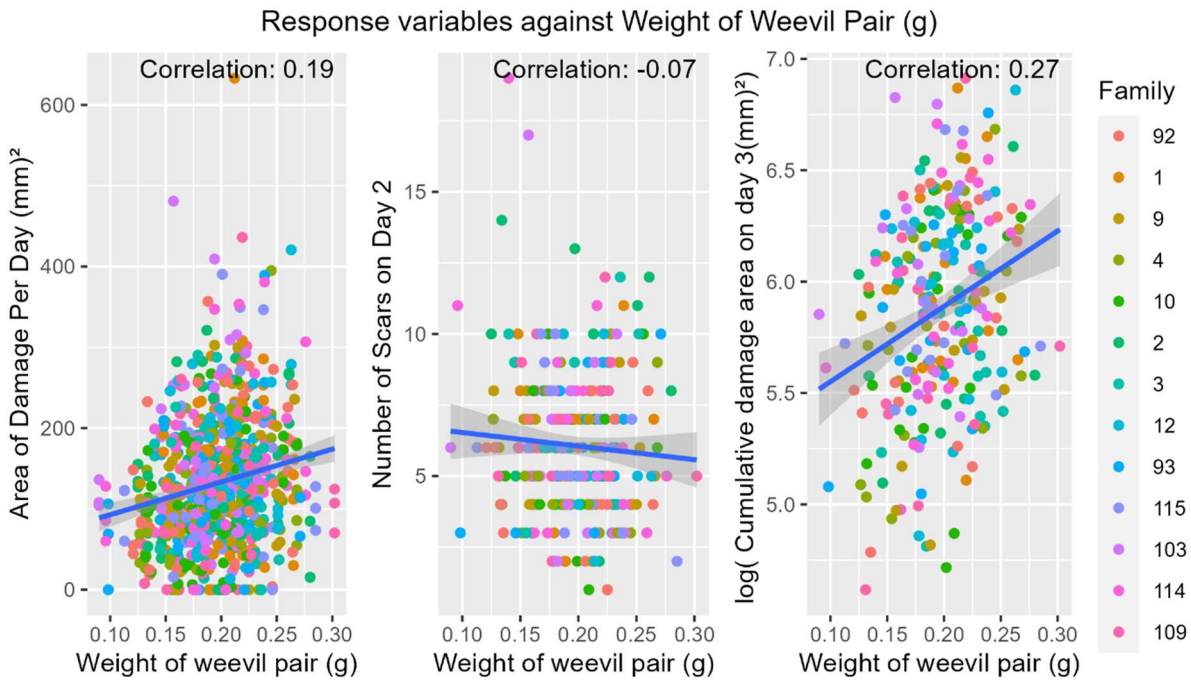


Figure 8 Variation across weights of weevil pairs against the metrics of damage assessment. Pearson's rank correlation coefficient is shown in the top corner and the line of best fit is shown in blue. Families are represented by different colours

Effect of experimenter

Visual inspection of the data Figure 9 showed that area per damage per day varied between the two experimenters who determined the damage area (Figure 9). Further investigation showed that one experimenter's measurements were 75% larger on average. This increase was consistent across days varying from 67% on day 1 to 79% on day 2. This observation demonstrates the need to include experimenter as a random effect in the models which look at area of damage. Each experimenter measured one tree of each family in each block, so the effect of the block and experimenter could not be added together. No difference was seen between the two experimenters for their measurements of number of scars.

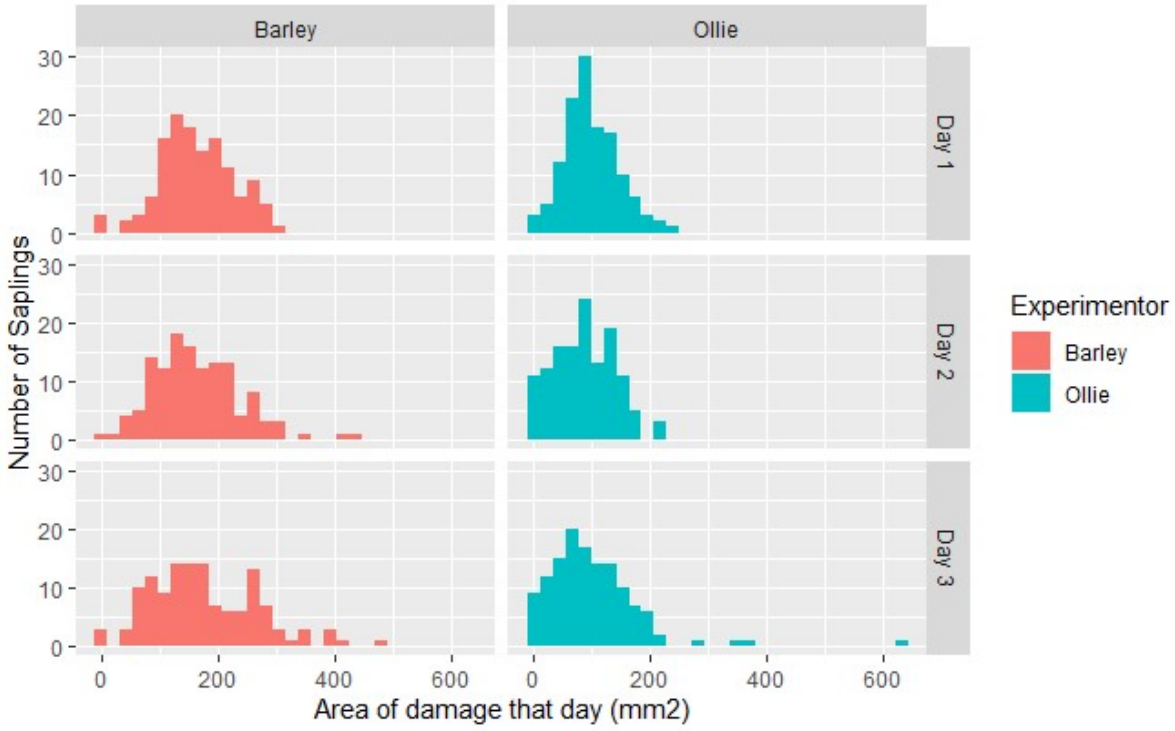


Figure 9 Frequency distribution of number of saplings with different amounts of damage, separated by experimenter (Ollie and Barley) and by day. Showing that the distributions differ between the two experimenters.

Effect of Family

Slight variations were seen between the means of the three response variables across the families. Visual inspection of the data indicated large variations between individuals within each of the families and relatively small variation among families, suggesting that family did not have much of an effect on any of the response variables (Figure 10).

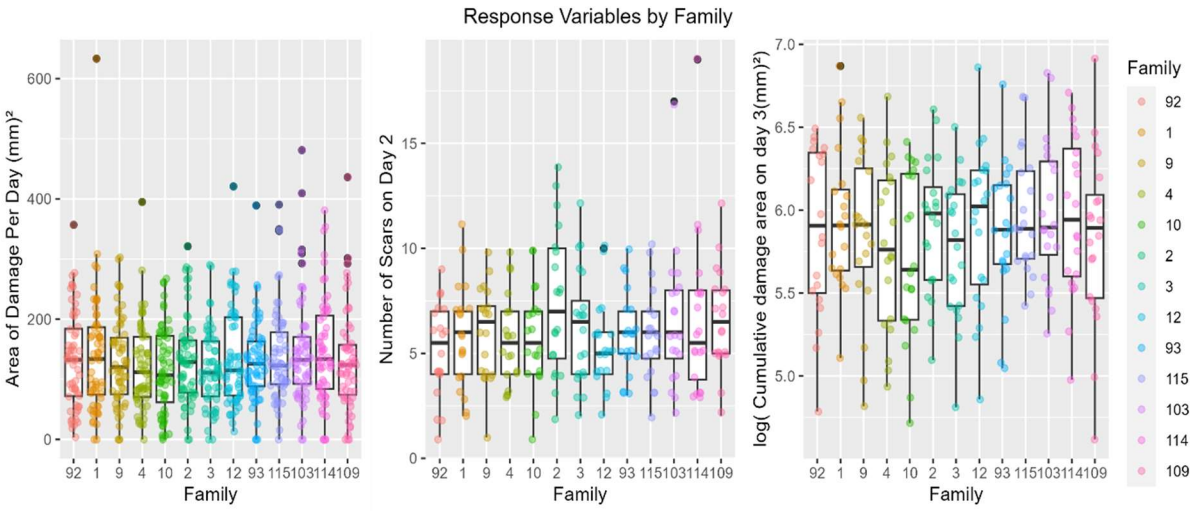


Figure 10 Variation across families against the metrics of damage assessment. Pearson's rank correlation coefficient is shown in the top corner and the line of best fit is shown in blue. Families are represented by different colours

Results of models

Several different models were considered to assess the effect of the major factors on weevil damage levels. Mixed models were made using either area of damage per day, with 780 observations (Model 1) or the damage area on day 3 (log-transformed) (Model 2) or number of scars on day 2 (Model 3) each with 260 observations. The results of the models using these three dependent variables are shown below in Table 4.

Dependent Variable		Area per day (Model 1)			Log Cumulative area on day 3 (Model 2)			Number scars on day 2 (Model 3)		
		X^2	df	p-value	X^2	df	p-value	X^2	df	p-value
Fixed effects	Family	13.7	12	0.319	16.0	12	0.192	14.3	12	0.279
	Weight of weevil pair	27.4	1	**1.68×10^{-7}**	33.5	1	**7.01×10^{-9}**			
	Day	2.30	1	0.129						
	Diameter at Base							18.7	1	**1.55×10^{-5}**
		σ^2	σ	n	σ^2	σ	n	σ^2	σ	n
Random effects	Subject	226	15.0	260						
	Block	228	15.1	10	0.0175	0.132	10	0.00634	0.0796	10
	Experimenter	2660	51.5	2	0.177	0.420	2	0	0	2
	Residual	4250	65.2		0.0885	0.300		0.15	0.38	
Marginal R^2 / Conditional R^2		0.036 / 0.443			0.061/0.706			0.110/ 0.147		

Table 4 Showing the results of the three main models. All numbers to 3 significant figures χ2 = chi squared value, df = degrees of freedom of variable, σ^2 = Variance, σ = Standard deviation, n = number of levels of random effect bold p values show a result where p < 0.05

Fixed effects

For area per day, we found that Family and Day did not significantly affect the area of damage. However, weevil weight did have a significant effect (p =1.68 $x10^{-7}$). Diameter at base, bud burst stage and many interactions terms were also tested and shown to not improve the fit of the model so were not included in any further analysis. Post hoc Tukey tests also showed there were no pairwise differences between any of the individual families. Conditional R^2 is 0.443 which means 44% of the variance in the data is explained by both fixed and random effects. The marginal R^2 is 0.036 meaning the fixed effects explain 3.6% of the variance. Visual inspection of the residuals showed mild heteroscedasticity, however log transforming the data was not possible due to the presence of 0's in the data and square rooting the data did not fix the problem. The residual variance component is larger than the subject variance component showing that there is more variation between different individuals than between different days for the same individuals.

Similarly, the family did not have a significant effect on cumulative damage area on day 3 (log transformed) (p= 0.1922) but again there was a significant effect of weight of weevil pair (p=7.01

x10^{-9}). Diameter at base, bud burst stage and many interactions terms were also tested and shown to not improve the fit of the model so were not included in any further analysis. Post hoc Tukey tests also showed there were no pairwise differences between any of the individual families. The conditional of R^2 0.706 means 71 % of the variance in the data is explained by both fixed and random effects. The marginal R^2 is 0.061 meaning the fixed effects explain 6.1% of the variance. Given the higher R^2 values and the lack of heteroscedasticity seen in the residuals this model was considered better and was further interrogated.

Effect size of each fixed effect can also be interrogated by working out the Partial (or adjusted) variance explained which measures the ratio of signal from said effect to noise. Partial Eta squared (η^2) and Cohen's f^2 are two of the indices used to measure effect size. In the model for cumulative area on day 3 (log transformed) η^2 and f^2 for family are both 0.07 and 0.08 respectively and η^2 and f^2 for weevil weight are 0.12 and 0.14 respectively. However, the confidence interval (CI) for family the effect size of family includes 0. This means that the observed data and model don't demonstrate enough evidence that the effect size is any different from 0 with a significance level of 0.05. Taken together, our results indicate that we cannot reject the null hypothesis that there is no effect of family. The two indices were also calculated at p values of 0.1 and 0.2, the CI for family did not include 0 at p=0.2. So, there is a 20% chance that the effect size is due to chance. The CI for the effect of weevil weight does not include 0 (lower bound 0.09 and 0.1); therefore, we can reject the null hypothesis and say at a significance level of 0.05, there is an effect of weevil weight on damage area and the effect size is around 0.12.

For the number of scars on day 2, diameter at base had a significant effect on the number of scars (p=1.549 x10^{-5}) Family did not, neither did weevil weight so they were not included in the final model. The conditional R^2 was 0.147 which means 15 % of the variance in the data is explained by both fixed and random effects. The marginal R^2 is 0.11 meaning the fixed effects explain 11% of the variance.

Random effects

Block, Experimenter and Subject are included in the model as random effects as they are part of the experimental design. They are all nuisance factors that the models aim to control for.

In the model of the number of scars in day 2, the variance of experimenter was recorded as 0, this lead to a singularity. Experimenter was then put into the model as a fixed effect instead (Oberpriller et al., 2022). Doing this did not improve the fit of the model so experimenter it was the excluded as a variable. On visual inspection no difference was seen in the number of scars recorded by either

experimenter. The variance of block was also low compared with the residual variance suggesting little variation between the blocks.

Discussion

This study was designed to assess the variability in resistance to *H. abietis* in the UK breeding population of *P. sitchensis,* the most widely planted conifer in the British Isles. Levels of damage were measured and used as a proxy for susceptibility which is the inverse of resistance. If differences in damage were observed across families, then the current UK breeding population of *P. sitchensis* could be taken forward to create a resistance breeding program to *H. abietis*. The main findings of this experiment were that the *P. sitchensis* family had no significant impact on the damage area caused by *H. abietis*. In contrast, there was a significant impact of the weight of the weevil. These factors have both been investigated in other studies in other conifers across Europe. I will discuss my results in this context and consider the implications of these results for integrated pest management of *H. abietis* in the UK.

Models were created looking at three separate variables: area consumed per day (mm^2), log of cumulative area consumed on day 3 (mm^2), and number of scars. The model for number of scars per day explained less than 15% of the variance of the data. Before analysing the results, I had wondered if there would be a negative correlation between number of scars per day and total damage area. If this were the case it would suggest more resistant trees might be in some way less palatable to *H. abietis* and therefore they would move around more to try to find an area that was more palatable. This was not the case, and a slight positive correlation was seen on day 1 and 2. This data shows that high numbers of scars do not demonstrate reduced palatability nor are they a good proxy for damage area.

Of the two area models, cumulative area on day 3 gave the higher marginal and conditional R^2 (0.061 and 0.706 compared with 0.036 and 0.443 for area per day), meaning more of the variance in the data was explained by the model. This could be because one day's worth of damage is too little for family differences to be expressed, and the detection of differences is hampered due in part to the large variation between individuals. Even taking the total area on the third day did not give a detectable effect. In other studies, 4-5 days was suggested when using excised branches (Zas et al., 2019). However, three days was selected for this study as we did not want the rate of *H. abietis* feeding to be limited by the small size of the cage.

Impact of covariates

We found a significant effect of weevil weight on area of damage which is consistent with other studies showing initial weevil mass positively corelated with feeding rate (Wainhouse et al., 2004). We opted to put one male and one female weevil on each tree to avoid a sex-bias in the experimental design (Bylund et al., 2004). Tree diameter did not affect area consumed. This is a

useful observation as it suggests that if resistance is found it is likely to be compatible with breeding for juvenile growth traits.

Our results also showed a significant effect of the experimenter. It is often stated that in linear mixed effects models it is important to have more than 5 levels (Arnqvist, 2020). There is some evidence that supports this claim (Harrison, 2015); however, there are also more recent studies which show that as few as two levels can be appropriate and effective as long as the correct random effects structure is used (Oberpriller et al., 2022). A simulation was used to assess if the number of levels of the random factors affects the parameter estimates for the fixed terms and their respective uncertainties (Gomes, 2022). The authors showed that if sample size is large and no inferences are intended to be drawn from those variables, fewer than five levels is acceptable for random effects. Given our large sample size and that experimenter is seen as a nuisance variable to be controlled for only, it is acceptable to include experimenter as a random effect despite it only having two levels. We controlled for experimenter in our model but in future experiments it may be controlled at the data collection stage by having a single or both experimenters taking measurements on all plants or using a different method of measuring damage.

Impact of family

We found no significant effect of family in any of the three models. The high level of variation between individuals hiding the effect of family is a plausible explanation for this results. In some studies, *H. abietis* were starved before they were exposed to the trees and only actively feeding individuals were used (Chen et al., 2021). Starving might have reduced the variation between individuals in the present study, as all weevils would have potentially adopted a similar feeding behaviour.

Reports from field studies often show conflicting results depending on the measure of resistance. Two studies in *P. abies* both show a significant family effect when analysing binomial survival data (Zas et al., 2017) or ordinal *H. abietis* damage class (0%, 0-1%, 1-5%, 5-30%, 30-50%, 50+%) (Puentes et al., 2018) but not total debarked area. Similarly, in *P. Pinaster* and *P. radiata* field trials, ordinal wound class gave a significant effect of family (Zas et al., 2005, 2006, 2008) but debarked area measured in a greenhouse experiment did not (López-Goldar et al., 2018). One reason for this disparity could be that the high levels of variation in debarked area impedes the detection of family differences. Another reason may be variations in levels of *H. abietis* pressure between experiments. We also measured debarked area directly; therefore, a different measure of resistance may allow for family effects to be detected, if present. Alternatively, more families may be needed to detect

differences; other studies mentioned above had 23-52 half-sib families whereas our study only used 13 full-sib families.

Studies reporting an effect of family (Puentes et al., 2018; Zas et al., 2005, 2006, 2008, 2017) all used field trials; therefore, they were choice experiments. These are more representative of real-world conditions where the weevils may choose among many different seedlings. However, field trials do not reveal the extent of repulsion compared to attraction to individual saplings. Interestingly, there were no differences among families in the damage area in no-choice experiments comparing 17 half-sib families of *P. pinaster* (Sampedro et al., 2011) or 48 half-sib families of *P. pinaster* (López-Goldar et al., 2018) and on cut sections stem on 34 half-sib families of P. radiata (Moreira et al., 2013). These later studies suggest that *H. abietis* feed at a similar rate across families where no choice is given. In contrast, in a field trial the effects of attraction to one family will be added to the effects of resistance from another. One additional consideration is that all no-choice studies tested far fewer individuals, with 6-18 saplings per family compared to around 100 individuals per family in the field trials.

The lack of genetic diversity within the UK breeding population of *P. sitchensis* is a further potential cause of the lack of a significant effect of family. Although seed was originally brought to the UK from across the native range, most the current breeding population is derived from one area, namely the island of Haida Gwaii, which is better adapted to the sites in the north of England and Scotland where *P. sitchensis* is more commonly planted (Samuel et al., 2007). Initially 2800 plus trees were selected from *P. sitchensis* stands on a range of sites across the UK (Fletcher, 1992). The current *P. sitchensis* breeding population is made up of 360 individuals selected for three top economic traits: growth rate, stem form and wood density All the families used in our study were full-sib crosses from individuals among those 360 trees. In *P. pinaster,* a study assessing variation in damage area both among families and populations found a significant effect of population but not of family (López-Goldar et al., 2018). All our families are effectively from one population; this could be why no effect of family is detected. In addition, the *P. sitchensis* from Haida Gwaii are known to be particularly susceptible to another bark eating insect, the white pine weevil, despite resistance to this weevil existing in other populations (Alfaro et al., 2013). A potentially low level of *H. abietis* resistance is also suggested by the comparatively low levels of some terpenes known to be important for resistance to the white pine weevil in the UK breeding population (Tumas et al., 2021).

Implications for Integrated Pest Management

The lack of a significant effect of family on area of damage done by *H. abietis* on *P. sitchensis* reported in this study does not support the development of a resistance breeding program from the current UK breeding population. If one is to be developed, it may first need to collect *P. sitchensis*

materials from a wider genetic background to be able to exploit more naturally-occurring resistance. However, the finding that weevil weight has a significant impact on damage area suggests that weevil weight as well as population density should be measured when looking at further ways to control *H. abietis.*

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Chapter 3: The genetic variability of resistance to *Hylobius abietis* in *Picea sitchensis* from across its native range in a field environment.

Abstract

The UK Breeding population of *Picea sitchensis* is thought to originate from a single provenance within its native range – Haida Gwaii, selected for its climatic similarity to the UK. A previous study demonstrated a lack of genetic variability in resistance to the bark eating insect pest *Hylobius abietis,* within the UK breeding population of *P. sitchensis.* Here, we compared *P. sitchensis* from a range of provenances across the native range based on damage score and mortality in a field study, as proxies for the inverse of resistance.. We saw that provenance significantly impacted mortality at the end of one growing season ($\chi 2=37.4$, df=17, p=0.0029), suggesting that although the two species did not coevolve there are varied levels of resistance to *H. abietis* throughout the *P. sitchensis* population. Provenances of *P. sitchensis* from areas known to have increased resistance to *Pissodes strobi* also showed increased resistance to *H. abietis*, indicating that the resistance mechanisms may be shared. These results indicate the potential of developing a successful resistance breeding program to *H. abietis* as has previously been done against *P. strobi.*

Introduction

Picea sitchensis (Bong.) Carriére (Sitka spruce) is the third largest crop in the UK by land area, the most planted tree species and accounts for 60% of the volume of softwoods harvested annually (J. Moore, 2011) contributing around £1bn a year to the UK (Forest Research, 2020). *P. sitchensis* is native to the costal rainforest on the west coast of North America from southern Alaska to northern California (Figure 11) (Peterson & Martin, 1997). *H. abietis* is a major pest to conifers in Europe which kills an average of 50% of planted conifer saplings if untreated (I. Willoughby et al., 2017). Due to climate change, it is likely that densities of *H abietis* will increase at higher latitudes such as in Scotland (Nordlander et al., 2017). All the current pesticides used to combat *H. abietis* are deemed as 'highly hazardous pesticides' (Forestry Stewardship Council, 2019) and are being phased out (Purdy, 2023; I. H. Willoughby et al., 2022). There is currently an integrated pest management programme for *H. abietis* (I. H. Willoughby et al., 2022) which does include several non-chemical options; however, resistance breeding is not currently a component of this programme. No differences were seen in *H. abietis* resistance between families belonging to the UK breeding population (Chapter 2). Given the potential lack of genetic diversity within the UK breeding population of *P. sitchensis,* it was

hypothesised that that more genetic variability may be observed in the resistance to *H. abietis* by expanding the analysis to include provenances from across the native range.

The UK breeding population is made up of 360 individuals thought to originate from a single provenance – Haida Gwaii selected for their growth and form traits (Lee et al., 2013). Given its narrow base, this population is unlikely to reflect the full genetic diversity of *P. sitchensis* and is expected to have low levels of genetic variation (Nei et al., 1975). As a non-native tree, there is also no opportunity for gene flow from the native range. Therefore, it may be beneficial to integrate new germplasm from a broader range of provenances into the breeding population so there is enough variation in resistance to allow for pest resistance selection.

The lack of variation in *H. abietis* resistance within the UK breeding population (Chapter 2) is consistent with some previous no choice studies in *Pinus radiata* (Moreira et al., 2013) and *Pinus pinaster* (López-Goldar et al., 2018; Sampedro et al., 2011). In the no-choice experiment in *P. pinaster,* there was a significant effect of population, but no family effect was observed within population (López-Goldar et al., 2018). However, several field studies showed significant additive variation in resistance to *H. abietis* in mainland Europe as observed in *Picea abies* (Puentes et al., 2018; Zas et al., 2017), *P. pinaster* (Zas et al., 2005) (Zas et al., 2006) *P. radiata* (Moreira et al., 2013).

A successful resistance breeding program to *Pissodes strobi* (Peck.) (The white pine weevil) has been developed in *P. sitchensis* (Alfaro et al., 2013). Three areas in British Columbia were identified as the source of resistant genotypes: Haney, the Big Qualicum River and the Skeena River (King et al., 2004; Ying, 1991). *P. strobi* has not colonised Haida Gwaii, the origin of the UK breeding population, and *P. sitchensis* from that area is considered highly susceptible to *P. strobi* (Alfaro et al., 2013). Differences between the resistant and susceptible *P. sitchensis* genotypes have been investigated to elucidate the underlying mechanisms for resistance to *P. strobi.* Potential mechanisms include: traumatic resin cell number and constitutive resin cell size (O'Neill et al., 2002; Whitehill et al., 2019), the terpene composition of the oleoresin (Robert et al., 2010) and the density, morphology and distribution of stone cells (Whitehill, Henderson, Schuetz, et al., 2016). Though *P. strobi* and *H. abietis* have different modes of attack, both consume the inner bark of *P. sitchensis* and damage the vasculature, leading to stem girdling. Therefore, we hypothesised that potentially some of the resistance mechanisms could be shared between the two species. The possibility of overlapping resistance mechanisms has been raised in other conifer species; for example, some populations of *P. pinaster* were consistently reported to have resistance to both the fungus *Fusarium circinatum* and *H. abietis* (Elvira-Recuenco et al., 2014; López-Goldar et al., 2018).

Investigating novel ways to prevent damage from *H. abietis* is crucial, given the imminent reduction in available pesticides, the likelihood of increasing *H. abietis* numbers due to climate change, and the UK forestry industry's heavy reliance on *P. sitchensis.* The lack of variation between families in the UK breeding population in our previous study suggests a lack of genetic variability in resistance traits; therefore, this study looked at material from across the native range.

There were two hypotheses at the start of the study:

"There are varied levels of naturally occurring heritable resistance to *H. abietis* across the native range of *P. sitchensis.*"

"Resistance to *P. strobi* may confer resistance to *H. abietis* if they have shared resistance mechanisms".

The objective of this study to examine levels of resistance to *H. abietis* between various provenances of *P. sitchensis* from across the native range, some of which are known to have high levels of resistance to *P. strobi*. To do this, we ran a field trial in Scotland in an area predicted to have a high density of *H. abietis* infestation. Damage score and mortality were measured over an entire growth season as emergence of *H. abietis* took place. Damage score and mortality were used as a proxy for susceptibility, which is the inverse of resistance. We then analysed the differences in damage score and mortality between provenances over time and at set time points, and examined whether the origin of the materials may impact resistance.

Materials

Plant material

The plant materials was saplings from: 1) 18 different provenances across the native range (Figure 11, Table 5); 2) crosses between parent-trees that were resistant or susceptible against to white pine weevil (*P. strobi*) (Table 6).

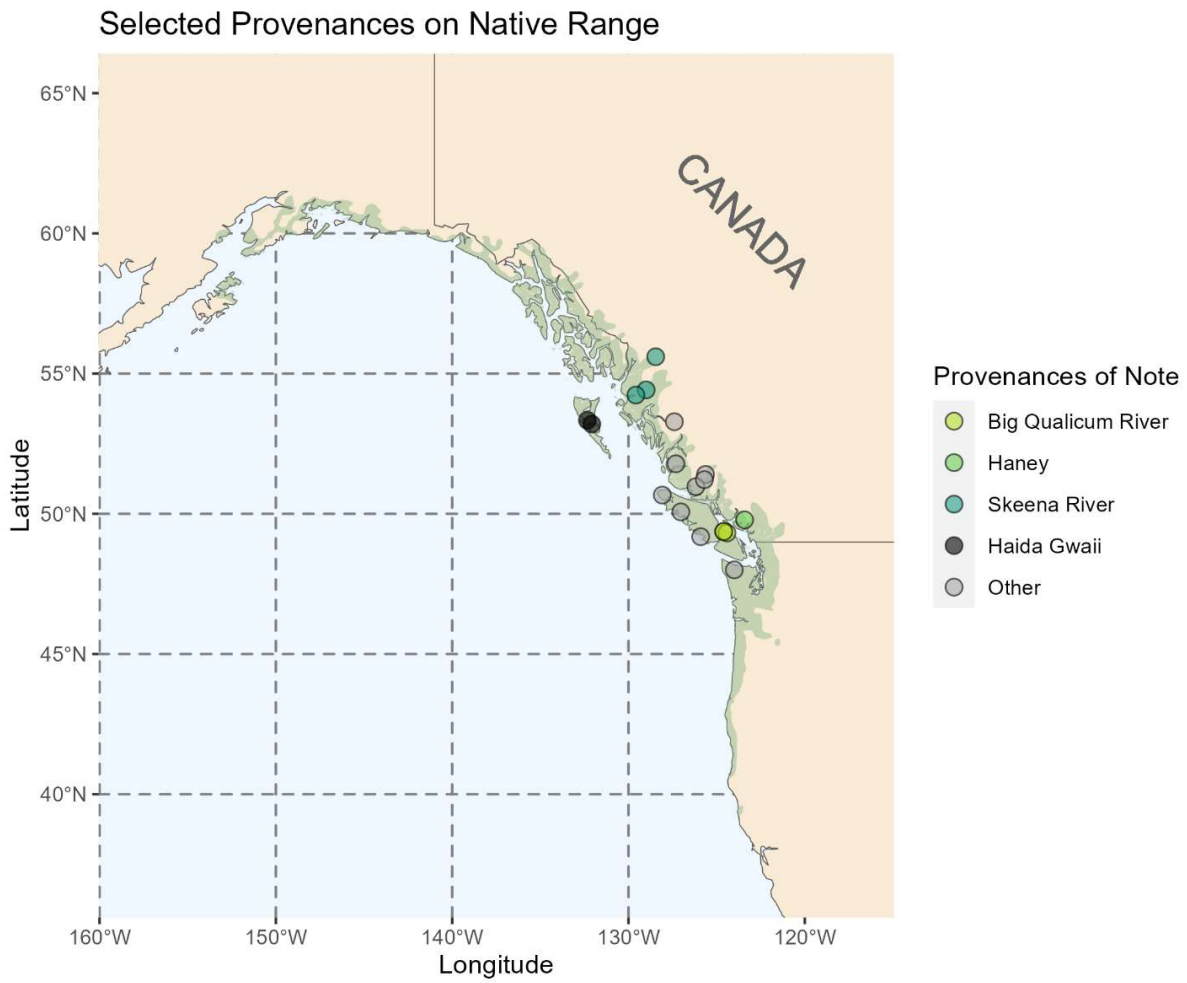


Figure 11 Map showing native range of P. sitchensis (pale green) and the locations of the 18 provenances selected for the field trial. Haida Gwaii is where the UK breeding material comes from, three areas known to have high resistance to the white pine weevil (P. strobi) are Big Qualicum River, Haney and Skeena River.

Seeds from provenances across the native range were collected from wild stands (Figure 1), all seed lots were collected from at least 10 parent trees between 1966 and 1998, and the germination rate of the lots had been checked regularly. Originally, 20 provenances were going to be used and more

saplings per provenance; however, germination levels were low and the 18 shown on the map were retained.

Provenance	No. saplings Planted	No. saplings analysed	No. saplings per block	Provenance of Note	Collection Latitude (N)	Collection Longitude (W)	Collection Elevation (m)
993	30	29	1	Other	50.07	127.03	61
1496	60	60	2	Other	49.18	125.91	30
1900	30	30	1	Other	51.78	127.32	61
1964	30	30	1	Haida Gwaii	53.20	132.08	152
2134	60	59	2	Other	50.67	128.08	30
2214	30	30	1	Haney	49.78	123.42	152
2775	30	30	1	Skeena River	54.42	129.00	61
4523	30	28	1	Other	51.40	125.63	1050
4728	30	29	1	Other	50.97	126.18	1
4744	30	30	1	Other	51.22	125.70	750
4751	30	30	1	Haida Gwaii	53.33	132.33	475
4799	30	28	1	Skeena River	55.60	128.46	305
4895	30	29	1	Skeena River	54.23	129.58	30
7946	60	57	2	Other	48.00	124.00	100
32404	60	60	2	Other	53.28	127.40	450
40437	60	60	2	Big Qualicum River	49.37	124.60	25
40438	30	27	1	Big Qualicum River	49.32	124.42	75
45004	30	30	1	Big Qualicum River	49.37	124.60	25

Table 5 Information about the 18 Provenances selected.

In addition, 11 full-sib families representing three types of crosses were included in the trial (Table 6). These were 60 individuals from six different crosses of two parents trees both known to be highly resistant to *P. strobi*, 30 individuals from two crosses of parent trees known to be highly susceptible to *P. strobi*, and 30 individuals from three crosses from parents that are part of the UK breeding population. The latter are deemed susceptible as UK breeding population seed originally came from Haida Gwaii where *P. sitchensis* are known to have low levels of naturally occurring resistance to *P. strobi*.

Cross type	Cross	Label	No. saplings Planted	No. saplings analysed	No. saplings per block
Resistant	860x1234	R1	10	10	2 Resistant
Resistant	849x1253	R4	10	10	
Resistant	1114x1253	R8	10	10	
Resistant	889x1075	R18	10	10	
Resistant	1075x1156	R22	10	10	
Resistant	898x1210	R25	10	10	
Susceptible	209x38	S2	11	11	1 Susceptible
Susceptible	253x316	S26	20	15	
Susceptible - UK	PF118TE	118	10	10	1 Susceptible UK
Susceptible - UK	PF65TE	65	10	10	
Susceptible - UK	PF150TE	150	10	9	

Table 6 Information on the 11 full sib crosses used for exploratory analysis in the field trial. These crosses are either known to be resistant or susceptible to the white pine weevil (P. strobi)

The trial used saplings grown from seed in grow cells in a polytunnel at Forestart (Shrewsbury, Shropshire, UK) from April 2021 (for the crosses) and July 2021 (for the provenance material) until March 2023, when they were planted in at the field site. The saplings were planted using a planting spade; the root plug was inserted just below the ground level and secured in place by pressing it down with boots. Each tree was marked with a yellow plastic label with the tree type and the blocks were marked with corner-poles and pink tape.

20 saplings died from other causes and were excluded from the analysis.

Field site specification

The experimental site was set up in March 2023, in a forest clear-fell within Galloway Forest (British National Grid NX 6460 7348). The site has a gentle north-facing slope. Galloway is known to have medium to high *H. abietis* population feeding pressure, and this is enhanced by the last planting being 100% *P. sitchensis*. The site was felled Nov 2021 - Jan 2022. The latter felling dates should help to ensure that the adult *H. abietis* population is not too high between April and July 2023 and then increases as emergence commences during autumn 2023. This normally ensures that damage

initially accumulates slowly during the monitoring period – April-June 2023 - so that differences between the treatments can be measured before the trees are killed.

The trial area was established on a homogeneous part of the clear-fell site so that tree plots are consistently amongst *P. sitchensis* stumps of relatively uniform density, running along 3 felling lines and have avoided putting tree plots into “boggy” areas as these are likely to be devoid of, or have reduced, weevil densities.

Experimental set up

The site was set up in a fully randomised complete block design with 30 blocks. Each block contained 29 trees, 870 trees in total, most types were represented once pre block , and some were represented twice. Sapling type was randomly allocated by drawing individuals of each type from a bag and placing them at random while planting. The trees were planted at 1 x 1m spacing in 5x6 grid with a 2m gap between blocks. A minimum 10m buffer strip surrounded the whole experimental planted area.

Damage measurements

Damage level was scored on a scale of 8 levels:

0 no damage

1 one small area of damage (up to 20mm2)

2 several small areas of damage

3 one large area of damage (over 20mm2)

4 several large areas of damage (each over 20mm2)

5 girdled/ dead

6 excessive damage beyond girdling

7 dead by other means



Figure 12 Damage experienced by saplings during field trial. Green arrows show damage area, pink arrow shows H. abietis actively feeding, green circle shows area of healing with new bark formation. A would be scored 4 as despite the large number of areas of damage it has not been girdled, unlike B which has been girdled and would eventually die.

Variables noted during the experiment included: date of first observation of bud burst, browning or loss of needles, tip death, presence of sap on the stem, presence of pests – aphids, caterpillars and Hylobius, and microsite aspects (presence of water, moss on the ground, abundance of weeds).

Assessments were done on 9 randomly selected blocks every week from the 22nd of March until the 2nd of June to monitor *H. abietis* emergence. Damage levels stayed low with less than 10% of saplings receiving any damage until the 19th of May. Full assessments of all 30 blocks were performed at five time-points each around a month apart: 19th May, 11th June, 25th July, 7th Sept, and 29th Sept. These full assessments were the data analysed in the results section, as the early assessments would have led to 0 inflated data.

Trees were considered dead for the mortality analysis if they scored a 5 or 6 as once girdled the trees will die, but this can take a few days.

The height of the trees was measured with a tape measure to the nearest cm from the soil to the tip of the needles on the tallest shoot, both at the start of the experiment (after planting and before they had begun to bud burst) and at the end, if the seedling was still alive.

Tissue samples

Needle samples were taken from all saplings on the 11th of June onto silica Gel for preservation. These were taken from the freshest needles at the tip of the saplings. These have not been used in this study but could be used in later work.

Statistical analysis

All coding was done in R (R Core Team, 2023). Graphics were made using the ggplot2 package (Wickham et al., 2023)

Models of damage score data were made using clmm from the ordinal package(Christensen, 2023). Models for mortality data were analysed using the glmer function with family as 'binomial' from the lme4 package (Bates et al., 2023). Models for height were made using lmer from the lme4 package (Bates et al., 2023). Different combinations of fixed and random effects were selected in each final model. Fixed effects considered were provenanceor cross, height and day and their interaction as well as latitude longitude and elevation of provenance, and random effects were Block and Sample ID. Sample ID was explicitly nested in block using unique name combinations.

Glmer and lmer models were validated by visually inspecting the residuals against the fitted values, using the DHARMa package (Hartig & Lohse, 2022) to simulate and plot scaled residuals as well as to check for within-group deviation from uniformity and the Levene-test was used for homogeneity of variance across treatments.

In the clmm model which looked at the impact damage score over all time points it was not possible to test the proportional odds assumption using the current version of clmm. This model had two random effects, both Block and SampleID, and both significantly improved the fit of the model, so they could not be excluded. The damage score models that only looked at the data on individual time points were made using the clmm2 function instead. This function only allows for one random effect, but as there were no repeated measurements in these models only block was the only random effect. To test the proportional odds assumption, each fixed effect was removed from the main model and instead added as nominal (Christensen, 2018). Subsequently, the two models were compared, if the model with the nominal effect was significantly better then the predictor was left as a nominal and was therefore a partial proportional odds effect.

Model selection was done using a mixed-effects model comparison using the likelihood ratio test (Chisq) between the models using the anova function from the stats package. To do this, the models had to be refitted to maximum likelihood (ML) estimation instead of restricted maximum likelihood (REML) to obtain appropriate degrees of freedom for the test statistics.

Post hoc testing of the glmer and lmer models was done using the emmeans package (Lenth et al., 2024) for differences between each provenance or cross mean through multiple comparisons using the Tukey adjustment and to get an estimate mean for each provenance or cross.

Results

The damage score and mortality data are analysed and presented separately for provenance material (n=676), and the exploratory full-sib crosses (n=115).

Provenance Material

Level of *H. abietis* damage over time

The 18 different provenances (Table 5) each had between 27 and 60 individuals. The level of damage increased over time (Table 7). Only 15% of the saplings showed any bark damage at the first complete assessment on the 19th of May. This increased to 91% by the third assessment on the 25th of July and to 99.7% by the final assessment. Only two saplings showed no signs of damage, with one sapling from each of the provenances 1496 and 7946. Very few saplings were scored as 0-3 after the third time point; these categories are very similar, and perhaps the scoring system does not have equally sized bins.

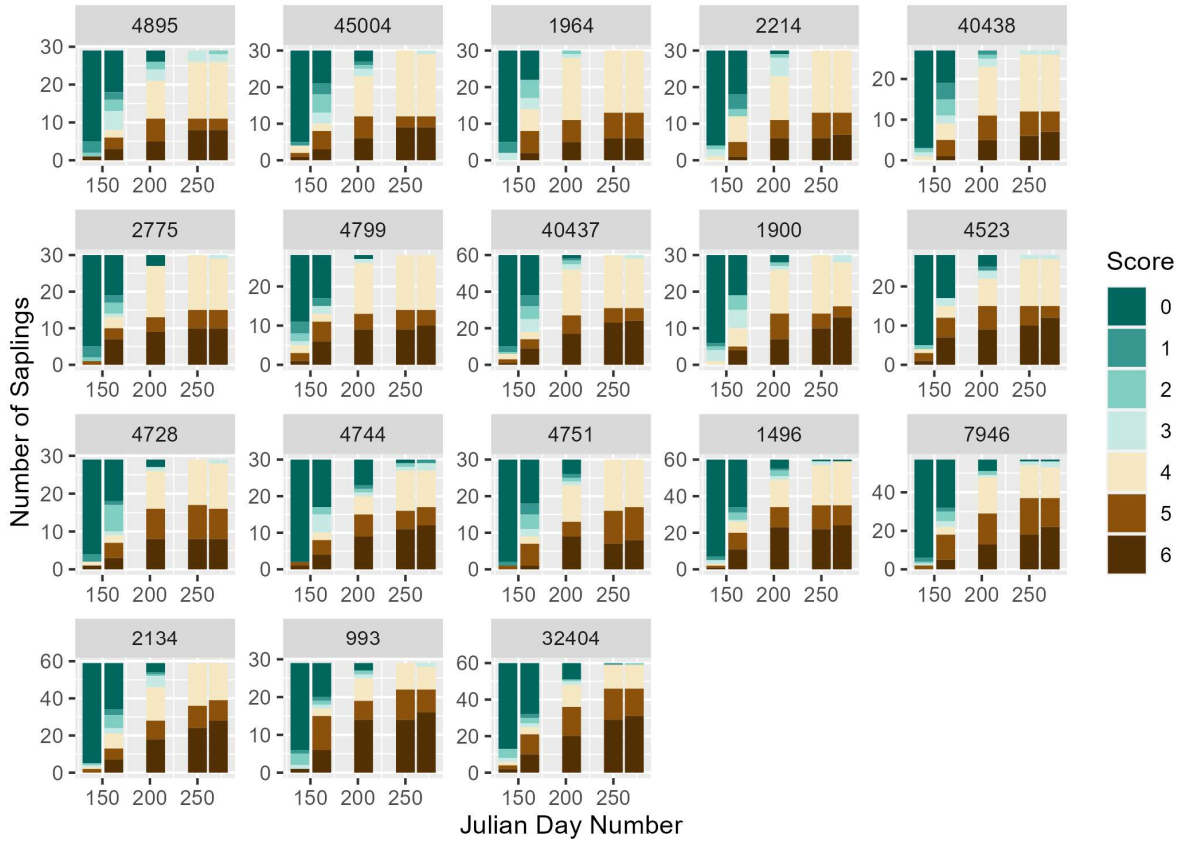


Figure 13 Number of saplings in each damage class at each time point. Each provenance is shown in a separate facet. Facets are ordered from lowest to highest damage score.

Visual inspection of the data suggested that Provenances varied in the amount of damage accumulated (Figure 13), but the effects were not significant. Cumulative link mixed models (CLMMs)

were created to allow the analysis of ordered non-continuous data such as the score data here. However, when likelihood ratio tests were performed, including a provenance term in the model did not significantly improve the fit of the data (p=0.25). Time did significantly improve the fit of the model (p=2.2e-16); all the damage scores increased as *H. abietis* emergence took place. There was also a clear time-by-provenance interaction (p = 0.00012), showing that the progression of damage level over time varied between provenances. There was also a significant time-by-height interaction (p=0.014). Inspection of an interaction plot (not shown) showed that there was no relationship between damage score and height on four of the time points, but there was a positive relationship on the third time point (25th July), so taller saplings were more damaged. There was the greatest spread of data on the 25th of July which could be why this relationship is only visible then.

Fixed effect	*χ2*	*df*	*Pr(>Chisq)*
Time	2260	1	**< 2.2e-16**
Provenance	20.5	17	0.251
Time :Provenance	47.1	17	**0.000120**
Height	2.14	1	0.143
Time :Height	6.03	1	**0.0140**

Table 7 Results of likelihood ratio tests using Cumulative link mixed models (CLMMs) To examine effect of Time Provenance Height and their interactions on damage score. All numbers to 3 significant figures χ^2 = chi squared value, df = degrees of freedom of variable Pr(>Chisq) p value of chi squared test, bold p values show a result where p < 0.05.

The models were applied using the probit link as when models made using different link functions it had the highest log likelihood suggesting it provides the best description of these data (Christensen, 2015). Block and sample ID were both included as random effects.

Level of *H. abietis* damage at two individual time points

At the final time point (29th of September), the impact of provenance, height and their interaction were significant (Table 8). On the 25th of July, the spread of the spread of damage scores was the greatest but there was no significant impact of provenance, height or their interaction.

As these models only had one random effect block, each fixed effect could be tested to see if it complied with the proportional odds assumption. For these models the proportional odds assumption was upheld for provenance but not for height and the provenance by height interaction, so these two terms were added as partial proportional effects using nominal.

Fixed effect	*χ2*	*df*	*Pr(>Chisq)*
Provenance	33.6	17	**0.00947**
Height	50.8	1	**3.31×10^{-9}**
Provenance:Height	106	17	**0.0359**

Table 8 Results of likelihood ratio tests using Cumulative link mixed models (CLMMs). To examine effect of Time Provenance Height and their interactions on damage score on the final time point (29th September). All numbers to 3 significant figures χ^2 = chi squared value, df = degrees of freedom of variable Pr(>Chisq) p value of chi squared test, bold p values show a result where p < 0.05.

Tree mortality

Mortality showed a large and significant variation between provenances (Table 9). Mortality was calculated at the end of the experiment on the 29th of September and plants were considered dead if they had a damage score of 5 or 6. Mortality ranged from below 40% to over 75% between provenances (Figure 14). Likelihood ratio tests showed that including provenance in the model significantly improved the fit (χ2 = 37.4, p= 0.00293) (Table 9), indicating a very clear effect of provenance on mortality. Post hoc Tukey tests showed a significant difference between provenance 4895 and 32404 (p= 0.0395), and between 32404 and 45005 if the significance level was relaxed to 0.1 (p=0.057) (Figure 14). The mean mortality normalised across blocks was 0.370 for 4895, 0.391 for 45005 and 0.783 for 32404.

Fixed effects	*AIC*	*χ^2*	*df*	*Pr(>Chisq)*
Provenance	908.34	37.4	17	**0.00293**
Height	908.69	1.65	1	0.199
Provenance:Height	918.24	24.5	17	0.108

Table 9 Results of likelihood ratio tests using Generalised linear mixed effects models (glmers) with a binomial distribution. To examine effect of Provenance Height and their interaction on mortality. All numbers to 3 significant figures, AIC = Akaike information criterion, χ^2 = chi squared value, df = degrees of freedom of variable Pr(>Chisq) p value of chi squared test, bold p values show a result where p < 0.05.

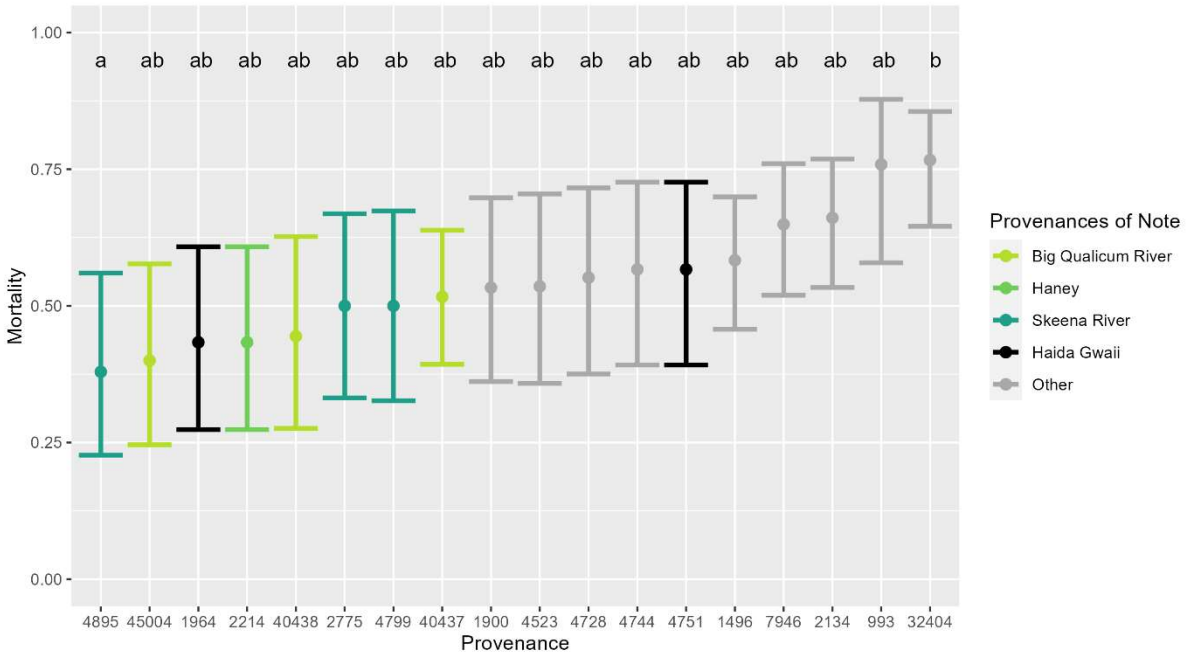


Figure 14 Mortality of provenances. Error bars show 95% confidence intervals calculated using the Wilson method for calculating confidence intervals of a binomial distribution. Letters of significance from post hoc Tukey tests shown above. Big Qualicum River Provenances are deemed superior and have previously been shown to have higher resistance to P. strobi. Haney and Skeena River provenances are from areas where resistance to P. strobi has previously been detected but these exact seedlots have not been previously tested. Haida Gwaii is where the UK breeding population is known to originate from.

The data indicated that lower mortality from *H. abietis* is consistent with reports of higher resistance to *P. strobi* (Alfaro et al., 2013; Alfaro & Ying, 1990). Mortality following *H. abietis* damage was plotted on a map to explore geographic trends (Figure 15, B). The three seedlots from Big Qualicum River previously demonstrated to have increased resistance to *P. strobi* showed comparatively low levels of mortality in the present study. Furthermore, the overall distribution of resistant and susceptible provenances from previous studies map to the same areas as the provenances with low mortality from *H. abietis* (Figure 15). Three areas have been highlighted as sources of resistant genotypes to *P. strobi:* Haney, just north of Vancouver, British Columbia, the Big Qualicum River area on eastern Vancouver Island and the Skeena River area of British Columbia's mainland (Alfaro et al., 2013; Alfaro & Ying, 1990). Although the exact seedlots used here from the Haney area and Skeena river have not been tested for their resistance to *P. strobi,* I still included them in the further analysis as provenances of note in some of my statistical models.

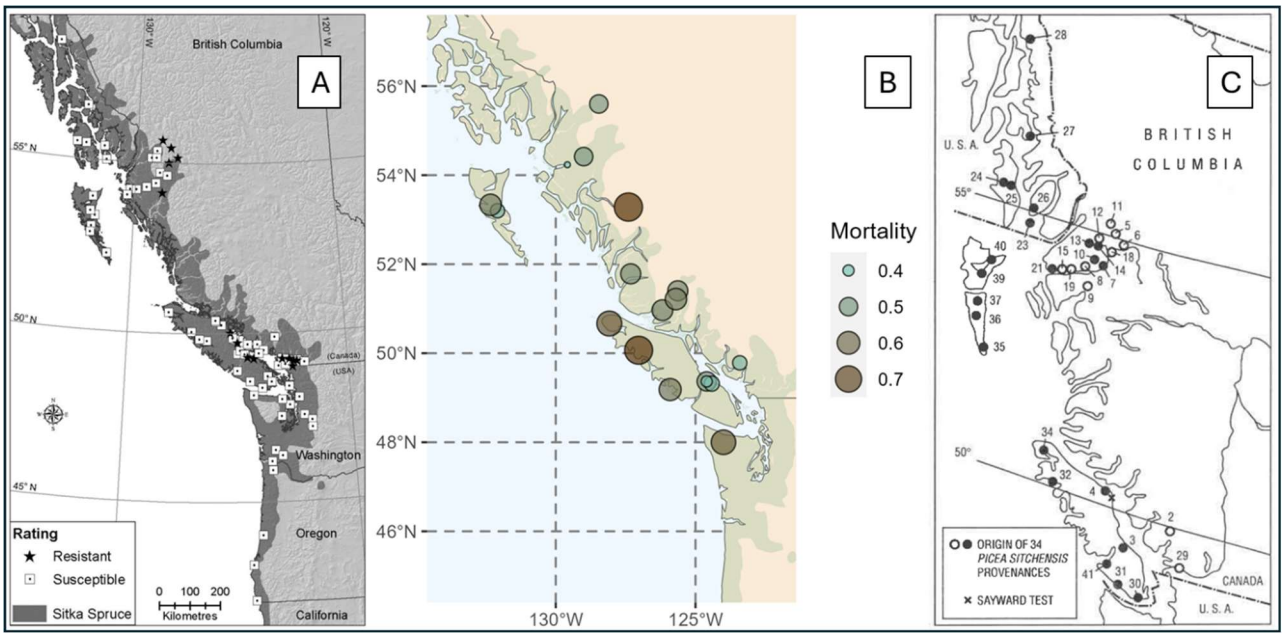


Figure 15 A: Figure from (Alfaro et al., 2013) showing results of multiple provenance trails looking for resistance to P strobi, stars show resistant provenances and dots show susceptible provenances. B: Mortality of provenances in this field trial. Smaller more blue dots have lower mortality and larger more brown dots show higher levels of mortality. C: Figure from (Alfaro & Ying, 1990) showing results of first provenance trial looking for resistance to P. strobi, open circles show provenances with lower levels of attack filled circles show provenance with higher levels of attack. There appears to be a colocalization between provenances with lower H. abietis mortality and areas where resistance to P. strobi has been found.

The model including only provenance as a fixed effect had a conditional R^2 of 0.16 and a marginal R^2 of 0.068 showing that provenance explained 6.8% of the variance. Despite the low R^2, the fixed effects are significant then the model is still worth interrogating (Ozili, 2023). The among block standard deviation is 0.60 which is a similar order of magnitude to the treatment effects (-0.89 to 0.91) showing that blocks vary a lot relative to the magnitude of treatment. This spatial variation in mortality is likely due to varying *H. abietis* pressure and microsite aspects. The model did not violate any of the assumptions (Figure 16). Levene's test for homogeneity of variance showed that the residuals were equally distributed for provenance (F= 1.17 p=0.285) and block (F= 0.702, p= 0.878).

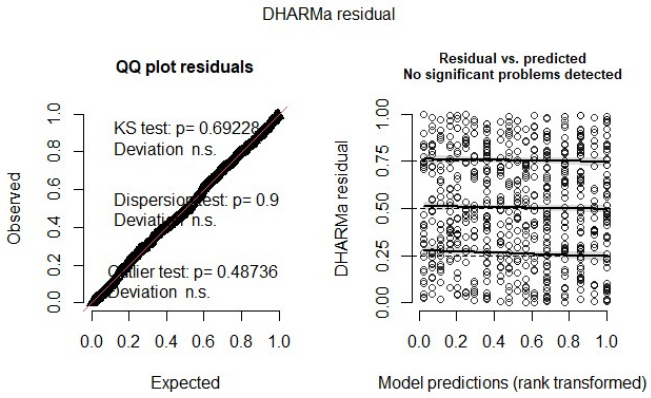


Figure 16 The simulated residuals produced by DHARMa package for the from a generalised mixed effects model of Mortality ~ Provenance + (1|Block). Where provenance is a fixed effect and block is a random effect and the distribution is binomial. Showing no over dispersion, outliers or deviation from the expected distribution.

Models were also made which included a scaled height fixed effect and the interaction between scaled height and provenance. Neither of these fixed effects significantly improved the fit of the model (Table 9) so they didn't have a significant effect on mortality. However, they did not increase the AIC by a large relative amount and the provenance height interaction had a reasonably large χ2 and low p-value (24.5 and 0.11 respectively). These models had higher conditional and marginal R^2, 0.16 and 0.072, when height was included along with provenance and 0.21 and 0.12 when provenance height and their interaction was included. Mortality decreased as height increased for most provenances but not all; all but one of the 'resistant to *P. Strobi*' provenances also showed decreased mortality as height increased (Figure 17). Height was scaled by subtracting the mean from each value and dividing by the standard deviation. The variable was scaled to allow the model to converge.

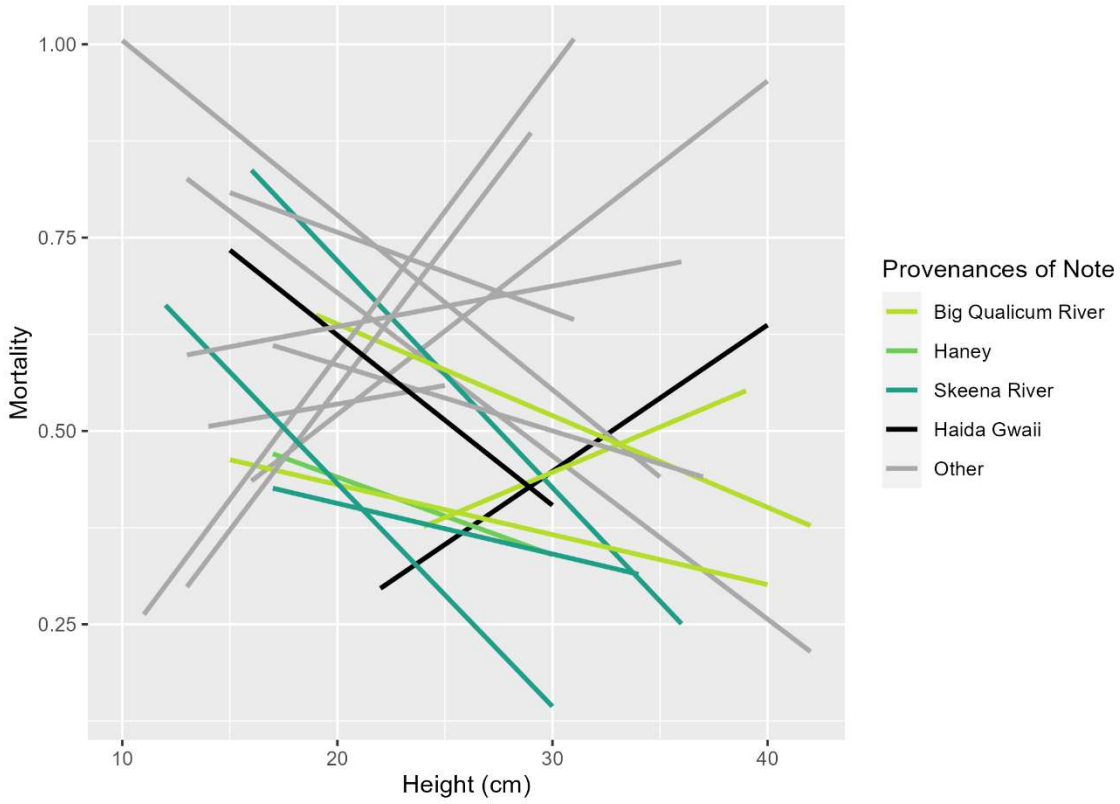


Figure 17 Mortality against height for each provenance demonstrating the Provenance-by-Height interaction. For most provenances as height increases mortality decreases however there are seven exceptions

The three provenances from Big Qualicum River had significantly less mortality than the remaining provenances. Including pools, which were comprised of these three provenances and of the other 15 provenances in the model significantly improved fit (χ^2 =5.36 p=0.021), showing that group had a significant impact on mortality. Average mortality, controlled for blocks, was 46.8% for the Big

Qualicum River provenances and 58.9% for the others. Also, comparison of the pool of the three Big Qualicum provenances and a pool of three random other provenances was repeated 1000 times. The Big Qualicum provenances showed lower mortality 955 times and significantly lower mortality ($p<0.05$) 398 times.

I next compared the seven provenances from one of the three areas with high *P. strobi* resistance (Haney, Big Qualicum River and Skeena River) to the remaining 11 provenances and again found significantly lower damage (Figure 18). Including these two groups (of 7 and 11 provenances) significantly improved the fit of the model ($\chi^2 = 16.6$, $p= 4.61x10^{-5}$) showing that group had a significant impact on mortality. Mortality in the seven provenances from areas with high *P. strobi* resistance was 0.457 and 0.627 for the other 11, giving a difference in mortality of 17% (Figure 18). Models were also made using three groups, the seven provenances from areas known to be resistant to *P. strobi*, the two Haida Gwaii provenances and the remaining nine other provenances known to be susceptible to *P. shhhhhhhhhhtrobi*. The Haida Gwaii provenances were not significantly different from either other of the other groups but those were different to each other (Figure 18).

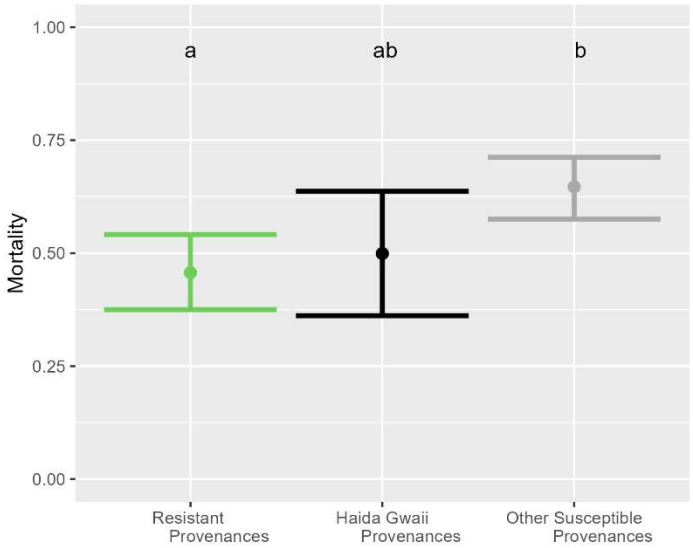


Figure 18 Mortality of the two pooled groups of seven 'resistant' provenances – which have shown resistance to P. strobi (Big Qualicum River) or are from areas which have (Haney and Skeena River) and 11 'susceptible' provenances . Error bars show 95% confidence intervals calculated using the Wilson method for calculating confidence intervals of a binomial distribution. Letters of significance from post hoc Tukey tests shown above.

Geographical Variables

Latitude, longitude, and elevation of the provenance did not have a significant impact of mortality or damage score (not shown); however, they all had a significant impact on height of the saplings (

Table 10). Plants originating from more northern latitudes and higher elevations were smaller, which is expected due to adaptation to shorter growing periods (Salk, 2020). The residual standard deviation is large (5.47) compared with the magnitudes of the effects which shows that there is large variation between individuals (Figure 19).

	Effect	χ^2	*Pr(>Chisq)*
Latitude	-0.718	18.965	**1.33 x10^{-5}**
Longitude	0.583	33.399	**7.51 x10^{-9}**
Elevation	-0.00652	53.917	**2.09 x10^{-13}**

Table 10 Results of likelihood ratio tests using linear mixed effects models (lmers). To examine effect of collection Latitude, longitude and elevation on height at start of experiment. All numbers to 3 significant figures, Effect = change in height(cm) for every one unit of the fixed effect (degree for latitude and longitude and m for elevation), χ^2 = chi squared value, Pr(>Chisq) p-value of chi squared test, bold p-values show a result where p < 0.05. All variables have one degree of freedom.

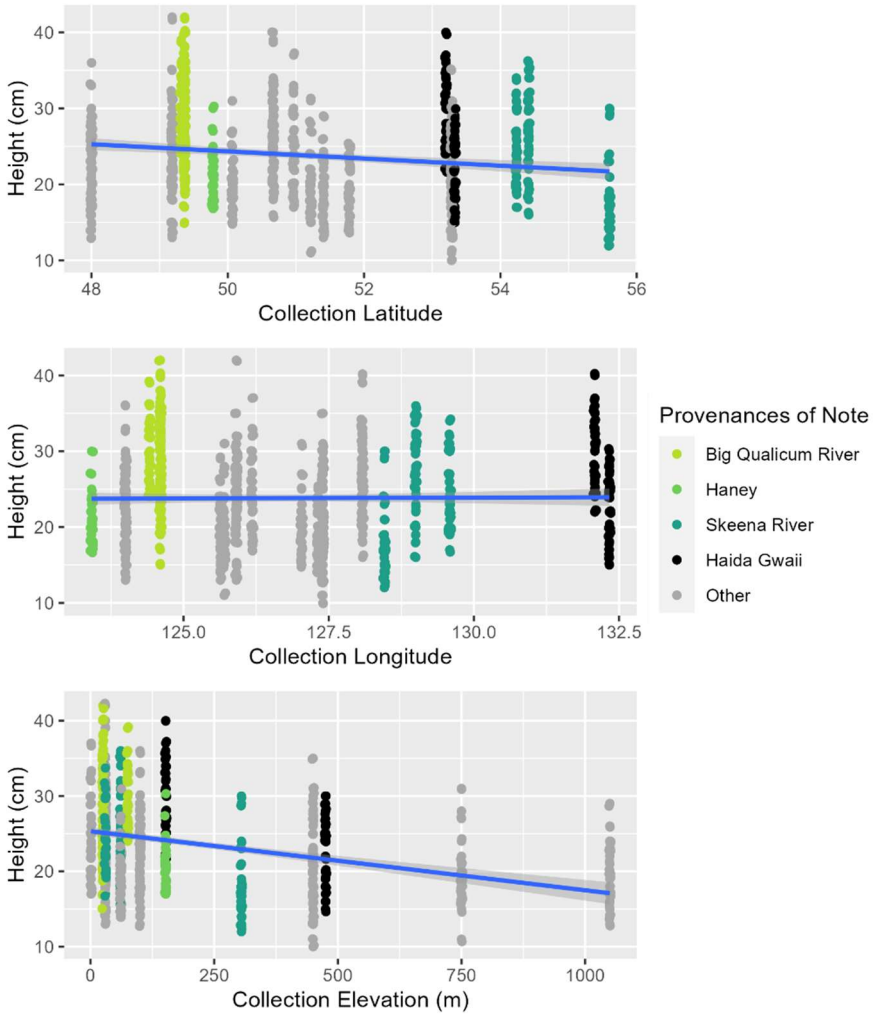


Figure 19 Height(cm) at start of experiment against latitude longitude and elevation. One dot per tree, large variation can be seen within provenance. A negative slope can be seen for Latitude and elevation showing that trees from colder more northerly or higher provenances are shorter

P. strobi resistant and susceptible full-sib crosses

The study also analysed levels of damage and mortality in 12 full-sib crosses in addition to the 18 provenances (Table 6). There were 60 and 30 full-sib F1 progenies from crosses between individuals of high resistance or between individuals of high susceptibility to *P. strobi,* respectively (eight crosses in total, see methods). Similarly, there were 30 full-sib progenies from crosses in the UK breeding population (considered susceptible as the population originates from Haida Gwaii which is known to have high susceptibility to the *P. strobi*). The small numbers were due to low germination rates and the data were only included as exploratory results. In each block there were two 'resistant cross' individuals, one 'susceptible cross' individual and one 'susceptible - UK' individual.

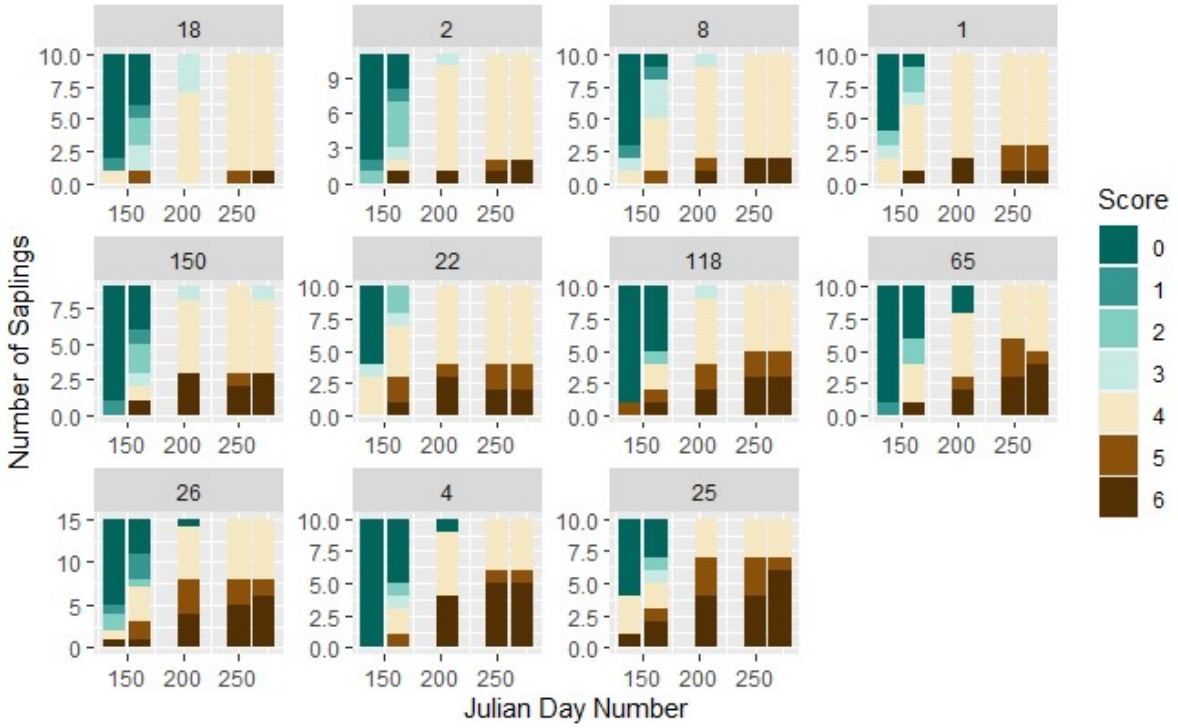


Figure 20 Number of saplings in each damage class at each time point. Each cross is shown in a separate facet. Facets are ordered from lowest to highest damage score.

The level of damage increased over time and appeared to plateau on the third time point as seen in the provenance materials (Figure 20). The damage appeared to vary between the crosses (Figure 20) but the differences were not significant, when likelihood ratio tests were performed on the cumulative link models including cross, or cross type (Table 11). This outcome is potentially due to the small sample size (n=10-20).

Fixed effect	*LR stat*	*df*	*Pr(>Chisq)*
Time	430	1	**< 2.2e-16 ****
Cross	13.5	10	0.198
Time :Cross	34.0	10	**0.000186 *****
Height	1.10	1	0.294
Time :Height	6.51	1	**0.0108 ***

Table 11 Results of likelihood ratio tests using Cumulative link mixed models (CLMMs) To examine effect of Time Cross Height and their interactions on damage score. All numbers to 3 significant figures χ^2 = chi squared value, df = degrees of freedom of variable Pr(>Chisq) p value of chi squared test, bold p values show a result where p < 0.05.

In contrast, time and its interactions with cross type, and height significantly improved the fit of the model (Table 11). The time by cross interaction (p = 0.000186) shows that the rate of damage accumulation varies between crosses. Tree mortality varied from 10% to 70% between crosses (Figure 21) but cross did not significantly improve the fit of the model and therefore these differences were not significant (χ2 = 16.6, p = 0.0840). When pooled into 'resistant' and 'susceptible' groups, the 'resistant' group had a slightly lower mortality of 38% compared to 42% for the 'susceptible' group but these differences were not significant (χ2 = 0.155, p = 0.694). When pooled into 'resistant', 'susceptible' and 'susceptible - UK' groups, the 'resistant' group had a mortality of 38.1%, the 'susceptible' group was 38.5%, and the 'susceptible - UK' group was 44.7% but these differences were not significant (χ2 = 0.373, p = 0.830).

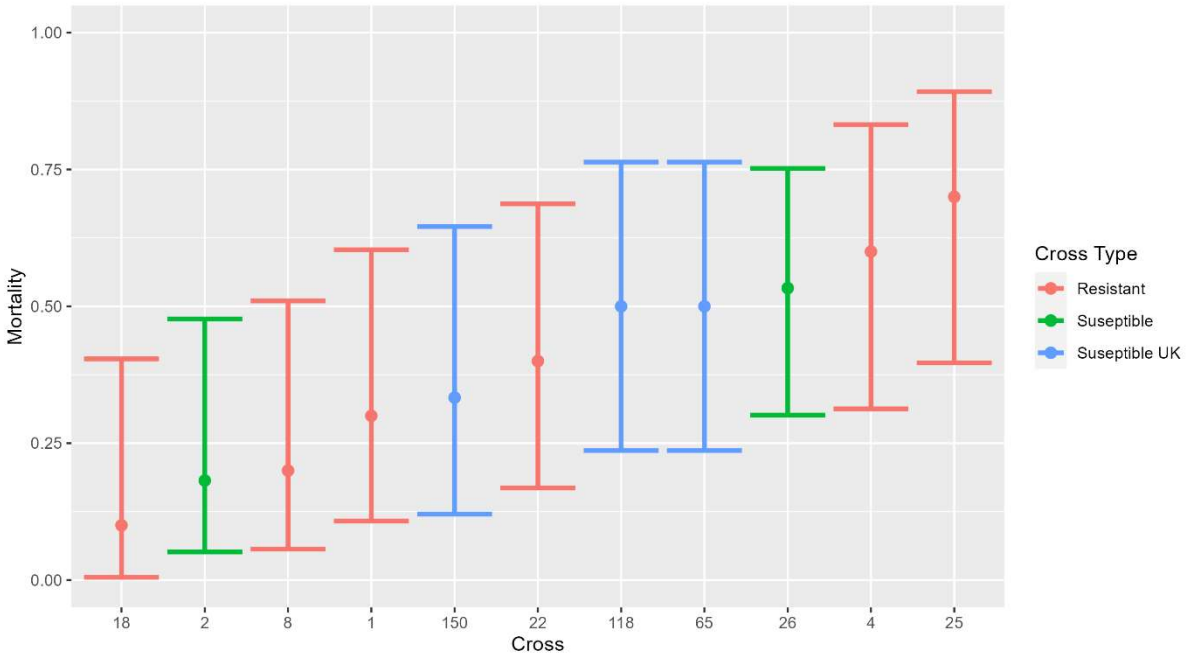


Figure 21 Mortality of crosses. Error bars show 95% confidence intervals calculated using the Wilson method for calculating confidence intervals of a binomial distribution. Resistant crosses were full sib crosses between two clones known to have high resistance to P. strobi. Susceptible crosses were full sib crosses between two clones known to have high susceptibility to P. strobi. Susceptible UK crosses were full sib crosses of two clones form the UK Breeding population.

The scaled covariate height did not significantly improve the fit of the mortality data ($\chi 2 = 1.23$, $p = 0.268$); however, the height cross interaction did ($\chi 2 = 23.3$, $p = 0.00985$). This shows that the effect of height on mortality was dependent on the cross (Figure 22); for most crosses, taller trees had lower mortality, but three 'resistant' crosses showed the opposite.

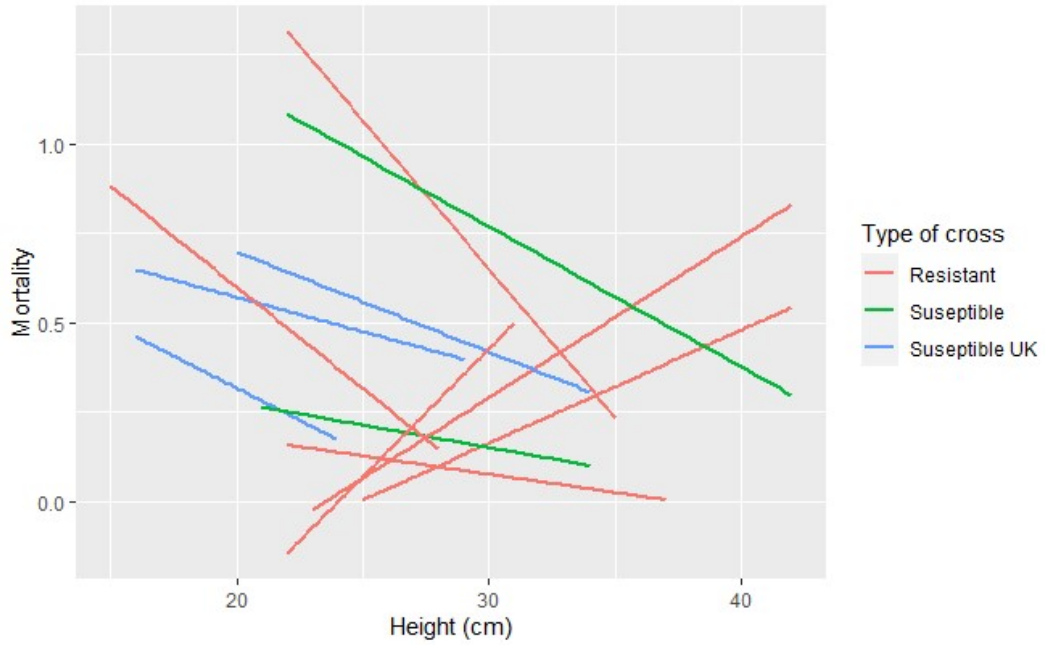


Figure 22 Mortality against height for each cross demonstrating the Cross:Height interaction. For most crosses, as height increases, mortality decreases; however, there are three exceptions.

Discussion

This study was designed to assess the extent of genetic variability in the resistance level to *H. abietis* in P. *sitchensis* from across its native range. Damage score and mortality were measured and used as a proxy for susceptibility which is the inverse of resistance. The patterns seen between mortality and provenances might allow selected provenances to be taken forward to create a resistance breeding program against *H. abietis*. The main findings were that the provenance had a significant impact on the mortality level in the *P. sitchensis* saplings caused by *H. abietis* and on the damage score at the end of the growing season. In addition, saplings from provenances in areas known for their resistance to *P. strobi* showed significantly lower mortality than those from other areas. Resistance to *H. abietis* has been studied in other conifers and a resistance breeding program to *P. strobi* has been successfully deployed in *P. Sitchensis.* I will discuss my results in the context of these previous findings and consider the implications of these results for *P. sitchensis* breeding in the UK and the integrated pest management of *H. abietis*.

Level of *H. abietis* damage

The damage score increases over time from very low to very high, but the rate of increase varies significantly between provenances over time demonstrated by a significant effect of the provenance by time interaction. The uneven *H. abietis* pressure experienced at the early time points, during emergence could account for the large among block standard deviation. Although *H. abietis* can fly vast distances after emergence they tend to walk to their maturation food source (Tan et al., 2011), which could cause spatial variation in the pressure experienced by saplings. The significant effect of provenance on the damage score of the final timepoint alone (Table 8) is likely because the emergence was complete and there was high pressure across the site. In other words, the differences between provenances had had time to accumulate as all the saplings had been exposed to *H. abietis*. In *P. abies,* comparison of two field trials showed that low *H. abietis* pressure failed to reveal differences between families whereas those with higher pressure did (Puentes et al., 2018). I would suggest that in future time and money could be saved by assessing the field trial fewer times, potentially only once at the end of each season. The differences seen between provenances suggest genetic differences in susceptibility and resistance to *H. abietis*.

The clmm function from the ordinal package is the only R package which allows for the analysis of ordered non-continuous data with more than one random effect, which is why it was used here; however, as a new package it does not yet have all the functionality of the clmm2 package it supersedes. As a result, it was not possible to test the proportional odds assumption for the fixed effects of the model looking at damage score over time. The proportional odds assumption states

that unless stated otherwise all fixed effects in the model should maintain the same 'slope' estimate between each pair of scores across two response levels. As the assumption did not hold for height or the provenance x height interaction for the models from only individual time points, it appears unlikely that it would in a model of damage score over time. Therefore, the output of the model may be invalid. In addition, clmms are not commonly used it is hard to make comparisons to other studies in the same system.

Tree Mortality

In conifers, field trials have shown significant variation in mortality caused by *H. abietis* between families (either full-sib or half-sib groups) rather than provenances. In *P. abies* studied in Sweden, mortality after two growing periods ranged from 3-20% Zas et al., 2017) lower than in the present study. However, the *H. abietis* population is much denser in the UK than Sweden (R. Moore, personal communication, 03 2024) as indicated by damage levels of 48% and 99.7% of saplings, in Sweden and in the present study, respectively. In a different exotic conifer, *P. radiata,* mortality varied significantly between families from 18%-48% and weevil pressure was similar with 94% of seedlings damaged (Zas et al., 2008). In our study, we counted mortality as any tree where girdling (removal of the bark over the entire circumference in at least one position on the stem) had occurred; therefore, this would also include what Zas et al. (2005) referred to as leader loss which was significantly different between families in *P. pinaster* ranging from 10 to 43% (Zas et al., 2005). Given the high *H. abietis* pressure in our trial, the significant variation on mortality levels between provenances is a promising finding for pest management. It would be interesting to see how many of these trees would survive a second growing season. Only one study was found in the literature which looked at variations in *H. abietis* damage across populations as well as families. No difference was detected between families in damage level in a no choice experiment in *P. pinaster,* however a difference in damage level was detected between populations (López-Goldar et al., 2018). This is similar to our observations *in P. sitchensis*, that is, no difference in damage levels between different families (Chapter 2) but differences in damage and mortality between provenances.

Interestingly, some populations of *P. pinaster* were shown to have consistent resistance both to the exotic fungal pathogen *Fusarium circinatum* and and *H. abietis* (Elvira-Recuenco et al., 2014; López-Goldar et al., 2018). This could be because similar defence mechanisms are able to defend the trees for both pests. A positive correlation was reported between the density of green spruce aphid and the amount of bark removed by *H. abietis* in a no choice cafeteria bioassay on *P. sitchensis* from 9 different provenances across the native range (Day et al., 1999); however, the *H. abietis* results were preliminary and not confirmed in addition to using bioassays method later shown to not be

representative of living saplings (Zas et al., 2019). In our study the data indicated that lower mortality from *H. abietis* is consistent with reports of higher resistance to *P. strobi.*

P. strobi is a severe pest of *P. sitchensis* in North America where both the host and the insect pest are native. The *P. strobi* larvae hatch from eggs laid in the terminal leaders of adult trees and mine down eating the phloem. This girdles and kills the previous year's leader forking the stem and making it unsellable (Alfaro, 1989). This is a very different method of damage of *H. abietis* where the adult insects eat the bark of saplings, however both damage the vasculature of the plant and lead to part of the tree being girdled.

Naturally-occurring resistance to *P. strobi* was studied after two *P. sitchensis* individuals were detected as potentially having resistance to *P. strobi* in the 1940s (Alfaro, 1989; Silver, 1968). After a series of provenance, full-sib, half-sib and clonal trials, three areas in British Columbia were identified as sources of resistant genotypes: Haney, the Big Qualicum River which both have very high incidence of *P. strobi* and the Skeena River which is the area of natural hybridization between Sitka and white spruce known as the introgression zone (King et al., 2004; Ying, 1991). The present study showed that materials from provenances from these three areas had significantly less mortality from *H. abietis,* suggesting that similar defence strategies increase resistance to both pests. *P. strobi* has not colonised the islands of Haida Gwaii (formerly Queen Charlotte Islands) and *P. sitchensis* from that area is considered highly susceptible to *P. strobi* (Alfaro et al., 2013). However, in our study the two provenances from Haida Gwaii don't have significantly different mortality compared to either the resistant provenances or the susceptible ones. This suggests that potentially, although some defence strategies may defend trees against both pests not all defence strategies are applicable to both.

Resistant and susceptible *P. sitchensis* genotypes have been investigated to try to elucidate the underlying mechanisms for resistance to *P. strobi.* The mean resistance to *P. strobi* of provenances in the introgression zone (Skeena River area) have been shown to be positively associated with traumatic resin cell (which form resin ducts) number and constitutive resin cell size (O'Neill et al., 2002). The increased flow of oleoresin from more or bigger resin ducts increases resistance to *P. strobi* by encasing the larvae and therefore restricting their movement (Whitehill et al., 2019). The oleoresin also increases resistance to *H. abietis* as it means most feeding is superficial and doesn't penetrate the inner bark (Wainhouse & Brough, 2007). The shallower wounds are less likely to impact the vasculature or cause girdling. Treatment with exogenous Methyl Jasmonate which increases resistance *H. abietis* in *P. pinaster* is also shown to increase resin content by 40% (Sampedro et al., 2011).

The monoterpene and diterpenoid profile of the oleoresin also differs between trees that are resistant or susceptible to *P. strobi* (Robert et al., 2010). Dehydroabietic acid (DRA) was strongly associated with resistance to *P. strobi* from all areas of resistance and (+)-3-carene and terpinolene were associated with resistant trees originating from the Haney area. This demonstrates that not all mechanisms of resistance are shared between the resistant provenances. Monoterpenes correlated with resistance to *H. abietis* based on the observation that *P sitchensis* saplings grown from rooted cuttings receive less damage than seedlings and have higher levels of monoterpenes (Kennedy et al., 2006). In addition, higher levels of monoterpenes were correlated with methyl jasmonate application which increased resistance to *H. abietis* in *P. sylvestris* and *P. abies* (Lundborg et al., 2016) A study of DRA, and (+)-3-carene and terpinolene levels across families form the UK breeding population showed high levels of variation in the three terpenes, given that the UK breeding population is deemed to be from a single source, Haida Gwaii (Tumas et al., 2021). In the UK breeding population, DRA concentration was similar to that of populations resistant to *P. strobi* however (+)-3-carene was closer to susceptible populations and terpinolene was below that of native populations.

Resistant clones of *P. sitchensis* from Haney have higher densities of stone cells and more G lignin in their previous year susceptible shoots than susceptible trees from Haida Gwaii (Whitehill, Henderson, Schuetz, et al., 2016). *P. strobi* lay their eggs in the previous year susceptible shoots so, the differences in abundance, morphology, distribution and cell wall composition create a strong physical barrier against larval feeding, which increases resistance (Whitehill, Henderson, Strong, et al., 2016). The position of this barrier and the extent to which it is localised is likely to have different consequences for the resistance against these two damaging weevils due to their feeding habits. In contrast to *P. strobi*, *H. abietis* can feed anywhere on the saplings and is most likely to do so at the base; therefore, this method of resistance is unlikely to also support resistance against *H. abietis*.

Our study indicated that provenances with lower mortality from *H. abietis* were consistent with reports of higher resistance to *P. strobi.* However, this was not the case for the F1 crosses, where RxR crosses show significantly less mortality to *P. strobi* than SxS crosses (Alfaro et al., 2004) , as we did not see any differences in mortality between the two cross types. This could be due to the small sample size, or it could be because the mechanism of resistance in the specific resistant parents does not provide resistance to *H. abietis*, as is likely the case with stone cells.

Impacts and future directions

Further studies could be of large benefit, to extend our understanding to a larger number of provenances and different conditions to control for any environment effects. It would be especially interesting to expand the experiment with full-sib crosses with more diverse crosses and individuals'

size to see if differences in resistance can be bred and potentially elucidate mechanisms that are effective against *H. abietis*. These studies could all be done more efficiently only taking measurements later in the season once emergence has finished and the *H. abietis* population has peaked. Given the limitations of using clmms it would be better to either record mortality or damage area as a continuous variable so they could be analysed with more conventional methods.

Selection of the most resistant individuals and crossing then to produce an F1 generation to test if the resistance is heritable may eventually allow for the development of a resistance breeding program as has been done for *P. strobi* (Alfaro et al., 2013). The consistency of resistance to *H. abietis* and *P. strobi* demonstrates the potential benefit of testing the UK breeding population for variation to known resistance markers such as resin ducts, oleoresin quality and composition and challenging any groups with these markers to see if resistant individuals can be detected within the UK breeding population. One approach to rapidly screen existing materials from different provenances for weevil resistance, is to use excised branches immediately placed in water (Zas et al., 2019). Finding significant differences in mortality to *H. abietis* between provenances of *P. sitchensis* shows that though the two did not coevolve, there are varied levels of resistance across the population of *P. sitchensis*. This observation suggests that incorporating germplasm from elsewhere in the native range into the UK breeding population could allow for increasing relative resistance. Mortality decreased as height increased for most provenances but not all. All but one of the 'resistant to *P. Strobi'* provenances also showed decreased mortality as height increased, demonstrating that increased resistance doesn't have to come with a trade-off for reduced growth.

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Chapter 4: Molecular response to attack by *Hylobius abietis* and the effects of priming in *Picea sitchensis*.

Abstract

Picea sitchensis saplings are severely affected by *Hylobius abietis* attack, which results in high mortality and heavy financial losses for forest industries if pesticides are not used. There is evidence that previous attacks by pathogens, mechanical wounding, and methyl jasmonate can all prime conifer defences to increase resistance. However, this has not been tested in relation to *P. sitchensis* and *H. abietis.* We conducted a damage response experiment followed by RNA-Sequencing to study the effects of a *H. abietis* attack in both naïve and previously attacked *P. sitchensis* saplings. Although similar damage levels were found in the naïve and previously attacked saplings, there were a small number of biologically insightful transcriptome changes, including the differential regulation of several genes involved in defence signalling. In naïve trees, in response to the attack, we saw rapid induction of many genes in the bark but in the needles, there were fewer differentially expressed genes, which responded more slowly, suggesting a small but longer term systemic response.

Introduction

Picea sitchensis (Bong.) Carriére (Sitka spruce) in the UK and several other conifers across Europe are heavily attacked by *Hylobius abietis* (L.), and untreated saplings have an average mortality 50% (I. Willoughby et al., 2017) which is well above the commercially acceptable threshold of 10%. Current clear-cut forestry practices augment the *H. abietis* pressure. The cut stumps release volatiles, which attract the adult weevils to the clear cut on mass and provide an ideal breeding substrate. The newly planted saplings provide foodstuff for the freshly emerged adults (Leather et al., 1999). Priming conifer defences with previous pathogen attacks, mechanical wounding or methyl jasmonate has been shown to increase resistance to a range of pests and pathogens, including the pathogenic bluestain fungus *Ceratocystis polonica* (Christiansen et al., 1999), *Ips typographus* (Mageroy, Christiansen, et al., 2020) and *H. abietis* (Berggren et al., 2023). These findings offer an exciting new avenue for increasing resistance to *H. abietis* in *P. sitchensis* as an additional part of the existing integrated pest management plan (I. H. Willoughby et al., 2022) which is crucial as current pesticides are being removed from the market. Defence priming has not been studied in *P. sitchensis*, nor has the transcriptional response of *P. sitchensis* to attack by *H. abietis*. RNA sequencing (RNA-Seq) analysis uses high throughput sequencing to allow the detection of transcript abundance, giving fine-scale detail of host responses by identifying differentially expressed genes (Stark et al., 2019). To determine if *P. sitchensis* defences can be primed a damage assessment experiment is required.

Subsequently running an RNA-Seq analysis of primed and naïve trees in their response to attack could help to unravel some mechanisms behind resistance priming.

As sessile organisms, plants have evolved a range of both constitutive (always present) and inducible (activated after attack commences) defences to pests and pathogens. Activating induced defences prior to attack might allow us to improve plant resistance. Induced defences caused by prior pathogen exposure or herbivory have been shown to increase resistance in conifer trees including *Picea abies* (Christiansen et al., 1999) and *Pinus radiata* (Bonello et al., 2001) and flowering plants such as *Brassica oleracea* (Mattiacci et al., 1994). Insect herbivory can induce defence responses which are associated with increased resistance, such as increased alkaloid concentrations (Baldwin, 1988), increased terpenes levels and numbers of traumatic resin ducts (Miller et al., 2005), oleoresin concentration (Zas et al., 2011) and upregulation of defence-related genes (Kovalchuk et al., 2015). These responses may be systemic and, therefore, detectable in other undamaged parts of the plant. In potato and tomato leaves herbivory rapidly induced accumulation of a proteinase inhibitor in both damaged leaves and their neighbours (Green & Ryan, 1972). Jasmonic acid related compounds have been proposed as one of the key signalling molecules in transmitting herbivory signals throughout the plant (L. Li et al., 2002).

In addition to constitutive and induced defences some induction stimuli can lead to acquired resistance (AR), which causes enhanced resistance to pests and pathogens for long time periods of many years (Wilkinson et al., 2019). AR can involve: 1) the prolonged upregulation of inducible defences and/or 2) defence priming. The prolonged upregulation of inducible defences occurs after defences are induced by stimuli and are continually upregulated for a long period of time. For example, the increase in traumatic resin ducts which causes the accumulation of oleoresins in response to herbivory in *P. sitchensis* (Miller et al., 2005). Although the prolonged upregulation of inducible defences can effectively increase resistance, it is also costly to the plant as resources allocated to defence have to be diverted away from growth and reproduction (Wilkinson et al., 2019). Defence priming is a more cost-effective form of AR. Priming occurs after a plant is exposed to a priming stimulus, which activates defence responses very minimally or briefly. This priming stimulus allows defence responses to be more strongly and rapidly activated when they are subsequently challenged. Defence priming is less costly to the plant than the prolonged upregulation of inducible defences as they don't have to maintain the response. For example, exposure of *A. thaliana* to (R)-β-homoserine primed ethylene and jasmonate defences increases resistance to a necrotrophic pathogen without stunting plant growth (Buswell et al., 2018). Determining if AR is due to the prolonged upregulation of inducible defences or defence priming can

be complicated, Martinez-Medina et al., (2016) state that for something to be defined as priming it must have an element of memory, i.e. a way to store the information between the priming stimulus and the subsequent challenge, a low fitness cost and lead to a more robust defence and ultimately better performance.

There is a need to add new strategies to the *H. abietis* integrated management plan in order for *P. sitchensis* and other conifers to remain commercially viable while using effective clear-felling approaches if no appropriate pesticides are available on the market. Developing the knowledge base surrounding *P. sitchensis* response to *H. abietis* is of particular interest in the UK and could provide vital resources for the discovery of novel plant protection strategies. This study aimed to look for increased resistance to insect pests caused by priming and measure and analyse the transcriptional response to attack in primed and naïve plants.

There were three hypotheses at the start of the study:

"*P. sitchensis* will respond systemically to *H. abietis* exposure"

"Priming caused by prior *H. abietis* exposure will increase resistance to *H. abietis* later on"

"Prior *H. abietis* exposure will cause differential gene expression"

The objectives of this study were to test these hypotheses, see if the analysis of the RNA-seq data could suggest some mechanisms behind systemic responses to attack and priming in *P. sitchensis*, and develop resources for future studies such as genes of interest. To do this, we ran a greenhouse experiment using UK breeding population material with three treatments: no exposure, a single exposure to *H. abietis*, or two periods of exposure. Needle and bark tissues were sampled over a time course to be analysed by RNA-seq, to allow us to compare transcript abundance between the treatments in different tissues over time.

Methods

I studied the gene expression response of *P. sitchensis* to a single or a double attack by *H. abietis* by using an experiment with the following three treatments: no exposure (Cont), a single three-day exposure to *H. abietis* (W1), or two three-day periods of exposure a month apart (W2) (Figure 23). The plants were rooted cuttings from six clonal lines: they were placed in four blocks within the same greenhouse compartment using a complete randomised block design. Tissues were sampled at the same three time points for each treatment and was destructive so samples could be taken only once from each individual plant. The clonal lines acted as biological replicates, and the blocks acted as technical replicates.

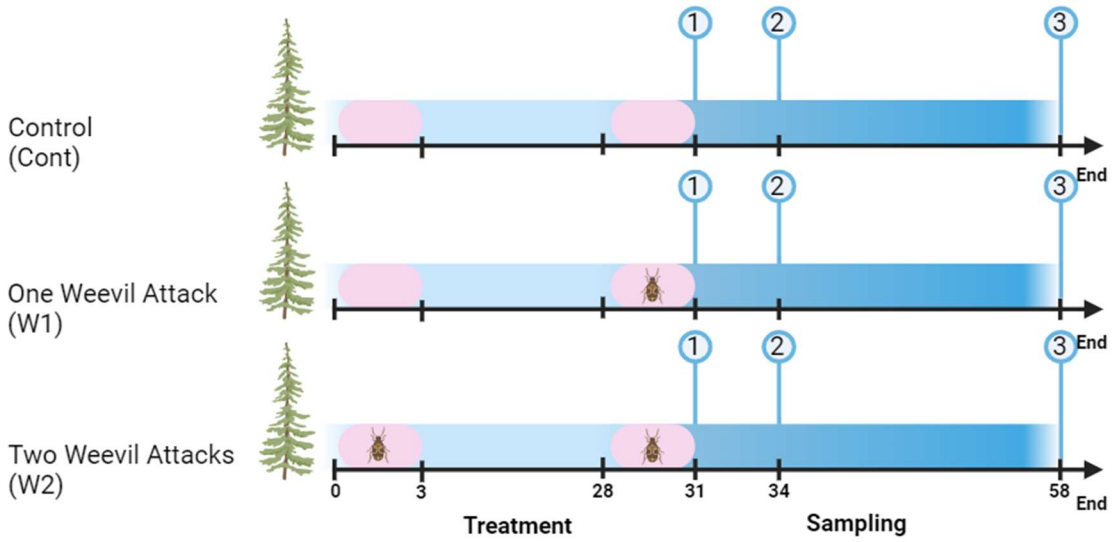


Figure 23 Experimental set-up showing three treatments, control, one weevil attack (W1) where the sapling is exposed to H. abietis for 72 hours from day 28 to day 31 and two weevil attacks (W2) where the sapling is exposed to two 72 hour periods of weevil attack one on day 0 to day 3 and one 25 days later from day 28 to day 31. Bark and needle tissues were sampled at three time-points, directly after exposure TP1 (day 31), three days after exposure TP2 (day 34), and 27 days after exposure TP3 (day 58).

Insects

Adult *H. abietis* were collected from the new North Highland Forest District (Scotland, UK) in an area known to have high population levels. *P. sitchensis* billets were cut and placed at the location. The billets were assessed, and *H. abietis* were collected twice a week throughout the season to replenish the experimental stock. The weevils were then posted to Oxford, where they were stored in an incubator (Aralab Bio FitoClima 600 PLH-V) on a 14°C and 60% Humidity 12 hours light: 9°C and 60% Humidity 12 hours dark cycle. They were fed and cleaned every two weeks on fresh *P. Sitchensis* branches collected from adult trees at Bagley Wood (Oxfordshire, UK) and provided with water from damp tissue paper.

All *H. abietis* were weighed to the nearest 0.001g using a professional digital miniscale (TL series). The sex of the weevil was also recorded by inspecting the underside of the weevil; the males have a

slight depression on their last sternite. (Wilson et al., 1966). In total, 216 *H. abietis* were used in the experiment, with no individual being used more than once i.e. either in treatment W1 or W2. *H. abietis* were starved for 24 hours prior to being exposed to the seedlings. If any died, they were removed and replaced with a different individual from the stock.

Plant material

The experiment used a total of 216 *P. sitchensis* rooted cuttings; these were from six different clonal lines, each from a different family in the UK breeding population. The cuttings were taken from the parent plants in April 2020 and inserted into 5cm x 5cm x 15cm root trainer trays and grown in a polytunnel at Forestart (Shropshire, UK) until September 2021, then a 20°C greenhouse in Oxford on a 16:8 hour light:dark cycle. In February 2022, the experimental cuttings were transferred to 3L square pots with 2.9 L of M2 pot and bedding, medium nutrient, professional growing media (Levington advance, UK) and the greenhouse temperature was increased to 25°C to promote growth. The experiment was performed in July and August 2022.

Stem diameter just above the collar was measured with electronic digital callipers (LDS1021, Linear tools) to the nearest 0.1mm. Sapling height was measured with a tape measure to the nearest cm from the soil to the tip of the needles on the tallest shoot when they were held up straight.

H. abietis exposure experiment

During the two weevil exposure periods (days 0-3 and 28-31) each sapling was placed within clear plastic tubes with sealed ends of 4-inch diameter for 72 hours (Figure 24). Each tube also contained damp tissue paper to provide the *H. abietis* with water and the soil was covered in a 1cm thick layer of sand to prevent insect escape. To expose the required trees to attack a single adult *H. abietis* was placed on the base of the stem of the sapling and then left for 72 hours. During the first exposure period (days 0-3) only W2 saplings were exposed to the *H. abietis* during the second exposure period (days 28-31) both W1 and W2 saplings were exposed (Figure 23). The debarked area was calculated by comparing each feeding scar to graded millimetre templates and totalling the areas (Berggren et al., 2023). The debarked area was measured at the end of each feeding period. For W2 the debarked area was calculated as the total after the second exposure minus the total after the first exposure.

Models were made using lmer function from the lme4 package (Bates et al., 2023). The dependent variable was the damage area, which was square rooted to correct for heteroscedasticity. The fixed effects were Treatment, Clonal line, Treatment: Clonal line interaction, weight of *H. abietis* and diameter at the base. The random effects were experimenter, block and sex of *H. abietis*. All random variables are explicitly nested using unique name combinations. Models using the DHARMa package

(Hartig & Lohse, 2022) to simulate and plot scaled residuals, and the Levene-test was used for homogeneity of variance across treatments. Model selection was done using the lmerTest package (Kuznetsova et al., 2020). A mixed-effects model comparison using the likelihood ratio test (Chisq) between the models using the anova function from the car package (Fox et al., 2023).



Figure 24 Experimental set-up: during each exposure period, every sapling was placed within clear plastic tubes with sealed ends of 4-inch diameter for 72 hours. One adult H. abietis *was placed on the base of the stem of the appropriate saplings and then left for 72 hours Each tube also contained damp tissue paper to provide the* H. abietis *with water and the soil was covered in a 1cm thick layer of sand to prevent insect escape.*

Sampling for RNA sequencing

Bark and needle tissues were sampled at three time points, directly after exposure TP1 (day 31), three days after exposure TP2 (day 34), and 27 days after exposure TP3 (day 58; Figure 23). Previous studies had also demonstrated biologically interesting changes in transcription in other species in samples taken directly after exposure and three days later (Kovalchuk et al., 2015). Taking samples at 27 days after exposure allowed us to look at more long term changes on time scales that could be commercially relevant, as often treatments are applied up to a month before planting out. Examining the TP3 sample between W1 and Cont could also inform us about the potential differences in transcription between W1 and W2 before exposure (Day 28). Needles were taken from the freshest growth area at the tip of the saplings. Bark samples were taken from within one cm of the largest feeding scar, normally near the base of the sapling, by scoring the bark with a razor blade and pealing

the bark and cambium off the sapwood. All samples were placed into Eppendorf tubes, immediately flash frozen in liquid nitrogen, and then preserved in a freezer at -80°C.

RNA isolation and sequencing

In total, 36 needle and 36 bark samples were selected for RNA sequencing plus 4 technical replicates (4 bark samples were placed on both plates). Therefore, each treatment had four biological replicates at each time point. Due to the low recovery of RNA, it was only possible for the needle and bark samples to be from the same sapling in 16 of the cases, and 20 of the samples came from two different biological replicates of the same clonal line.

RNA was isolated from 50 mg of bark or 100 mg of needle tissue. Frozen tissue samples were homogenised in a 2 mL tube with two sterile steel 3 mm ball bearings using a Tissuelyser II (Qiagen Valencia, CA, USA) at 30,000 revolutions per minute for 2 minutes. Samples were kept on dry ice when not in the freezer and the Tissuelyser blocks were pre-frozen in the -80°C freezer to prevent defrosting.

RNA was isolated by using the Monarch Total RNA Miniprep kit (NEB #T2010, Ipswich, MA, USA) as per the protocol for plant samples. Residual gDNA was removed through enzymatic DNase I treatment. The RNA was eluted into 50 µl of nuclease-free water. Sample quantitation, integrity and purity were all measured using a 5400 Fragment Analyzer System (Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed on an Illumina NovaSeq 6000 (Illumina, CA, United States) doing paired-end, 150 base pair sequencing to achieve a minimum of 6 G of data per sample by Novogene (Cambridge, UK).

RNA sequence analysis

The quality of the raw sequences was assessed using FastQC 0.11.9 (Andrews, 2018). Raw sequences were pseudo-aligned onto the reference transcriptome of *P. sitchensis* (NCBI GenBank under the BioProject PRJNA421925 currently under embargo) (Rizzuto et al., 2023) using kallisto 0.44.0 (Bray et al., 2016) with 1000 bootstraps.

The transcriptome used contained 42976 transcripts and was deemed to be 81.3% complete, 78.7% fragmented, and 2.6% missing. Annotation for the transcriptome of *P. sitchensis* was searched against the protein homologues of *Arabidopsis thaliana* (Araport11) (Cheng et al., 2017) using blastx 2.11.0+ (Camacho et al., 2009)with default parameters. . 41758 transcripts from the transcriptome had an annotation from the Araport11 database and were used to form an annotation table.

However, as *P. sitchensis* is not a model species and the transcriptome has not been formally annotated we cannot be sure what is an isoform and what is a gene. The differentially expressed gene analysis considers all the transcripts in the transcriptome, and the *Arabidopsis thaliana* annotations are only used for the enrichment analysis.

Initially, all samples were analysed together to perform a PCA to look for clustering of the samples by tissue type, timepoint, and treatment. Subsequently the two tissue types were analysed separately to create differentially expressed genes (DEGs) lists within each tissue independently. The analysis of DEGs was done using the DESeq2 package in R (Love et al., 2014) to generate log2 fold changes with adjusted p-values. The DESeq model was ~group, where the group referred to each group of 4 samples for each TPxTreatmentxTissue combination. Subsequently 12 pairwise comparisons were performed on pairs of contrasts to identify differentially expressed genes. These comparisons were done using Wald tests with a Benjamini Hochberg correction for a p-value of 0.05 and a significant |log2foldchange| >1. This means only genes significantly differentially expressed by a factor of 2 were selected. To identify genes differentially expressed due to attack, I compared the samples from Cont and W1 at each TP. To identify genes differentially expressed due to priming, I compared the trees W1 and W2 at each TP. The enrichment of Gene ontology (GO) terms was determined using go_enrich from the clusterprofiler package (Yu et al., 2012), and a Benjamini Hochberg correction was applied with the significance threshold set at a p-value of 0.05. Gene model descriptions for the DEG lists were downloaded from The Arabidopsis Information Resource (TAIR), on www.arabidopsis.org, using the bulk data retrieval tool (Berardini et al., 2015).

All coding was done in R (R Core Team, 2023). PCA graphics were made using the ggplot2 package (Wickham et al., 2023). Venn diagrams were made using venn.diagram from the VennDiagram package (H. Chen, 2022)

Results

Damage response

Similar damage areas were recorded in the treatments W1 and W2, and across the clonal lines tested (Table 12). When controlling for block, experimenter, and sex of *H. abietis*, the mean damage area for W1 was 163 mm^2 and 168 mm^2 for W2. Each clonal line was from a separate family and no effect of family was also seen in Chapter 2. Visually, there appeared to be the least damage and the lowest variation in damage area for family 2 (Figure 25). Unlike in Chapter 2, there was no significant effect of *H. abietis* weight on the damaged area, nor was there a significant effect of sapling diameter or family treatment interaction.

Fixed effect	*χ2*	*df*	*Pr(>Chisq)*
Treatment	0.061	1	0.805
Clonal Line	3.45	5	0.630
Treatment: Clonal Line	6.59	5	0.253
Diameter	2.32	1	0.128
H. abietis Weight	0.561	1	0.453

Table 12 Results of likelihood ratio tests using linear mixed effects models (lmers). To examine the effect of Treatment, Clonal Line, Treatment:Clonal Line interaction, Diameter and weight of H. abietis on the square root of damage area. χ^2= chi squared value, df = degrees of freedom of variable, Pr(>Chisq)= p value of chi squared test.

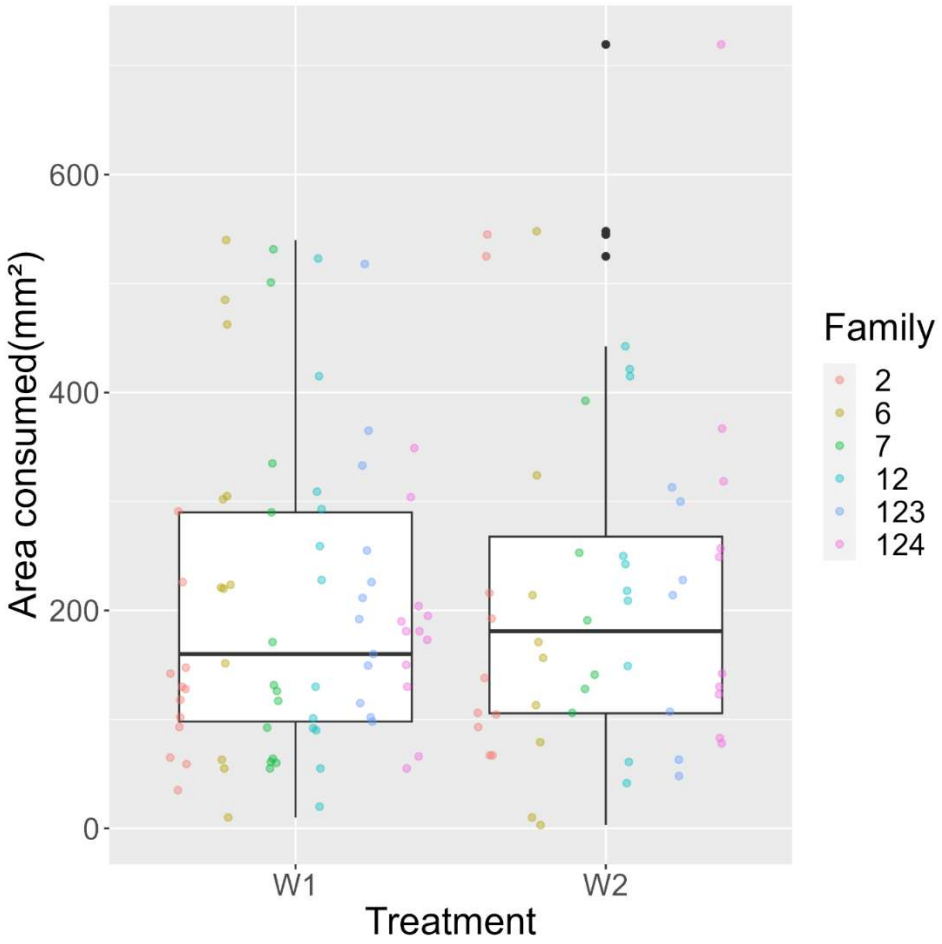


Figure 25 Boxplot showing area of bark consumed in the second exposure period for both treatments W1 and W2, mean of W1 is slightly lower than that of W2 (163mm2 and 168mm2). High levels of between-sample variation are seen, each data point is represented with colours showing different families.

RNA sequencing and read mapping

RNA sequencing of the 76 samples (36 needle samples, 36 bark samples + 4 bark technical replicates) produced a total of 2098 million 150-base-pair paired-end reads with a median of 23 million reads per sample (Table 13). All samples had over 20 million reads and therefore meet high standards for differential gene expression analysis as power increases with sequencing depth of up to 20 million reads (Ching et al., 2014). The number of reads per sample varied from 20 to 100 million per sample, though only three samples had over 50 million reads. The variation in read number is potentially due to input samples not being correctly normalised. The over-represented libraries were not noticed as outliers; therefore, they were kept in the analysis. There were also non-negligible levels of adapter contamination in some samples, but these samples did not appear as outliers in the PCAs, so they were kept in the analysis. Adapters were not trimmed, as soft clipping using modern aligners is sufficient (Liao & Shi, 2020). A median of 80% of the reads uniquely aligned to the reference transcriptome of *P. sitchensis* (Rizzuto et al., 2023). A total of 41,861 transcripts were identified across all samples.

	Total Reads per Sample	Reads aligned (%)	Pseudo-aligned reads per Sample
Minimum	20,169,449	70.4	16,065,217
Maximum	100,011,617	83.7	73,503,390
Median	23,636,630	80.3	19,000,124

Table 13 RNA Sequencing summary statistics for the 76 samples. All samples had a sequencing depth of over 20 million reads.

To check if the two sequencing runs were comparable, four samples were sequenced twice, once on each of the two plates sent to Novogene. A PCA of the eight samples shows that they are highly correlated, two of the pairs are virtually indistinguishable (Figure 26). This shows the similarity of the results and supports the plates being analysed simultaneously as was done for the exploratory data analysis.

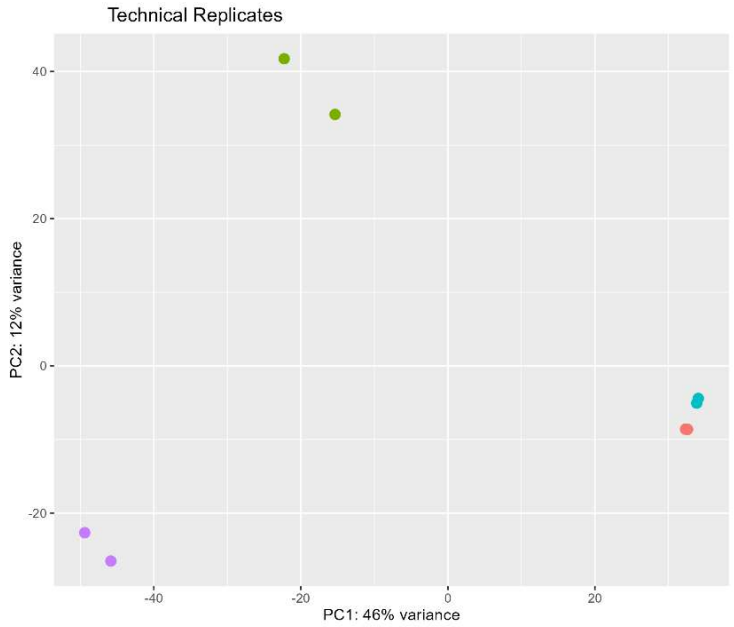


Figure 26 Principal component analysis of the regularised log transformation of the gene expression in the two technical replicates of the four samples that were sequenced on both plates, showing the clustering of the pairs of samples together. Each colour is a different sample.

Differential expression across the dataset

Visual inspection of a PCA on the whole data set showed a clear differentiation between the samples from the two tissue types (Figure 27). Within the bark samples, some clustering was apparent by treatment type, with W1 and W2 separating from the control samples. This pattern was not observed within the needle samples. The needle samples had much higher within-group variability than the bark samples. Therefore, sub-setting the data set into the two tissue types and then running the DESeq on the two groups separately allows for more sensitivity than examining all the samples simultaneously.

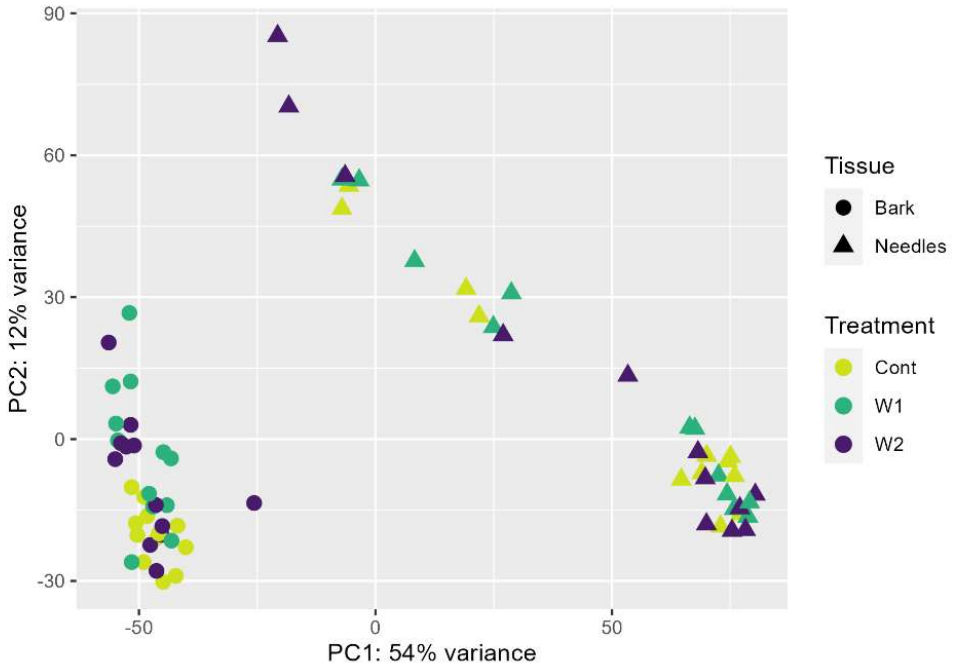


Figure 27 Principal component analysis of the regularised log transformed gene expression data for the 72 samples showing clustering by tissue type as well as clustering by treatment within the bark samples. Colours represent different treatments; shapes represent different tissue types.

The same patterns were seen in the unsupervised hierarchical clustering of the 500 genes with the most significant variation in abundance (Figure 28). The two tissue types are clustered separately (seen on the top x-axis). Within the bark samples, there are two clear clusters (C1 and C2). C1 is made up of the samples from W1 and W2 (1 and 2 weevil exposures) mostly from TP1 and TP2, and C2 is made up of the Cont samples (0 weevils) as well as some samples from W1 and W2 in TP3. This clustering potentially shows that the effects of the two treatments have ended by TP3. A subset of the 500 genes appears to be upregulated in C1 compared with C2 (labelled DEGs). There appear to be two clusters within the needles; however, these clusters are not grouped by time point or treatment.

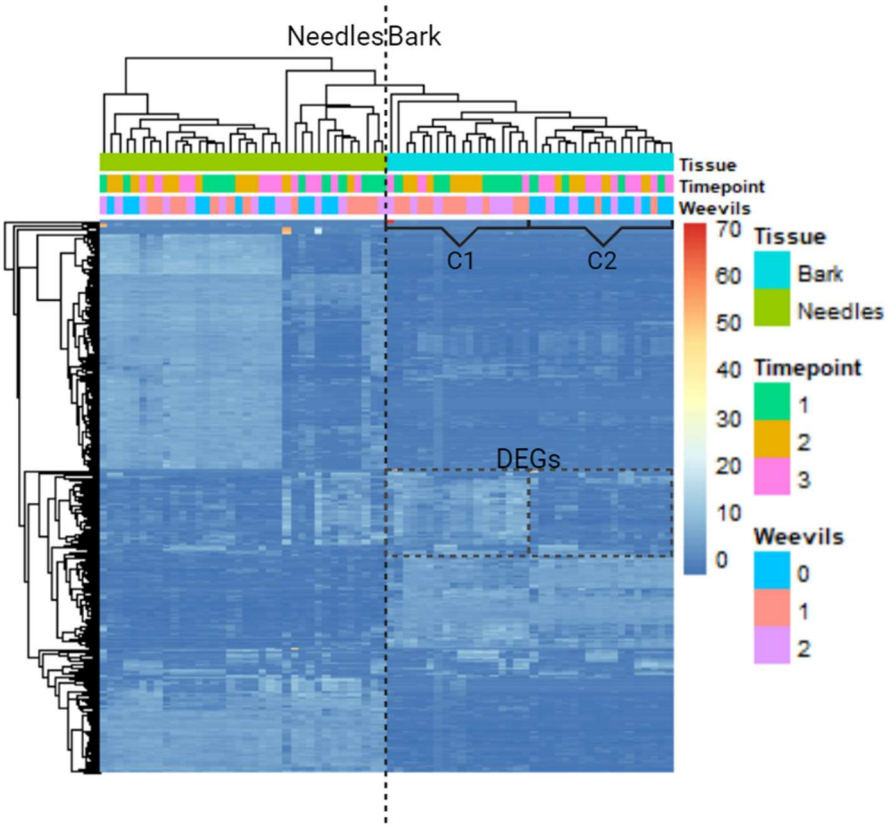


Figure 28 Hierarchical cluster analysis of the 500 genes with most significant variation in abundance showing that the normalised counts of different genes (rows) varied across the samples (columns). Rows and columns are also grouped in hierarchical clustering. Samples clearly cluster by tissue type with needles samples appearing on left and needles on right. Within the bark samples, there are two clusters. C1 and C2, C1 contains samples from W1 and W2 at TP1 and TP2, and C2 is the Cont samples and some W1 and W2 samples in TP3.There is a group of genes which are differentially expressed between these two clusters (DEGs)

Constitutive differences between Needles and Bark

Of the 41,861 transcripts identified in this analysis, 20,962 (50%) genes are significantly differentially expressed between tissue types when controlling for treatment and timepoint. Nearly half of these (9550) had a fold change of 2, of which 6780 were upregulated in bark and 2770 were upregulated in needles.

A subset of the samples was analysed to assess the constitutive differences between the two tissues, including only the control samples. Within the control samples, there were 41,098 different transcripts, 15,636 (36%) of which were differentially expressed between the two tissue types when controlling for timepoint. Over a third (5413) of these had a fold change of 2 between the two tissue types, 2127 transcripts were upregulated in bark, and 3286 transcripts were up-regulated in needles.

To study the functional differences between the tissues' constitutive transcriptomes, the Gene Ontology (GO) annotations for the DEGs were obtained. The lists were analysed for overrepresentation of biological processes, and the top 10 most significantly overrepresented terms were examined (Figure 29). The terms overrepresented in bark included phenylpropanoid metabolic

and biosynthetic processes (Figure 29A). Phenylpropanoid metabolism is associated with the synthesis of lignin, and higher lignin concentrations are required to provide additional rigidity in the bark. The phenylpropanoid pathway is also an important factor in defence against insect pests in spruce, and several genes in this pathway have been shown to be differentially expressed between genotypes of *Picea glauca* known to be resistant to *Pissodes strobi* (Porth et al., 2011). The gene concept network plot (not shown) showed that many of the genes involved in phenylpropanoid overlapped with those of secondary metabolic processes; these genes are not generally required for growth and cell maintenance but may, along with the genes annotated as 'response to oomycetes', be involved in constitutive defence. Floral whorl development GO terms may be linked to tissue patterning. The terms overrepresented in needles include many terms relating to photosynthesis, as would be expected. These terms are much more significantly upregulated than any terms in the bark (Figure 29B).

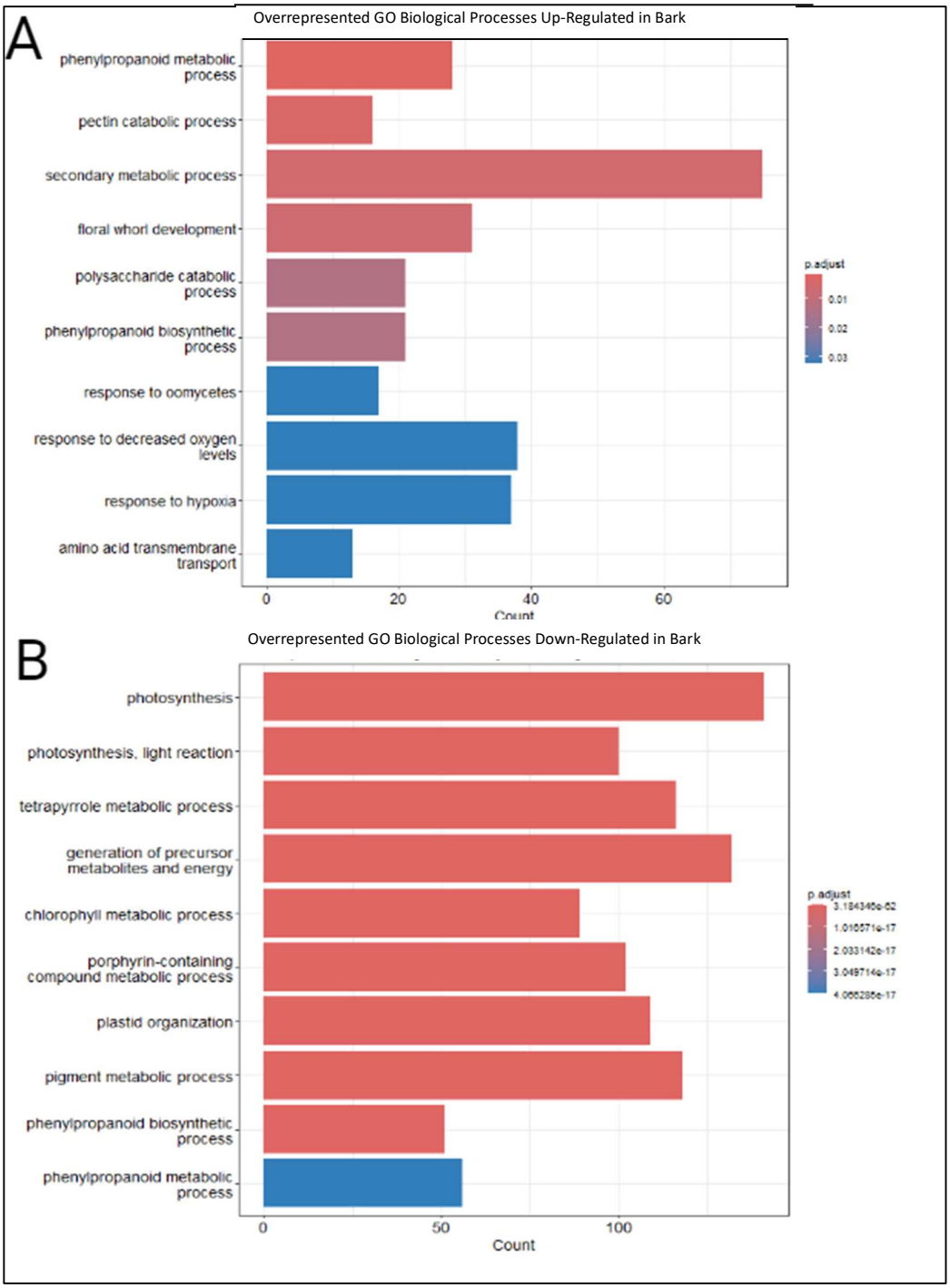


*Figure 29 Over-representation analysis of GO terms for biological processes. **A** all GO terms which are overrepresented in the list of DEGs that are upregulated in Cont bark and compared to Cont needles samples. **B** all GO terms which are overrepresented in the list of DEGs that are downregulated in Cont bark, compared to Cont needles samples. Only the top 10 most significantly represented terms are shown. Colour shows the significance of the over-representation, based on p values adjusted with a Benjamini Hochberg correction, scales are different between the two charts.*

Effect of Treatment on Gene Expression

Due to the higher within-group variability among needle samples than the bark samples, the data was split into two data sets and examined separately to allow for more sensitivity. Two PCAs were done within each tissue type, one for the effect of attack containing the W1 and Cont samples and

one for the effect of priming containing the W1 and W2 samples. In the PCA comparing W1 with Cont in bark samples, there was a clear separation between the two treatments along the x-axis (Figure 30A). There were two W1 samples grouped with the Cont samples, but these were both from TP3, potentially suggesting that the treatment effect may have begun to decrease by then. Within each treatment cluster there is also clustering by timepoint with TP3 clustering furthest left TP1 clustering furthest right.

In the bark PCA containing the W1 and W2 samples, there is no clear grouping by treatment but some grouping by timepoint, with most of the samples from TP1 clustering on the left-hand side and those from TP3 clustering on the right (Figure 30B). When the samples are coloured by clonal line (not shown) each clonal line clustered separately along the y-axis (PC2), showing clonal line explains a large amount of the 13% explained by PC1. This observation shows the importance of including representative biological repetitions in RNA sequencing analysis. PC2 explains much less of the variation than PC1 in both PCAs suggesting the effect of timepoint (B) and treatment and timepoint (A) are greater than that of clonal line. The two PCAs for needles showed no clustering by timepoint or treatment (not shown)

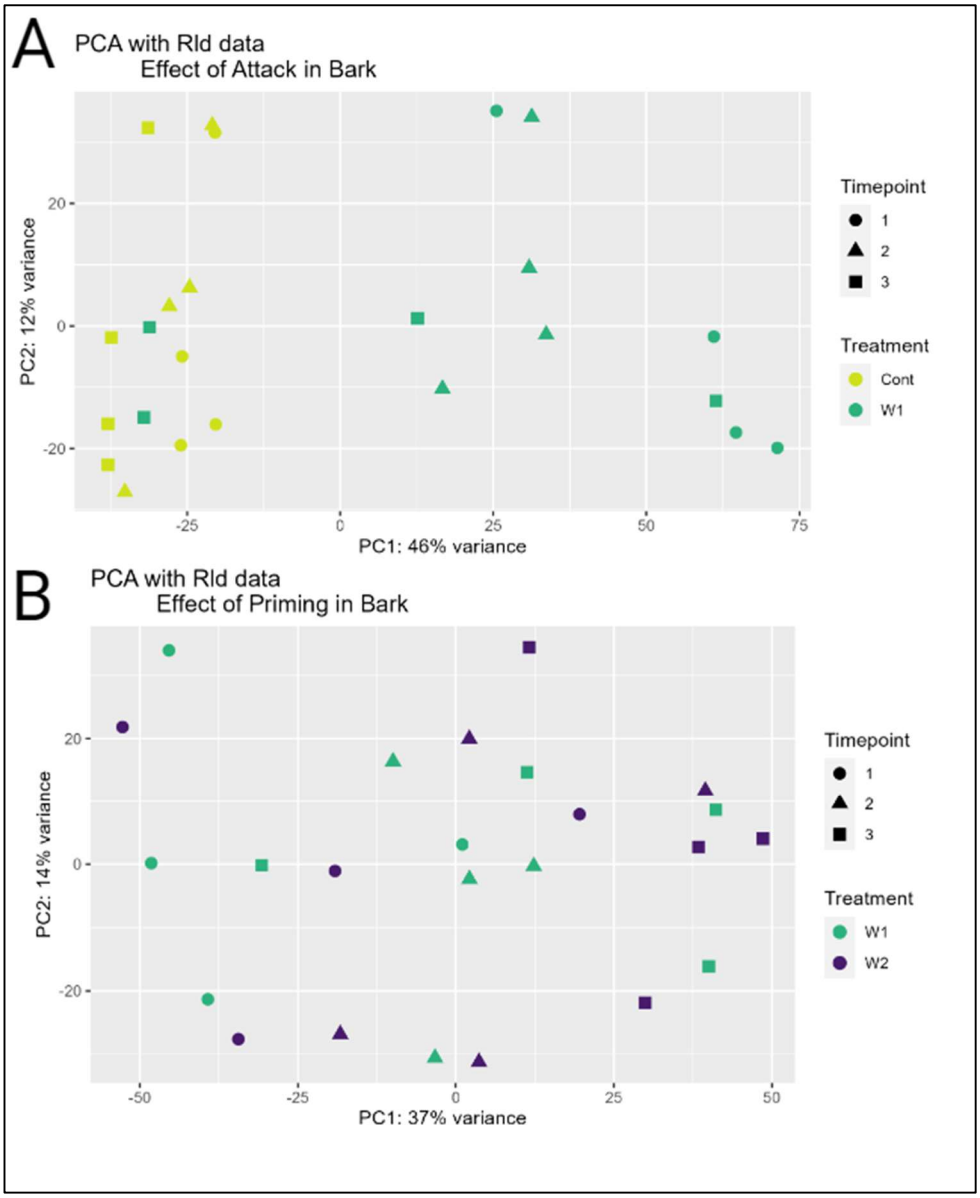


*Figure 30 Principal component analysis of the regularised log transformation of the gene expression data for the 36 bark samples. **A** Cont and W1 treatments with clustering by treatment as well as by timepoint within the treatment. **B** W1 and W2 treatments with clustering by timepoint but not treatment.*

Six pairwise comparisons were made in each tissue type, one for each time point and pair of treatments. In bark, comparing W1 and Cont, 497 separate transcripts (out of 40,768 – 1.3%) were significantly differentially expressed with a fold change of at least 2 over all time points. The most differentially expressed genes were detected at TP1, with 435 DEGs, 190 up-regulated, and 45 downregulated (Figure 31). There were also fewer downregulated genes at TP2 and TP3 (1:35 and 0:136, respectively). When comparing W1 and W2 in bark, there were only 14 separate transcripts in total (20 if the fold change requirement is removed), and in all but two of the instances, they were downregulated. The small numbers of DEGs between W1 and W2 show a very limited transcriptional response to priming by previous attacks. In needles, there were small numbers of DEGs at all

contrasts and time points (Figure 31). Unlike in bark in needles the number of DEGs between W1 and Cont was lowest at TP1, when there was 1 DEGs compared with 18 at TP2 and 25 by TP3. These patterns show there is a large and immediate molecular effect of attack localised to the bark, and the transcriptional effect is more restricted in the needles and increases over time. Priming appeared to have a very low-level effect on transcription within either tissue.

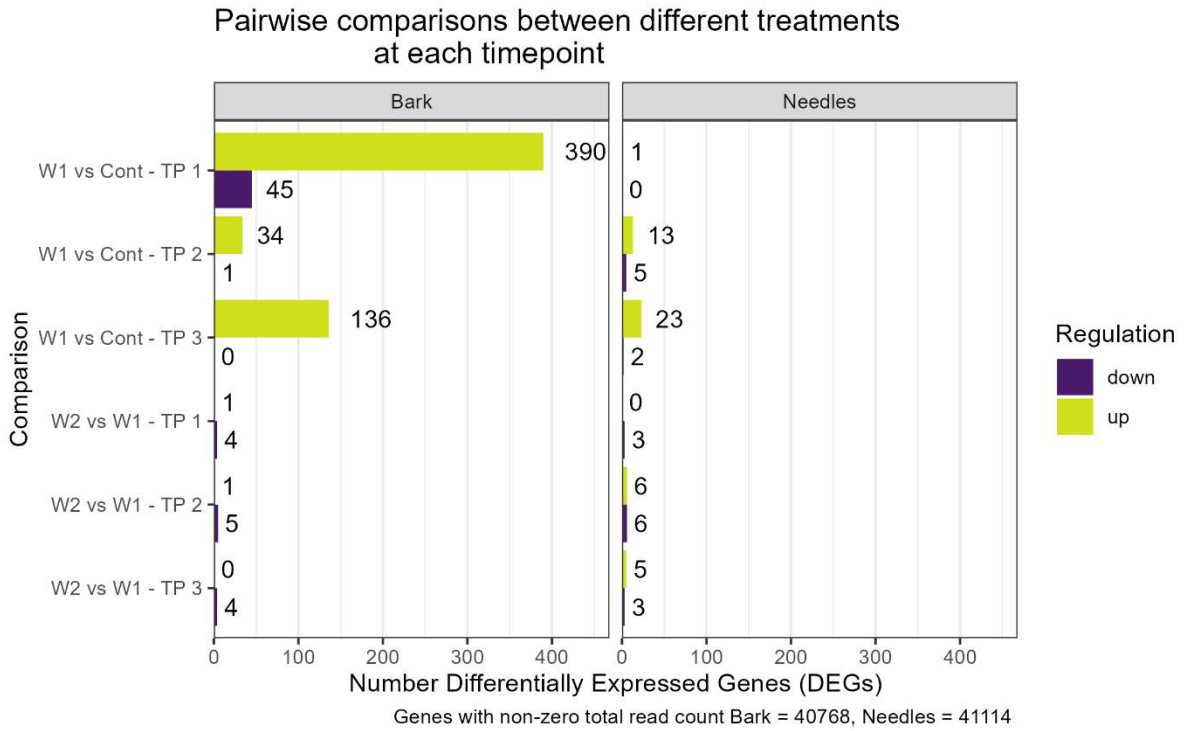


Figure 31 Number of DEGs between each treatment at each timepoint in each tissue. Comparisons were done using Wald tests with a Benjamini Hochberg correction for a p-value of 0.05 and a significant |log2foldchange| >1. Up-regulated genes shown in green down-regulated genes shown in purple.

Of the 497 DEGs in bark, 87 were differentially expressed at more than one TP, and 22 were differentially expressed at all three TPs (the gene list of the 22 DEGs shared between timepoints is in Appendix 1). Comparisons between the DEGs in the needles and the bark show that there are very few genes that are differentially expressed in both tissue types (Figure 32). Fold changes varied with the 35 highest log2 fold changes being 27-49. Interestingly, those with the highest fold changes were all in TP2 or TP3, with 23 of the 35 being from TP3.

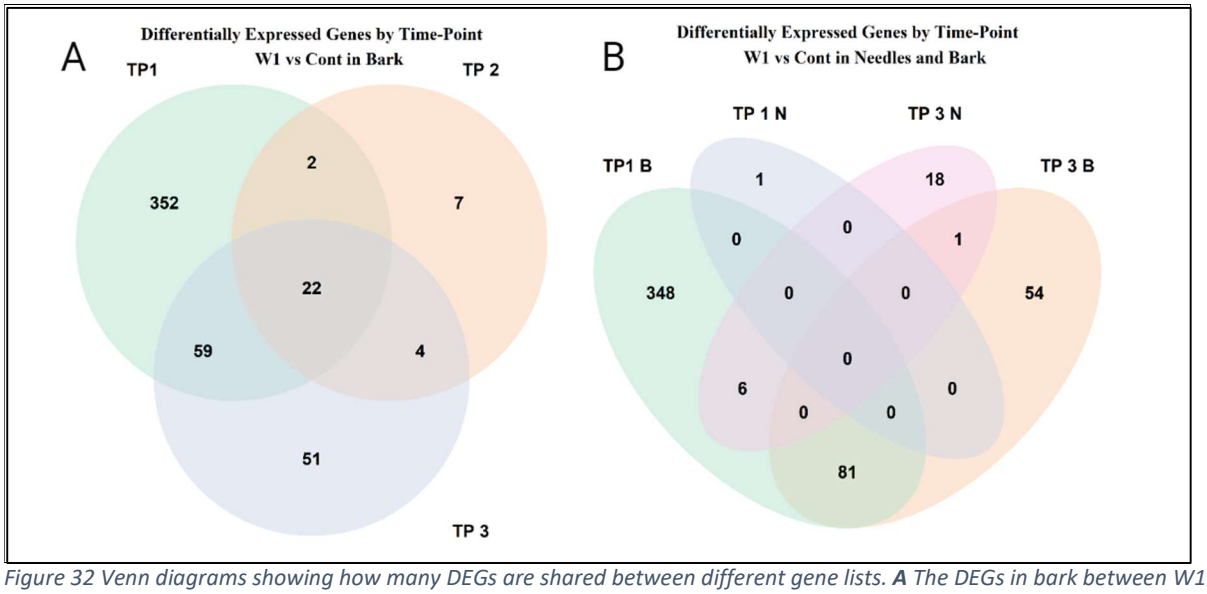


*Figure 32 Venn diagrams showing how many DEGs are shared between different gene lists. **A** The DEGs in bark between W1 and Cont and how they are shared between time points. **B** The DEGs between W1 and Cont at TP1 and TP3 in needles and bark, showing that very few genes are shared between the needles and bark.*

Gene Ontology Analysis

To interrogate the outcome of the transcriptional changes caused by the weevil attack and priming, TAIR IDs were allocated to the transcripts based on sequence similarity and the gene ontology terms were assigned to them separately. These terms were then assessed for overrepresentation against the background of all the experiment's transcripts. The number of differentially expressed transcripts with a particular gene ontology tag in that list is compared to the total number of transcripts with that tag in the experiment to determine if the term is overrepresented in the list.

Effect of attack

We examined four lists, up-regulated genes at TP1 and down-regulated at TP1 (Figure 33 A) upregulated genes at TP2 (Figure 33 B) and upregulated genes at TP3 (Figure 33 C). There were too few downregulated genes at TP2 and TP3 to perform the analysis. From the four lists, we report on the most significant overrepresented biological processes (up to a maximum of 15). Although TAIR IDs were not available for each transcript, they were for 96% of them.

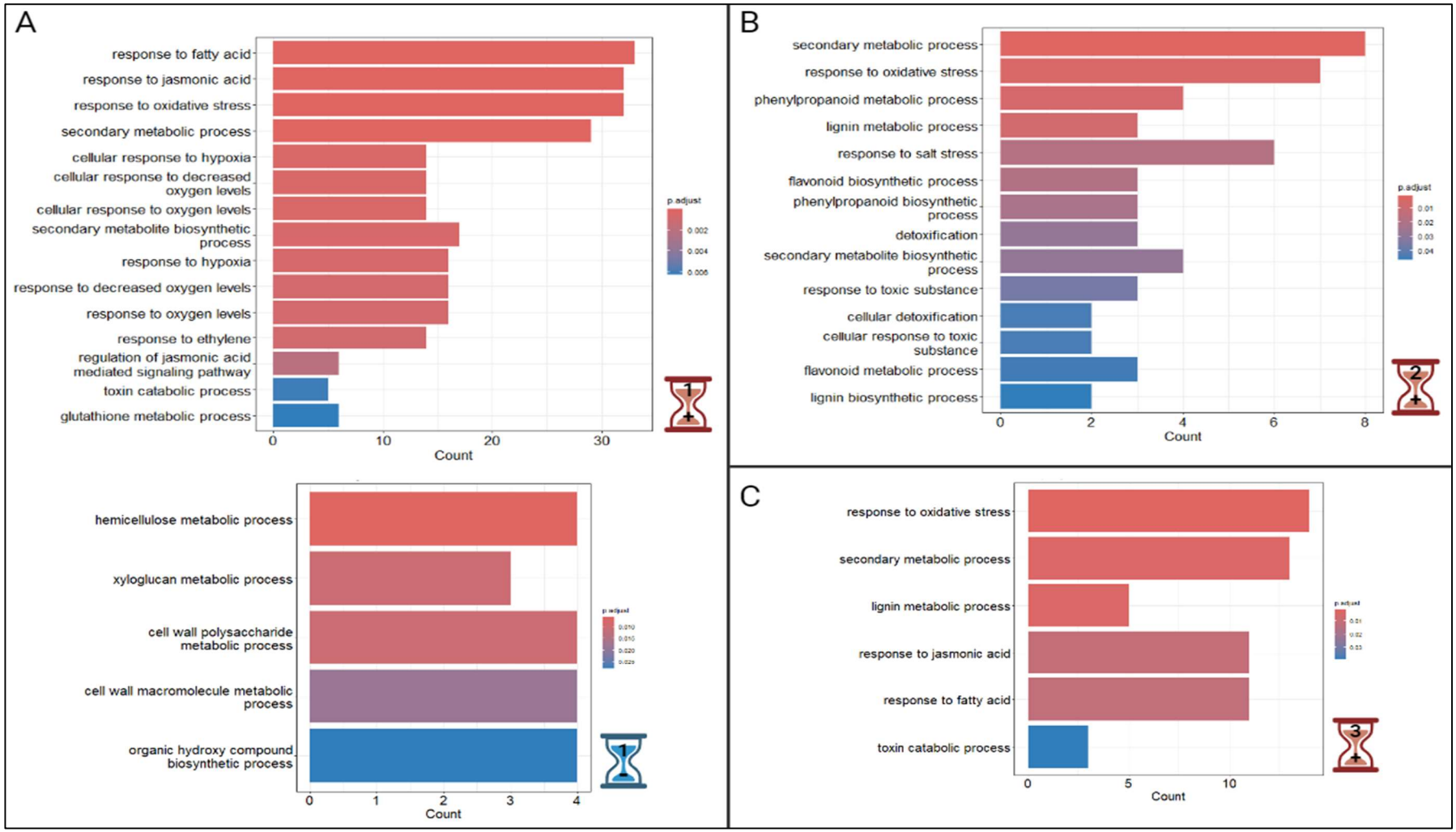


Figure 33 Over-representation analysis of GO terms for biological processes within DEG lists from bark samples between W1 and Cont treatment at each TP . Hours glass number reprenesnts TP and colour red shows upregulated genes and blue shows down regulated genes. ***A*** *TP1, top shows overrepresented in the list of DEGs that are upregulated bottom shows over represented terms that are downregulated.* ***B*** *TP2****,*** *Over-represented terms of upregulated DEGs, too few down regulated DEGs to perform analysis* ***C*** *TP3 Over-represented upregulated terms, there were no down regulated genes. Only the top 15 most significantly represented terms are shown. Colour shows the significance of the over-representation, based on p values adjusted with a Benjamini Hochberg correction, scales are different between the two charts.*

At TP1, several terms of interest were overrepresented in the upregulated list. The two terms with the highest number of DEGs and which were the most significantly overrepresented were response to fatty acid and response to jasmonic acid. As jasmonic acid is derived from a fatty acid most of these genes were shared between the two terms, (seen in gene concept network - not shown). The list of 32 DEGs with the GO term response to jasmonic acid was extracted and is in Appendix 2. The list of 29 with the GO term secondary metabolic process was extracted and is in Appendix 3 many of the genes are involved in the biosynthesis of lignin and glutathione. DEGs involved in the response to oxidative stress are also overrepresented, potentially as reactive oxygen species are associated with cell death. There were 5 terms which were over-represented in the list of downregulated transcripts at TP1 (Figure 33 A bottom). Four of these terms relate to the production of structural polysaccharides: hemicellulose metabolic processes, Xyloglucan metabolic process, cell wall polysaccharide metabolic process and cell wall macromolecule metabolic process, suggesting that the saplings downregulate some growth processes in response to attack.

At TP2 and 3, the gene lists were much smaller than TP1 but still contained many of the same overrepresented terms. At TP2, 8 of the 34 DEGs were related to secondary metabolites, including processes involving lignin, flavonoids and phenylpropanoids (Figure 33 B). The similarity of the overrepresented terms suggests that response to the attack is sustained throughout the experiment even though the damage itself is not.

Within the needles, there were so few DEGs that they were combined into a single list (Appendix 6) before being assessed for overrepresentation. Two terms emerged as significantly overrepresented auxin transport and regulation of jasmonic acid mediated signalling pathway. There were three genes linked to auxin transport PSIR_DN19611_c0_g1 (AUX1) an auxin influx transporter, the mRNA of which is cell to cell mobile, it was up-regulated at TP2 and TP3, PSIR_DN3876_c0_g1 (LAX1) which is also a auxin influx transported and is upregulated at TP3 and PSIR_DN44225_c3_g2 (ABCB4) a polar orientated auxin efflux transporter that redirects auxin from the root tip upregulated at TP3. There was also a further transporter upregulated at TP3, an ATP Binding Cassette transporter protein, but it is not specifically auxin-related. There were two Jasmonate Zim Domain proteins, PSIR_DN9245_c0_g1 (JAZ12) and PSIR_DN33361_c0_g2(JAZ 3), both up-regulated at TP3. JAZ proteins are known to be down-regulated by jasmonate and downregulate the transcription of jasmonate-responsive genes (Z. Li et al., 2021). Notably, there was also a terpene synthase (PSIR_DN43647_c0_g1, TPS14) that was significantly up-regulated at TP3. Terpenes are a major class of both volatile and non-volatile compounds, several of which are involved in plant defence and plant-to-plant signalling (Rosenkranz et al., 2021).

Effect of priming

To fully examine the DEGs between W2 and W1, the lists were remade without requiring the log2 fold change to be significantly greater than 1, as this represents a two-fold change in expression and is a stringent criterion. In the bark, there were 8 DEGs at TP1, 7 at TP2 and 6 at TP3, only two of which did not have TAIR IDs (Table 14) and these gave no significant results to the over representation analysis. However, there were two different orthologs of lipoxygenase 1 (LOX1), PSIR_DN29743_c0_g1 which was upregulated at TP1 and PSIR_DN29154_c0_g1 that was down-regulated in W2 at TP1 and TP2. Lipoxygenase catalyses a step in the Jasmonic acid biosynthesis pathway (Singh et al., 2022). There was also an ortholog of AZELAIC ACID INDUCED 1 (AZI1), PSIR_DN24303_c0_g1, which was downregulated at TP1. AZI1 is known to be involved in defence priming and regulates systemic acquired response in Arabidopsis (Riedlmeier et al., 2017). There were also several genes relating to sugar metabolism, a beta-glucosidase, two orthologues of a UDP-glycosyltransferase and two Galactose oxidases, all of which could be related to cell wall repair.

The needles also had no significantly over-represented terms within the W2 vs W1 DEG list (full list in Appendix 5). Within the list there was a SAGA complex subunit which regulates gene expression by affecting histone H3 acetylation, histone acetylation has been shown to be involved in priming defence as well as 'memory' heat and cold events (Nishio et al., 2024). In addition to several stress response genes, SAD2 (super sensitive to ABA and drought 2), jasmonate-zim-domain protein 12, a cationic peroxidase, a TIR-NBS-LRR sequence and F5H, which is involved in lignin biosynthesis.

Gene	L2FC	TP	TAIR ID	Gene Model Description
PSIR_DN43726_c0_g1	18	1	AT5G20950.2	Encodes a beta-glucosidase involved in xyloglucan metabolism.
PSIR_DN29743_c0_g1	18	1	AT1G55020.1	lipoxygenase, a defense gene conferring resistance Xanthomonas campestris. The mRNA is cell-to-cell mobile.
PSIR_DN28532_c0_g3	-1	1	AT5G46700.1	Encodes a transmembrane protein of the tetraspanin (TET) family, one of 17 members found in Arabidopsis. Required for the maintenance of both the radial pattern of tissue differentiation in the root and for the subsequent circumferential pattern within the epidermis.
PSIR_DN30378_c0_g1	-2	1	AT5G01710.1	methyltransferase
PSIR_DN29154_c0_g1	-16	1	AT1G55020.1	lipoxygenase, a defense gene conferring resistance Xanthomonas campestris. The mRNA is cell-to-cell mobile.
PSIR_DN24303_c0_g1	-18	1	AT4G12470.1	Encodes AZI1 (AZELAIC ACID INDUCED 1). Involved in the priming of salicylic acid induction and systemic immunity triggered by pathogen or azelaic acid. Targeting if AZI1 to chloroplasts is increased during SAR induction and that localization requires the PRR domain.It is involved in the uptake and movement of the azelaic acid signal. AZI1 uses a previously undescribed variant of the signal anchor proteins mechanism to target plastids. AZI1 uses a bipartite N-terminal signature: a non-cleavable TMD that anchors the protein to membranes, followed by a proline rich region with features that are shared with bona fide chloroplastic transit peptides. flg22 MAMP treatment strongly induces AZI1/EARLI1 protein levels and increases their relative enrichment in the plastid fraction.
PSIR_DN26018_c0_g1	-22	1	AT3G51630.2	Encodes a member of the WNK family (9 members in all) of protein kinases, the structural design of which is clearly distinct from those of other known protein kinases, such as receptor-like kinases and mitogen-activated protein kinases.
PSIR_DN26193_c0_g1	-24	1	NA	
PSIR_DN41935_c0_g1	19	2	AT3G53980.2	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
PSIR_DN29154_c0_g1	-16	2	AT1G55020.1	lipoxygenase, a defense gene conferring resistance to Xanthomonas campestris. The mRNA is cell-to-cell mobile.
PSIR_DN36180_c0_g3	-19	2	AT3G62000.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
PSIR_DN40399_c1_g2	-19	2	AT4G33300.2	Encodes a member of the ADR1 family nucleotide-binding leucine-rich repeat (NB-LRR) immune receptors.
PSIR_DN38604_c0_g1	-22	2	AT2G15490.3	UDP-glycosyltransferase 73B4
PSIR_DN38252_c2_g5	-22	2	AT2G15490.3	UDP-glycosyltransferase 73B4

PSIR_DN29821_c1_g1	-23	2	AT5G15410.2	'defense, no death' gene (DND1) encodes a mutated cyclic nucleotide-gated cation channel; Same as CNGC2 (article ID 229): Cyclic nucleotide gated channel, activated by cAMP, conducts K+ and other monovalent cations but excludes Na+, does not contain the GYG amino acid sequence found in other channels with this conductivity profile. Conducts Ca2+ into cells which is linked to the generation of NO and the NO signaling pathway involved in the innate immune response to pathogens. CNGC2 could be the key step mediating bulk Ca2+ influx into leaf cells after unloading from the vascular and have no direct roles in the leaf development and HR.
PSIR_DN34657_c0_g2	-4	3	AT5G42360.1	Galactose oxidase/kelch repeat superfamily protein
PSIR_DN26360_c0_g2	-7	3	AT4G13420.1	Encodes a protein of the KUP/HAK/KT potassium channel class that is upregulated in the roots by K levels.
PSIR_DN2628_c0_g1	-17	3	NA	
PSIR_DN21190_c0_g1	-34	3	AT1G19900.1	RUBY encodes a secreted galactose oxidase involved in cell wall modification.
PSIR_DN14519_c0_g1	-35	3	AT1G51990.2	O-methyltransferase family protein
PSIR_DN43684_c0_g3	-39	3	AT2G43580.1	Chitinase family protein

Table 14 DEGs in Bark between W2 and W1 samples, these genes are differentially expressed in response to priming. L2FC is the log2 fold change, gene model descriptions were downloaded from The Arabidopsis Information Resource (TAIR) on www.arabidopsis.org .

Discussion

This study was designed to test if insect attacks primed the defences of *P. sitchensis* saplings. We tested for defence priming based on the damage phenotype by comparing the saplings which had been previously attacked by *H. abietis* and naïve saplings experiencing their first exposure. Despite not seeing any significant difference in damage level between the primed and naïve saplings, we detected 20 transcripts that were significantly differentially expressed between the two treatments in the bark and 27 transcripts in the needles, including several genes of interest, e.g. two orthologs of lipoxygenase 1. We used the same experiment to examine the transcriptional response to attack by comparing the transcriptome of the saplings after a single attack (W1) to the control saplings (Cont). We saw a much larger response to attack in the bark with 497 DEGs and only 39 in the needles, suggesting a strong localised response and a weaker systemic response. It should be acknowledged that RNA sequencing measures transcript abundance; it cannot detect if changes in abundance are due to changes in the rate of transcription or rate of transcript turn-over, nor can it predict translation rate. I will discuss my results in the context of other studies on defence priming, often using methyl jasmonate, in a range of plant species against a range of pests, and consider what scientific advances would be beneficial to allow exploitation of defence priming for insect resistance in commercial settings.

Response of *P. sitchensis* to *H. abietis* attack

In bark, a total of 497 distinct transcripts (out of 40768 – 1.3%) were differentially expressed. By collecting the samples for both the W1 and Cont saplings at each time point, we could account for variation from unrelated abiotic factors and circadian rhythms. At the end of the 72 h *H. abietis* attack (TP1) there was the largest number of DEGs, with 435 DEGs, with over four times more up-regulated genes than down-regulated (390:45). In *Pinus sylvestris,* which had experienced 8 days of *H. abietis* attack, a much larger number of genes were also upregulated than downregulated (774 vs 64) (Kovalchuk et al., 2015). That study used a 36.4k microarray, which is not dissimilar in size to our 41k transcripts; however, it detected considerably more DEGs, potentially suggesting that *Pinus* sylvestris is more responsive to *H. abietis* attack. This difference is possible given that *P. sylvestris* is a native conifer and has coevolved with *H. abietis*, so it may be better able to detect its herbivore-associated molecular patterns (Snoeck et al., 2022). Similarly, resin accumulation induced by *H. abietis* damage was twice as high a native pine than in exotic pine (Zas et al., 2011). However, making direct comparisons between our study and that of Kovalchuk et al (2015) is difficult as they only had two biological replicates for attacked saplings and used older 6-year-old saplings, compared with our study with two-year-old rooted cuttings and 4 replicates per treatment at each time point. I potentially could have increased the number of detected DEGs due to attack by also considering

DEGs between W2 and Cont and then excluding any DEGs between W2 and W1 as was done by Mageroy et al (Mageroy, Wilkinson, et al., 2020); however, I felt this would complicate the design and it would have led to unequal groupings. Several of the putative TAIR ID annotations allocated to the top 25 or 35 most highly differentially expressed genes were shared between my study and the *P. sylvestris* study by Kovalchuk et al (2015). These were: a lipoxygenase (LOX5), which is involved in jasmonic acid biosynthesis, an alpha-dioxygenase (DOX1) involved in protection against oxidative stress and cell death, a serine-type endopeptidase inhibitor which is a defence related protease inhibitor this putitative annotation was allocated to 5 of their top 25 most differentially expressed transcripts, and a putative beta-1,3-endoglucanase. The large number of shared transcripts between these two short lists suggests a conserved stress response mechanism; no GO over-representation or pathway analysis was presented in the *P. sylvestris* study.

In the initial response (TP1), we observed many overrepresented terms to do with oxidative stress in the bark, potentially showing that there is a reactive oxygen species (ROS) burst. At TP2, three days after attack, many over-represented terms were related to secondary metabolism, specifically phenylpropanoid metabolism. Phenylpropanoids are classed as secondary metabolites but include compounds that are vital for plant survival as they are involved in both defence and structural support (Vogt, 2010). At TP2, along with flavonoids, phenylpropanoids could be part of an upregulation of induced defences. Some phenylpropanoid derived compounds such as lignin may also be involved in the repair of the herbivory damage. At TP3 there are fewer overrepresented terms showing that response becomes more uneven, although some of the same genes are still being differentially expressed and show a prolonged response.

Several studies in conifers show the transcriptional responses to various pests and pathogens. Similar to our results, with mechanical bark stripping and exogenous methyl jasmonate application , the number of DEGs decreased over time (Nantongo, Potts, Frickey, et al., 2022). Experiments using the pathogens *Phytophthora ramorum* (an oomycete) in *Larix decidua* and *Larix kaempferi* (Dun et al., 2022) and *Chrysomyxa rhododendri* (a fungus) in *Picea abies* (Trujillo-Moya et al., 2020) saw the number of DEGs increase over time, unlike ours. Interestingly, both pathogen studies reported similar GO Terms overrepresented as we found here, such as secondary metabolic process or response to jasmonic acid, but not for oxidative stress or oxygen levels, which were overrepresented in the upregulated genes at TP1 in our study. However, pathogens function differently from insects, and they both saw symptoms develop over time after infection, unlike in our study, where there was no further damage after the insect was removed Reactive oxygen species (ROS) bursts are triggered by both pathogens (Torres et al., 2006) and herbivory (Block et al., 2018; Bricchi et al., 2010). Although ROS bursts have mostly been studied in non-conifer plants, it also occurs in conifers (Bittner et al.,

2017; Durak et al., 2019). Our observation could suggest that *H. abietis* herbivory causes a ROS burst. Hence, our data show many related terms are overrepresented initially after exposure. ROS can both be directly toxic to insects (Bi & Felton, 1995) and act as regulators of defence responses; increased ROS concentration can cause salicylic acid accumulation, which induces defence responses (Mittler et al., 2011). The over-represented terms referencing oxidative stress could suggest that ROS play a role in activating defence pathways in *P. sitchensis.*

Changes in transcript abundance and in the proteome were detected in *P. sitchensis* in response to three different treatments: attack by stem-boring phloem feeders by *Pissodes strobi*, foliage eating by *Choristoneura occidentalis* and mechanical wounding of stem (Lippert et al., 2007; Ralph et al., 2006). Using a microarray representing 5500 unique genes, the authors observed a range of wound and biotic stress-induced genes, including genes involved in general metabolism, photosynthesis, transport, transcriptional regulation, response to stress, detoxification and redox processes, octadecanoid and ethylene signalling, secondary metabolism (Ralph et al., 2006). Many of these processes overlap with the ones overrepresented in our study, and the redox and detoxification functional group also suggests that these herbivores induce a ROS burst. In the proteomics study on the same materials, there was an increased abundance of a family of heat shock proteins (Lippert et al., 2007), which are thought to protect plants from ROS (ul Haq et al., 2019). Despite the increase in protein abundance, there was no variation in transcript level of these proteins, suggesting post-transcriptional control.

Our data indicated a low level of response to attack in the needles, suggesting a mostly localised response to *H. abietis* herbivory. Mechanical bark stripping to simulate browsing in *Pinus radiata* resulted in many DEGs in the bark but none in the needles, although there were changes in sugar concentrations in the needles, highlighting that changes in transcript abundance may give a partial picture of the defence response (Nantongo, Potts, Davies, et al., 2022; Nantongo, Potts, Frickey, et al., 2022).

We found that the of DEGs in the needles increased in number over time, potentially indicating the time required for the response to become systemic. The terms 'Auxin transport' and 'regulation of jasmonic acid mediated signalling pathway' were both significantly overrepresented among the DEGs, with all genes up-regulated at TP3, 27 days after the attack. The genes relating to the jasmonate-mediated signalling pathway were both annotated as Jasmonate Zim Domain proteins (JAZs), which are down-regulated by jasmonate and downregulate the transcription of jasmonate-responsive genes by facilitating repressive chromatin modifications (Z. Li et al., 2021). Given that jasmonate signalling is involved in defence responses: this finding is unexpected, as their

upregulation might attenuate the pathway. At TP2, one of the genes was downregulated, and the other was not differentially expressed, but at TP3, both were significantly upregulated. It could be that these genes are upregulated later to return the sapling to its baseline as part of a complex feedback system.

The DEGs involved in auxin transport were all auxin transporters, two influx transporters and a polar-orientated auxin efflux transporter that redirects auxin from the root tip. The mRNA of one of the transporters is known to be cell to cell mobile so could have been transcribed elsewhere. The upregulation of these three transcripts, suggests that at TP3, there is an increase in auxin flow into the needles. Auxin is a major plant hormone involved in both growth and defence and the defence growth trade off (Denancé et al., 2013) and it is mobile, so it could be involved in coordinating the systemic response. Auxin is also linked to plant defence in a variety of ways (Kazan & Manners, 2009) and it has been suggested that auxin and jasmonate signalling work synergistically while they act agnostically to salicylic acid signalling (Robert-Seilaniantz et al., 2007). These relationships may not be conserved in conifers where methyl jasmonate up-regulated genes are involved in both jasmonic acid and salicylic acid biosynthesis, suggesting the pathways are synergistic rather than antagonistic (Wilkinson et al., 2022). Auxin also peaks earlier than jasmonate in response to herbivory in *Nicotiana attenuata* (after only 5 minutes) and is required for some jasmonate dependent defences as shown in complementation studies (Machado et al., 2016). Auxin levels are also heightened in response to herbivory in maise and the response is detected systemically in the leaf tissue (Maag et al., 2016).

There is evidence of crosstalk between the jasmonate and auxin signalling-mediated pathways involving COI1 (CORONATINE INSENSITIVE1), which is required for the response to jasmonate and is part of a family of F-Box proteins, the remainder of which are auxin receptors. In response to a jasmonate derivative (jasmonyl isoleucine (JA-Ile)) COI1 enhances the degradation of JAZ transcription repressors which allows the activation of JA responsive genes. Another F-Box protein, the auxin receptors TIR1 works in a very similar way in the presence of auxin and both receptors, COI1 and TRI1, are required in *A. thaliana* for resistance to necrotrophic pathogens (Kazan & Manners, 2009). This crosstalk could be one explanation for the over-representation and the upregulation of auxin transporters and JAZs at TP3. Hormonal crosstalk is especially important in plants as it allows them to regulate defence-associated fitness costs (Denancé et al., 2013).

The upregulation of TPS14 was also notable, as terpenes are known to be involved in plant defence and plant-to-plant signalling. Different molecules may activate both salicylic acid and jasmonate

signalling pathways (Rosenkranz et al., 2021). Upregulation of TPS14 may suggest an increase in terpene production which could potentially prime defences at a community level.

Uncovering priming

Our findings highlight commonalities with other reports in response to pest attacks, suggesting conserved mechanisms between species. However, individual insect attacks studied in isolation are not very representative of the stresses faced by saplings in the field. In field settings, there are often multiple rounds of attack potentially from different species of pests and pathogens. Therefore, the next part of our study was designed to compare the localised and systemic responses over time between previously attacked and naïve saplings.

The concept of a previous attack inducing long-term defences isn't new. In 1993, it was shown that exposing *Picea abies* to a previous lower dose of a fungal infection and mechanical wounding increased resistance to the following inoculation with the same fungus (Christiansen et al., 1999). In contrast, an attack from *H. abietis* on *Pinus sylvestris* did not significantly impact the damage level in a subsequent attack (Y. Chen et al., 2021). Varied responses to previous mechanical wounding were observed depending on the method used (Y. Chen et al., 2021); however, exogenous spraying with methyl jasmonate did significantly increase to *H. abietis* resistance in a range of species (Huynh et al., 2024). These reports and the widespread observation of jasmonate responses to insect attack suggested there may be differences in the molecular response to an attack between previously attacked or naïve saplings. Similarly to Chen et al (2021), we observed no difference in damage area between W2 saplings that had been previously attacked and W1, which were naive before exposure. I theorise that the bark damage phenotyping may not have allowed for the observation of changes due to the high levels of variation in feeding damage between insects.

On the other hand, we observed small but biologically insightful transcriptomic changes between W2 and W1, with 20 DEGs in the bark and 27 in the needles. In other conifers, spraying with methyl jasmonate to establish long-term resistance induced large-scale transcriptome changes (Wilkinson et al., 2022) which were much larger transcriptional than those induced by bark stripping (Nantongo, Potts, Frickey, et al., 2022). Those studies examined RNA levels after treatment but not after subsequent triggering challenges, unlike in our study, where W2 samples were taken after the challenge. So, their transcriptome changes were more akin to those in our W1 saplings, which had only been attacked once.

Our study was designed with similar gaps between the end of the first exposure and the start of the second exposure (Day 3-Day 28) and TP1 and TP3 (Day 31-Day 58). Our experimental design allowed for comparisons between naïve saplings and saplings a month after attack. Given that at TP3, there

were 136 DEGs between W1 and Cont, it is reasonable to assume that there were similar transcriptomic differences between the W2 and W1 saplings on day 28. However, after the attack at TP1 (Day 31) there were only 5 DEGs between W2 and W1, I suggest that the effect of the second attack overrides the remaining signal from the first attack, causing the sapling response to predominantly mimic the single attack. This differs from results in a study that showed that *Picea abies* bark which had experienced previous MeJA treatment had a considerably different transcriptional response to mechanical wounding than naïve bark (~600 DEGs) (Mageroy, Wilkinson, et al., 2020) suggesting that MeJA primes more transcriptional responses than herbivory. However, a more rapid increase in terpenes was detected in response to wounding in the MeJA-treated plants, but there was no difference in the expression levels of terpene biosynthesis genes (Mageroy, Wilkinson, et al., 2020). So, despite the small number of DEGs we detected, there still could be large changes in the physiology of the saplings.

When comparing W2 and W1 our study identified two DEGs in bark which were annotated as lipoxygenase 1. One transcript was up-regulated at TP1 and one was down-regulated at both TP1 and TP2. The differences in expression suggest these orthologues may be under different regulation. These LOX transcripts are noteworthy because lipoxygenases are involved in jasmonic acid synthesis, and MeJA primes defences and increases resistance to a range of pests in conifers; therefore, the gene expression results suggest that previous attack may have priming effect. Similarly, in *P. sylvestris* a lipoxygenase was one of the most upregulated genes after exogenous spraying with methyl jasmonate to prime defences (Nantongo, Potts, Frickey, et al., 2022). In addition, silencing of a lipoxygenase gene in rice increased susceptibility to a chewing herbivore (Zhou et al., 2009). So, the differential expression of these two genes may demonstrate a priming of defences.

A transcript annotated as AZELAIC ACID INDUCED 1 was also down-regulated at TP1. AZI1 is involved in the regulation of systemic acquired resistance signal production as well as the perception of monoterpenes which have been shown to prime neighbouring plants (Riedlmeier et al., 2017). JAZ12 was upregulated at TP2, suggesting a down-regulation in genes related to jasmonate signalling. In the needles, a SAGA complex subunit, which regulates gene expression by affecting histone H3 acetylation, was downregulated at TP1. The subunit is part of a complex needed to acetylate the N terminal tails of histones, which increases transcriptional activation (Wu et al., 2021) . Therefore the down-regulation of this subunit could lead to gene silencing; the regulation of histone acetylation has been implicated in priming defence as well as 'memory' to heat and cold events (Nishio et al., 2024). The regulation of these genes all suggests a reduction in expression of genes involved in priming and an increase in gene silencing. Potentially, because the W2 plants have already been

primed by the first attack; therefore, with the second attack, genes involved in priming are down-regulated.

In our study 4% of transcripts were not assigned a TAIR IDs based on sequence similarity and remain unclassified, including three transcripts differentially expressed between W2 and W1 (PSIR_DN26193_c0_g1, PSIR_DN2628_c0_g1, PSIR_DN34144_c0_g1). These genes are potentially of interest as they could be specific to conifers or adapted to this particular response.

Future directions

There are several genes of interest that could be further investigated including the family of lipoxygenase genes. Two LOX1 homologs are differentially expressed in the bark between W2 and W1. A putative LOX5 gene was the second most highly differentially expressed gene between W1 and Cont, where its log2Fold change was 49 at TP3. In addition, there appears to be far more LOX genes in the *P. sitchensis* transcriptome (49 in total, 22 most homologous to LOX1) compared to *A. thaliana*, which only has six. Given their distinctive expression pattern and high copy number LOX genes could play a key regulatory role in conifer defence.

To take this study further, bark samples from more places up the stem may reveal the insights into the systemic response across the whole sapling. A longer time course would also be of interest to see if the increase in DEGs in the needles continues or is sustained.

One important step that needs to be addressed is the high level of between-sample variation in the damage assessment based on area of damage in the *H. abietis* herbivory assay. It would be highly beneficial if resistance biomarkers could be found and used in further work, such as the terpenoid metabolites dehydroabietic acid, (+)-3-carene, and terpinolene which are all associated with resistance to *P. strobi* in *P. sitchensis* and can therefore be used for screening resistance (Robert et al., 2010). It is also interesting to use transcriptomics to contrast responses to attack in resistant and susceptible saplings (see chapter 3) which may allow for the underlying pathways of resistance to be elucidated as was done with Pine Wood Nematode Resistance in *Pinus massoniana* (Liu et al., 2017). Many studies over the past 10 years compared the transcriptomic response of conifers to various pests and pathogens, or methyl jasmonate. In the future, more sophisticated experiments may be needed to further unravel the mechanisms for resistance, potentially through si or miRNA or looking at epigenetic changes. Some work has been done in this area in both A. thaliana (Wilkinson et al., 2023) and conifers (Wilkinson et al., 2021). There are still many questions, including how conserved these mechanisms are and how long-lasting they are.

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Appendices

Appendix 1

22 transcripts that are differentially expressed at TP 1 TP2 and TP3 between W1 and Cont. Log2 fold change (L2FC) significantly larger than 1 , p adjusted value of 0.05 using Benjamini-Hochberg correction. Gene model description from The Arabidopsis Information Resource (TAIR)'s bulk download tool (Berardini et al., 2015)

Gene	TAIR ID	Gene Model Description
PSIR_DN9142_c0_g1	AT1G03230.1	Eukaryotic aspartyl protease family protein
PSIR_DN21190_c0_g1	AT1G19900.1	RUBY encodes a secreted galactose oxidase involved in cell wall modification.
PSIR_DN14519_c0_g1	AT1G51990.2	O-methyltransferase family protein
PSIR_DN26941_c0_g1	AT1G55360.1	tRNA-splicing ligase (DUF239)
PSIR_DN38329_c0_g2	AT1G59700.1	Encodes glutathione transferase belonging to the tau class of GSTs. Naming convention according to Wagner et al. (2002). The mRNA is cell-to-cell mobile.
PSIR_DN41376_c1_g4	AT1G61720.1	Negative regulator of flavonoid biosynthesis, mutants accumulate flavonoid pigments in their seed coat, putative oxidoreductase. It is thought that a ternary complex composed of TT2, TT8 and TTG1 is necessary for correct expression of BAN in seed endothelium.
PSIR_DN24103_c0_g1	AT1G72060.1	Serine-type endopeptidase inhibitor
PSIR_DN33949_c0_g1	AT1G75470.1	Member of a family of proteins related to PUP1, a purine transporter. May be involved in the transport of purine and purine derivatives such as cytokinins, across the plasma membrane.
PSIR_DN14950_c0_g1	AT2G02103.1	Ta11-like non-LTR retrotransposon
PSIR_DN38023_c0_g1	AT2G41480.1	Encodes a cationic cell-wall-bound peroxidase homolog that is involved in the lignification of cell walls. Regulated by COG1, involved in seed longevity.
PSIR_DN43555_c0_g3	AT2G44480.5	beta glucosidase 17
PSIR_DN41147_c1_g1	AT4G11650.1	Osmotin-like protein; functions as a positive regulator in the generation of ABA responses and is under post-translational control.
PSIR_DN35001_c0_g2	AT4G19810.2	ChiC encodes a Class V chitinase that is a part of glycoside hydrolase family 18 based on CAZy groupings. It appears to primarily act as an exochitinase in vitro where it predominantly cleaves a chitobiose (GlcNAc)2 residue from the non-reducing end of a chitin oligosaccharide. However, it shows some minor endochitinase activity in vitro, as well. A putative 24 amino-acid signal peptide may direct this protein to the secretory system and it has been detected in cell wall apoplastic fluid. RT-PCR experiments demonstrate that ChiC transcript levels are increased in response to abscisisc acid, jasmonic acid, and NaCl stress. Microarray results also suggest that transcript levels rise in response to osmotic stress, two fungal pathogens, a bacterial pathogen, and the elicitor flagellin.The mRNA is cell-to-cell mobile.

PSIR_DN37842_c0_g2	AT4G29360.1	O-Glycosyl hydrolases family 17 protein
PSIR_DN35746_c0_g1	AT5G05340.1	Encodes a protein with sequence similarity to peroxidases that is involved in lignin biosynthesis. Loss of function mutations show abnormal development of xylem fibers and reduced levels of lignin biosynthetic enxymes.
PSIR_DN41804_c0_g1	AT5G07990.1	Required for flavonoid 3' hydroxylase activity. Enzyme abundance relative to CHS determines Quercetin/Kaempferol metabolite ratio. The mRNA is cell-to-cell mobile.
PSIR_DN34179_c0_g2	AT5G13330.1	Encodes a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 7 members in this subfamily.
PSIR_DN49386_c0_g1	AT5G15540.2	Encodes Adherin SCC2. Essential for viability. Required for normal seed development. Plays a role in the establishment of sister-chromatid cohesion and chromosome organization during meiosis.
PSIR_DN34261_c0_g1	AT5G48540.1	Receptor-like protein kinase-related family protein
PSIR_DN39896_c1_g1	AT5G51950.2	Glucose-methanol-choline (GMC) oxidoreductase family protein
PSIR_DN38341_c1_g1	AT5G54160.1	A caffeic acid/5-hydroxyferulic acid O-methyltransferase. Interacts with 14-4-3 proteins in yeast 2 hybrid assay. AtOMT1 (At5g54160) encodes a flavonol 3?-O-methyltransferase that is highly active towards quercetin and myricetin. The substrate specificity identifies the enzyme as flavonol 3?-methyltransferase which replaces the former annotation of the gene to encode a caffeic acid/5-hydroxyferulic acid O-methyltransferase The mRNA is cell-to-cell mobile.
PSIR_DN35154_c0_g3	AT5G64260.1	EXORDIUM like 2

Appendix 2

The transcripts that are upregulated in W1 compare to Cont at timepoint one, which have the gene ontology term “response to jasmonic acid”. Log2 fold change (L2FC) significantly larger than 1 , p adjusted value of 0.05 using Benjamini-Hochberg correction. Gene model description from The Arabidopsis Information Resource (TAIR)’s bulk download tool (Berardini et al., 2015)

Gene	L2FC	TAIR ID	Gene Model Description
PSIR_DN41160_c4_g1	4	AT1G07900.1	LOB domain-containing protein 1
PSIR_DN41335_c1_g1	7		
PSIR_DN35341_c0_g1	5	AT1G11050.1	Protein kinase superfamily protein
PSIR_DN38266_c1_g1	4		
PSIR_DN38266_c1_g3	5		
PSIR_DN39204_c0_g2	5	AT1G13360.3	Hypothetical protein
PSIR_DN43699_c0_g1	3	AT1G15520.1	ABC transporter family involved in ABA transport and resistance to lead.
PSIR_DN41201_c0_g1	4	AT1G17420.1	LOX3 encodes a Lipoxygenase. Lipoxygenases (LOXs) catalyze the oxygenation of fatty acids (FAs).
PSIR_DN33102_c0_g1	6	AT1G19210.1	Encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family.
PSIR_DN28748_c0_g3	10	AT1G48500.3	Jasmonate zim domain transcription factor family protein. Involved in freezing tolerance and JA induceed leaf senesence.
PSIR_DN75379_c0_g1	8	AT1G66160.1	CYS, MET, PRO, and GLY protein 1
PSIR_DN60410_c0_g1	8	AT1G73325.1	Kunitz family trypsin and protease inhibitor protein
PSIR_DN40112_c0_g1	7	AT1G74950.1	Key regulator in alternative splicing in the jasmonate signaling pathway, alone and in collaboration with other regulators.
PSIR_DN40216_c0_g1	11		
PSIR_DN24335_c0_g2	21	AT2G34810.1	FAD-binding Berberine family protein
PSIR_DN31328_c0_g1	22		
PSIR_DN41130_c1_g1	4	AT2G41380.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
PSIR_DN41130_c1_g2	4		
PSIR_DN40609_c0_g4	3	AT2G43320.2	
PSIR_DN24654_c0_g1	9	AT3G11180.1	One of 4 paralogs encoding a 2-oxoglutarate/Fe(II)-dependent oxygenases that hydroxylates JA to 12-OH-JA.
PSIR_DN43339_c0_g1	5		
PSIR_DN41552_c2_g2	5	AT3G15210.1	Encodes a member of the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family (ATERF-4). The protein contains one AP2 domain. Acts as a negative regulator of JA-responsive defense gene expression and resistance to the necrotrophic fungal pathogen Fusarium oxysporum and antagonizes JA inhibition of root elongation. The mRNA is cell-to-cell mobile.
PSIR_DN27847_c0_g1	6	AT3G17860.2	JAZs are direct targets of the SCFCOI1 E3 ubiquitin-ligase and JA treatment induces their proteasome-mediated degradation. Furthermore, JAZ3 negatively regulates the key transcriptional activator of JA responses, AtMYC2.

PSIR_DN40606_c1_g2	8	AT3G21680.1	hypothetical protein
PSIR_DN35745_c0_g2	4	AT3G55950.1	CRINKLY4 related 3
PSIR_DN27401_c0_g1	7	AT3G57450.1	hypothetical protein
PSIR_DN27401_c0_g2	6		
PSIR_DN38290_c0_g1	4		
PSIR_DN38290_c0_g2	6		
PSIR_DN39747_c0_g3	6	AT3G59340.1	solute carrier family 35 protein (DUF914)
PSIR_DN35737_c0_g2	9	AT4G16740.1	TPS3 Encodes an (E,E)-alpha-farnesene. This enzyme can also catalyze the formation of (E)-beta-ocimene as well as trace amounts of myrcene and other related compounds in vitro. Transcript levels for this gene increase in response to treatment with the jasmonic acid mimic coronalon or in response to the insect *Plutella xylostella,*
PSIR_DN40211_c1_g4	5	AT4G16740.2	
PSIR_DN35001_c0_g2	8	AT4G19810.2	ChiC encodes a Class V chitinase that is a part of glycoside hydrolase family 18. It appears to primarily act as an exochitinase. ChiC transcript levels are increased in response to abscisisc acid, jasmonic acid, NaCl stress, osmotic stress, two fungal pathogens, a bacterial pathogen, and the elicitor flagellin.The mRNA is cell-to-cell mobile.
PSIR_DN39624_c0_g2	6		
PSIR_DN42801_c0_g1	5	AT4G22530.2	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
PSIR_DN42801_c0_g5	5		
PSIR_DN36833_c1_g3	4	AT5G02230.2	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein
PSIR_DN28748_c0_g1	7	AT5G13220.1	Plants overexpressing At5g13220.3, but not At5g13220.1 showed enhanced insensitivity to MeJa.
PSIR_DN34179_c0_g2	7	AT5G13330.1	Encodes a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family.
PSIR_DN59527_c0_g1	9	AT5G20900.1	Jasmonate-zim-domain protein 12
PSIR_DN29742_c0_g1	7	AT5G21960.1	Encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family.
PSIR_DN42220_c0_g3	4	AT5G24080.3	Protein kinase superfamily protein
PSIR_DN34255_c0_g1	5	AT5G38700.1	Cotton fiber protein
PSIR_DN32386_c0_g1	9	AT5G42650.1	Encodes a member of the cytochrome p450 CYP74 gene family that functions as an allene oxide synthase. This enzyme catalyzes dehydration of the hydroperoxide to an unstable allene oxide in the JA biosynthetic pathway
PSIR_DN35154_c0_g3	6	AT5G64260.1	EXORDIUM like 2

Appendix 3

The transcripts that are upregulated in W1 compare to Cont at timepoint one, which have the gene ontology term "secondary metabolic process". Log2 fold change (L2FC) significantly larger than 1 , p adjusted value of 0.05 using Benjamini-Hochberg correction. Gene model description from The Arabidopsis Information Resource (TAIR)'s bulk download tool (Berardini et al., 2015)

Gene	L2FC	TAIR ID	Gene Model Description	Metabolite
PSIR_DN43206_c1_g2	10	AT1G10360.1	Encodes glutathione transferase belonging to the tau class of GSTs. Naming convention according to Wagner et al. (2002).	Glutathione
PSIR_DN35341_c0_g1	5	AT1G11050.1	Protein kinase superfamily protein	
PSIR_DN38266_c1_g1	4			
PSIR_DN38266_c1_g3	5			
PSIR_DN42622_c1_g9	18	AT1G22640.1	MYB-type transcription factor (MYB3) that represses phenylpropanoid biosynthesis gene expression	Phenylpropanoid
PSIR_DN38329_c0_g2	9	AT1G59700.1	Encodes glutathione transferase belonging to the tau class of GSTs. The mRNA is cell-to-cell mobile.	Glutathione
PSIR_DN39049_c0_g1	7	AT1G64160.1	Encodes a dirigent protein involved in the synthesis of (-)pinoresinol. Dirigent proteins impart stereoselectivity on the phenoxy radical coupling reaction yielding optically active lignans from two molecules of coniferyl alcohol.	(-)pinoresinol
PSIR_DN43104_c0_g2	4	AT1G65690.1	Encodes NHL6 (NDR1/HIN1-like 6). Plays an important role in the abiotic stresses-induced ABA signaling and biosynthesis, particularly during seed germination and early seedling development.	Abscisic acid
PSIR_DN34146_c0_g2	11	AT1G69930.1	Encodes glutathione transferase belonging to the tau class of GSTs.	Glutathione
PSIR_DN60410_c0_g1	8	AT1G73325.1	Kunitz family trypsin and protease inhibitor protein	
PSIR_DN37831_c0_g1	7	AT1G80820.1	Encodes a cinnamoyl CoA reductase isoform. Involved in lignin biosynthesis.	Lignin
PSIR_DN32042_c0_g1	3	AT2G19130.1	S-locus lectin protein kinase family protein	
PSIR_DN40786_c0_g2	3			
PSIR_DN43130_c1_g2	8	AT2G29480.1	Encodes glutathione transferase belonging to the tau class of GSTs.	Glutathione

PSIR_DN42144_c0_g2	8	AT2G30860.1	Encodes glutathione transferase belonging to the phi class of GSTs.	Glutathione
PSIR_DN32195_c0_g1	3	AT2G32030.1	Acyl-CoA N-acyltransferases (NAT) superfamily protein	
PSIR_DN39375_c0_g1	5	AT2G38060.1	Encodes an inorganic phosphate transporter (PHT4;2).	
PSIR_DN41130_c1_g1	4	AT2G41380.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	
PSIR_DN41130_c1_g2	4			
PSIR_DN38023_c0_g1	22	AT2G41480.1	Encodes a cationic cell-wall-bound peroxidase homolog that is involved in the lignification of cell walls.	Lignin
PSIR_DN43555_c0_g3	21	AT2G44480.5	beta glucosidase 17	Glucosinolate
PSIR_DN39371_c0_g1	7	AT3G22240.1	cysteine-rich/transmembrane domain PCC1-like protein	
PSIR_DN35745_c0_g2	4	AT3G55950.1	CRINKLY4 related 3	
PSIR_DN43006_c0_g1	4	AT4G01070.1	The glycosyltransferase is involved in metabolizing xenobiotica .The glucosyltransferase preferred UDP-xylose over UDP-glucose indicating its (additional) functioning as a xylosyltransferase in planta	Lignin
PSIR_DN39099_c0_g1	11	AT4G39230.1	Similar to phenylcoumaran benzylic ether reductase (PCBER), which catalyzes NADPH-dependent reduction of 8-5' linked lignans such as dehydrodiconiferyl alcohol to give isodihydrodehydrodiconiferyl alcohol.	Lignin
PSIR_DN39385_c0_g1	6	AT4G39330.1	Member of a family of cinnamyl alcohol dehydrogenases (CAD) that normally catalyze the last steps of monolignol biosynthesis Expressed predominantly in lignified tissues.	Lignin
PSIR_DN406_c0_g1	10	AT5G02090.1	Hypothetical protein	
PSIR_DN36833_c1_g3	4	AT5G02230.2	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein	Haloacid
PSIR_DN35746_c0_g1	8	AT5G05340.1	Encodes a protein with sequence similarity to peroxidases that is involved in lignin biosynthesis. Loss of function mutations show abnormal development of xylem fibers and reduced levels of lignin biosynthetic enxymes.	Lignin
PSIR_DN36448_c0_g7	9			
PSIR_DN41174_c0_g6	7			
PSIR_DN42977_c0_g5	6			
PSIR_DN42977_c0_g6	8			

PSIR_DN34179_c0_g2	7	AT5G13330.1	Encodes a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family. The protein contains one AP2 domain.	
PSIR_DN42220_c0_g3	4	AT5G24080.3	Protein kinase superfamily protein	
PSIR_DN38341_c1_g1	23	AT5G54160.1	Encodes a flavonol 3-O-methyltransferase that is highly active towards quercetin and myricetin. The mRNA is cell-to-cell mobile.	Flavonol Lignin
PSIR_DN35154_c0_g3	6	AT5G64260.1	EXORDIUM like 2	

Appendix 4

35 genes most highly differentially expressed between W1 and Cont. Log2 fold change (L2FC) significantly larger than 1, p adjusted value of 0.05 using Benjamini-Hochberg correction. Gene model description from The Arabidopsis Information Resource (TAIR)'s bulk download tool (Berardini et al., 2015)

Gene	L2FC	TP	TAIR ID	Gene Model Description
PSIR_DN31328_c0_g1	49	3	AT2G34810.1	FAD-binding Berberine family protein
PSIR_DN34586_c0_g1	49	3	AT3G22400.1	Encodes lipoxygenase5 (LOX5). LOX5 activity in roots facilitates green peach aphid colonization of Arabidopsis foliage by promoting green peach aphid feeding from sieve element and water consumption from xylem.
PSIR_DN12670_c0_g1	49	3	AT5G41310.1	P-loop nucleoside triphosphate hydrolases superfamily protein with CH (Calponin Homology) domain-containing protein
PSIR_DN31569_c0_g1	47	3	AT3G01420.1	Encodes an alpha-dioxygenase involved in protection against oxidative stress and cell death. Induced in response to Salicylic acid and oxidative stress. Independent of NPR1 in induction by salicylic acid. The mRNA is cell-to-cell mobile.
PSIR_DN18386_c0_g1	47	3	AT4G15093.1	catalytic LigB subunit of aromatic ring-opening dioxygenase family
PSIR_DN41147_c1_g5	46	3	AT4G11650.1	Osmotin-like protein; functions as a positive regulator in the generation of ABA responses and is under post-translational control.
PSIR_DN24335_c0_g2	45	3	AT2G34810.1	FAD-binding Berberine family protein
PSIR_DN40490_c0_g1	44	3	AT1G28140.1	Located in chloroplast and involved in change in response to H2O2.
PSIR_DN26667_c0_g2	44	3	AT1G03220.1	Eukaryotic aspartyl protease family protein
PSIR_DN43586_c1_g3	43	2	AT1G15000.1	serine carboxypeptidase-like 50
PSIR_DN42622_c1_g9	42	2	AT1G22640.1	MYB-type transcription factor (MYB3) that represses phenylpropanoid biosynthesis gene expression
PSIR_DN34261_c0_g1	42 39	3 2	AT5G48540.1	receptor-like protein kinase-related family protein
PSIR_DN26941_c0_g1	42 40	3 2	AT1G55360.1	tRNA-splicing ligase (DUF239)
PSIR_DN43555_c0_g3	41 39	3 2	AT2G44480.5	beta glucosidase 17
PSIR_DN38023_c0_g1	40 40	2 3	AT2G41480.1	Encodes a cationic cell-wall-bound peroxidase homolog that is involved in the lignification of cell walls. Regulated by COG1, involved in seed longevity.
PSIR_DN9142_c0_g1	40 38	3 2	AT1G03230.1	Eukaryotic aspartyl protease family protein
PSIR_DN43684_c0_g3	39	3	AT2G43580.1	Chitinase family protein
PSIR_DN41376_c1_g4	36 36	2 3	AT1G61720.1	Negative regulator of flavonoid biosynthesis, mutants accumulate flavonoid pigments in their

				seed coat, putative oxidoreductase. It is thought that a ternary complex composed of TT2, TT8 and TTG1 is necessary for correct expression of BAN in seed endothelium.
PSIR_DN14519_c0_g1	35 34	3 2	AT1G51990.2	O-methyltransferase family protein
PSIR_DN38341_c1_g1	35 34	3 2	AT5G54160.1	Encodes a flavonol 3?-O-methyltransferase that is highly active towards quercetin and myricetin. The mRNA is cell-to-cell mobile.
PSIR_DN21190_c0_g1	34 34	3 2	AT1G19900.1	RUBY encodes a secreted galactose oxidase involved in cell wall modification.
PSIR_DN43329_c0_g1	28	3	AT1G77520.1	O-methyltransferase family protein
PSIR_DN39476_c0_g1	27 27	3 2	AT1G72060.1	serine-type endopeptidase inhibitor
PSIR_DN39022_c0_g1	27	3	AT4G16260.1	Encodes a putative beta-1,3-endoglucanase that interacts with the 30C02 cyst nematode effector. May play a role in host defense.
PSIR_DN31725_c0_g1	27	3	AT5G44380.1	FAD-binding Berberine family protein

Appendix 5

DEGs between W2 and W1 in needles at each timepoint, p adjusted value of 0.05 using Benjamini-Hochberg correction. L2FC is log 2 fold change. Gene model description from The Arabidopsis Information Resource (TAIR)'s bulk download tool (Berardini et al., 2015)

Gene	L2FC	TP	TAIR ID	Gene Model Description
PSIR_DN19093_c0_g1	-6	1	AT2G19390.2	SAGA complex subunit. Regulates gene expression by affecting histone H3 acetylation.
PSIR_DN35114_c1_g3	-7	1	AT2G03880.1	Pentatricopeptide repeat (PPR) superfamily protein
PSIR_DN32907_c0_g1	-20	1	AT2G44910.1	Encodes a homeodomain protein whose expression displays a dependence on phyB for both red and far-red light response. Also involved in the shade avoidance syndrome.
PSIR_DN28419_c0_g1	-23	1	AT2G39130.2	Transmembrane amino acid transporter family protein
PSIR_DN19708_c0_g1	-31	1	AT5G07280.1	Encodes EMS1 (EXCESS MICROSPOROCYTES1), a putative leucine-rich repeat receptor protein kinase that controls somatic and reproductive cell fates in Arabidopsis anther.
PSIR_DN43947_c1_g5	23	2	AT4G10490.1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
PSIR_DN27126_c0_g1	23	2	AT1G69830.1	Encodes a plastid-localized α-amylase. Loss of function mutations show normal diurnal pattern of starch accumulation/degradation. Expression follows circadian rhythms.
PSIR_DN10341_c0_g1	22	2	AT2G31660.1	SAD2 (super sensitive to ABA and drought 2) encodes an importin beta-domain family protein likely to be involved in nuclear transport in ABA signalling. Mutation of SAD2 in Arabidopsis alters abscisic acid sensitivity. SAD2 expression was not induced by ABA or stress. Loss of function mutations in SAD2 exhibit increased tolerance for UV stress, increased production of UV protective secondary metabolites and suppression of nuclear localization of MYB4 (a repressor of UV stress response genes). Regulates microRNA activity. Defective trichome activity.
PSIR_DN59271_c0_g1	22	2	AT3G04560.1	nucleolar/coiled-body phosphoprotein
PSIR_DN38191_c0_g1	22	2	AT1G05260.1	Encodes a cold-inducible cationic peroxidase that is involved in the stress response. The mRNA is cell-to-cell mobile.
PSIR_DN9245_c0_g1	22	2	AT5G20900.1	jasmonate-zim-domain protein 12
PSIR_DN26088_c0_g1	16	2	AT5G53190.1	Nodulin MtN3 family protein
PSIR_DN42510_c0_g2	-5	2	AT5G36930.2	Disease resistance protein (TIR-NBS-LRR class) family

PSIR_DN59455_c0_g1	-6	2	AT5G04050.1	Essential maturase splicing factor required for splicing of nad1 introns 1, 3 and 4, holo‐complex I biogenesis, and embryo development.
PSIR_DN41459_c2_g2	-17	2	AT4G36220.1	encodes ferulate 5-hydroxylase (F5H). Involved in lignin biosynthesis.
PSIR_DN39958_c0_g2	-19	2	AT3G48320.1	putative cytochrome P450
PSIR_DN43160_c1_g1	-22	2	AT1G78370.1	Encodes glutathione transferase belonging to the tau class of GSTs. Naming convention according to Wagner et al. (2002).
PSIR_DN22718_c0_g1	-22	2	AT3G52850.1	Encodes the Vacuolar Sorting Receptor-1 (VSR-1)/Epidermal Growth Factor Receptor-like protein1(VSR-1/ATELP1). Binds vacuolar targeting signals. Involved in sorting seed storage proteins into vacuoles. The mRNA is cell-to-cell mobile.
PSIR_DN43964_c1_g3	-22	2	AT5G13700.1	Encodes a protein with polyamine oxidase activity.
PSIR_DN43964_c0_g1	-22	2	AT4G38600.2	encodes a member of HECT ubiquitin protein ligase family that is involved in trichome cell morphogenesis. Mutants in this gene exhibit supernumerary trichome branches and increased DNA content.
PSIR_DN29234_c0_g1	37	3	AT5G24860.1	encodes a small protein that regulates flowering and is involved in gibberellin signalling pathway. It is expressed in apical meristems immediately after the photoperiodic induction of flowering. Genetic interactions with flowering time and floral organ identity genes suggest that this gene may be involved in modulating the competence to flower.
PSIR_DN34144_c0_g1	32	3	NA	
PSIR_DN41459_c2_g2	17	3	AT4G36220.1	encodes ferulate 5-hydroxylase (F5H). Involved in lignin biosynthesis.
PSIR_DN28689_c0_g1	17	3	AT5G54930.2	AT hook motif-containing protein
PSIR_DN43293_c0_g2	15	3	AT2G46750.1	Encodes a homolog of rat L-gulono-1,4-lactone (L-GulL) oxidase that is involved in the biosynthesis of L-ascorbic acid.
PSIR_DN17678_c0_g1	-16	3	AT1G30950.1	Required for the proper identity of the floral meristem. Involved in establishing the whorled pattern of floral organs, in the control of specification of the floral meristem, and in the activation of APETALA3 and PISTILLATA. UFO is found at the AP3 promoter in a LFY-dependent manner, suggesting that it works with LFY to regulate AP3 expression. UFO may also promote the ubiquitylation of LFY.
PSIR_DN40022_c1_g6	-16	3	AT1G02870.1	nucleolar-like protein

PSIR_DN3980_c0_g1	-17	3	AT2G26490.1	JGB contains seven WD40 repeats and is highly conserved in flowering plants. Overexpression inhibits pollen germination. suggesting JGB is a negative regulator of pollen germination
PSIR_DN32907_c0_g1	-17	3	AT2G44910.1	Encodes a homeodomain protein whose expression displays a dependence on phyB for both red and far-red light response. Also involved in the shade avoidance syndrome.

Appendix 6

The transcripts that are upregulated in W1 compared to Cont at all time points in the Needles. Log2 fold change (L2FC) significantly larger than 1 , p adjusted value of 0.05 using Benjamini-Hochberg correction. Gene model description from The Arabidopsis Information Resource (TAIR)'s bulk download tool (Berardini et al., 2015)

Gene	L2FC	TP	TAIR ID	Gene Model Description
PSIR_DN43868_c2_g2	18	1	AT5G05390.1	putative laccase, a member of laccase family of genes (17 members in Arabidopsis); involved in copper-iron crosstalk, root-to-shoot iron partitioning.
PSIR_DN34144_c0_g1	28	2	NA	
PSIR_DN36395_c1_g2	24	2	AT2G03350.1	DUF538 family protein (Protein of unknown function, DUF538)
PSIR_DN43964_c1_g3	23	2	AT5G13700.1	Encodes a protein with polyamine oxidase activity. The mRNA of this gene is only expressed in very low amounts in the organs where it was detected (light-grown plants).
PSIR_DN43964_c0_g1	23	2	AT4G38600.2	encodes a member of HECT ubiquitin protein ligase family that is involved in trichome cell morphogenesis. Mutants in this gene exhibit supernumerary trichome branches and increased DNA content.
PSIR_DN22718_c0_g1	22	2	AT3G52850.1	Encodes the Vacuolar Sorting Receptor-1 (VSR-1)/Epidermal Growth Factor Receptor-like protein1(VSR-1/ATELP1). Binds vacuolar targeting signals. Involved in sorting seed storage proteins into vacuoles. The mRNA is cell-to-cell mobile.
PSIR_DN43160_c1_g1	22	2	AT1G78370.1	Encodes glutathione transferase belonging to the tau class of GSTs. Naming convention according to Wagner et al. (2002).
PSIR_DN19611_c0_g1	20	2	AT2G38120.1	Encodes an auxin influx transporter. AUX1 resides at the apical plasma membrane of protophloem cells and at highly dynamic subpopulations of Golgi apparatus and endosomes in all cell types. AUX1 action in the lateral root cap and/or epidermal cells influences lateral root initiation and positioning. Shoot supplied ammonium targets AUX1 and inhibits lateral root emergence. The mRNA is cell-to-cell mobile.
PSIR_DN18313_c0_g1	20	2	AT4G02220.1	zinc finger (MYND type) family protein / programmed cell death 2 C-terminal domain-containing protein
PSIR_DN43782_c1_g3	20	2	AT5G27060.2	receptor like protein 53
PSIR_DN36978_c1_g4	19	2	AT5G52070.3	Agenet domain-containing protein

PSIR_DN24467_c0_g1	17	2	AT1G43160.1	encodes a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family (RAP2.6). The protein contains one AP2 domain. There are 7 members in this subfamily.
PSIR_DN406_c0_g1	17	2	AT5G02090.1	hypothetical protein
PSIR_DN13244_c0_g1	17	2	AT5G09970.1	Member of CYP78A family. Paralog of CYP78A5 and appears to function in a shoot meristem maintainence pathway with LAMP1 that parallels AMP1/CYP87A5.
PSIR_DN26088_c0_g1	-16	2	AT5G53190.1	Nodulin MtN3 family protein
PSIR_DN9245_c0_g1	-17	2	AT5G20900.1	jasmonate-zim-domain protein 12
PSIR_DN27126_c0_g1	-17	2	AT1G69830.1	Encodes a plastid-localized α-amylase. Expression is reduced in the SEX4 mutant. Loss of function mutations show normal diurnal pattern of starch accumulation/degradation. Expression follows circadian rhythms.
PSIR_DN43947_c1_g5	-17	2	AT4G10490.1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
PSIR_DN59271_c0_g1	-20	2	AT3G04560.1	nucleolar/coiled-body phosphoprotein
PSIR_DN3876_c0_g1	22	3	AT1G77690.1	Encodes an auxin influx carrier LAX3 (Like Aux1) that promotes lateral root emergence. Auxin-induced expression of LAX3 in turn induces a selection of cell-wall-remodelling enzymes, which are likely to promote cell separation in advance of developing lateral root primordia.
PSIR_DN19611_c0_g1	22	3	AT2G38120.1	Encodes an auxin influx transporter. AUX1 resides at the apical plasma membrane of protophloem cells and at highly dynamic subpopulations of Golgi apparatus and endosomes in all cell types. AUX1 action in the lateral root cap and/or epidermal cells influences lateral root initiation and positioning. Shoot supplied ammonium targets AUX1 and inhibits lateral root emergence. The mRNA is cell-to-cell mobile.
PSIR_DN51689_c0_g1	20	3	AT2G30800.1	Has RNA or DNA helicase activity and expressed specifically in tapetum and vascular tissue. First identified member of a new group of the mle helicase group of the DEAH family.
PSIR_DN38091_c0_g3	20	3	AT3G28415.1	ABC transporter family protein
PSIR_DN13915_c0_g1	19	3	AT1G30040.2	Encodes a gibberellin 2-oxidase that acts on C-19 gibberellins. AtGA2OX2 expression is responsive to cytokinin and KNOX activities.
PSIR_DN43017_c0_g2	19	3	AT2G35630.1	Member of the MAP215 family of microtubule-associated proteins required to establish interphase arrays of cortical microtubules.Mutants have defects in cytokinesis during pollen development. Vegetative phenotypes observed in temperature

				sensitive mutants include left-handed organ twisting, isotropic cell expansion and impairment of root hair polarity. The mRNA is cell-to-cell mobile.
PSIR_DN44225_c3_g2	19	3	AT2G47000.7	Encodes an auxin efflux transmembrane transporter that is a member of the multidrug resistance P-glycoprotein (MDR/PGP) subfamily of ABC transporters. Functions in the basipetal redirection of auxin from the root tip. Exhibits apolar plasma membrane localization in the root cap and polar localization in tissues above and is involved in root hair elongation.
PSIR_DN43947_c1_g5	19	3	AT4G10490.1	
PSIR_DN20821_c0_g1	19	3	AT1G61110.1	NAC transcription regulator. Regulates endosperm cell expansion during germination.
PSIR_DN33361_c0_g2	19	3	AT3G17860.1	JAZs are direct targets of the SCFCOI1 E3 ubiquitin-ligase and JA treatment induces their proteasome-mediated degradation. Furthermore, JAI3 negatively regulates the key transcriptional activator of JA responses, AtMYC2. The C-terminal portion of JAZ3, including the Jas domain, appears to be important for JAZ3-COI1 binding in the presence of coronatine.
PSIR_DN43647_c0_g1	18	3	AT1G61680.2	terpene synthase 14
PSIR_DN18313_c0_g1	18	3	AT4G02220.1	zinc finger (MYND type) family protein / programmed cell death 2 C-terminal domain-containing protein
PSIR_DN32907_c0_g1	18	3	AT2G44910.1	Encodes a homeodomain protein whose expression displays a dependence on phyB for both red and far-red light response. Also involved in the shade avoidance syndrome.
PSIR_DN41454_c0_g3	18	3	AT5G09970.1	Member of CYP78A family. Paralog of CYP78A5 and appears to function in a shoot meristem maintainence pathway with LAMP1 that parallels AMP1/CYP87A5.
PSIR_DN354_c0_g1	18	3	AT2G26490.1	JGB contains seven WD40 repeats and is highly conserved in flowering plants. Overexpression inhibits pollen germination. suggesting JGB is a negative regulator of pollen germination
PSIR_DN43740_c0_g4	18	3	AT5G65550.1	UDP-Glycosyltransferase superfamily protein
PSIR_DN40192_c0_g1	18	3	AT2G45430.1	Encodes a nuclear localized AT hook domain containing protein that can bind AT rich DNA in vitro. Overexpression of the gene results in delayed flowering. Is likely to act redundantly with AHL18, AHL27 and AHL29 in the regulation of flowering. It is also involved in both photo- and skotomorphogenesis.

PSIR_DN28419_c0_g1	17	3	AT2G39130.2	Transmembrane amino acid transporter family protein
PSIR_DN13244_c0_g1	17	3	AT5G09970.1	Member of CYP78A family. Paralog of CYP78A5 and appears to function in a shoot meristem maintainence pathway with LAMP1 that parallels AMP1/CYP87A5.
PSIR_DN62118_c0_g1	17	3	AT5G10540.1	Zincin-like metalloproteases family protein
PSIR_DN40022_c1_g6	16	3	AT1G02870.1	nucleolar-like protein
PSIR_DN9245_c0_g1	16	3	AT5G20900.1	jasmonate-zim-domain protein 12
PSIR_DN17678_c0_g1	16	3	AT1G30950.1	Required for the proper identity of the floral meristem. Involved in establishing the whorled pattern of floral organs, in the control of specification of the floral meristem, and in the activation of APETALA3 and PISTILLATA. UFO is found at the AP3 promoter in a LFY-dependent manner, suggesting that it works with LFY to regulate AP3 expression. UFO may also promote the ubiquitylation of LFY.
PSIR_DN41459_c2_g2	-16	3	AT4G36220.1	encodes ferulate 5-hydroxylase (F5H). Involved in lignin biosynthesis.
PSIR_DN34856_c0_g1	-22	3	AT3G07780.3	Encodes a nuclear PHD finger protein that is functionally redundant with OBE2 and plays an important role in the maintenance and/or establishment of the root and shoot apical meristems.The mRNA is cell-to-cell mobile.

Chapter 5: General Discussion and Conclusions

Picea sitchensis is the most widely planted commercial conifer in the UK and contributes over £1bn annually to the UK economy (Forest Research, 2020). Current management practices have augmented the *H. abietis* population (Leather et al., 1999) and it is only predicted to increase further with climate change (Inward et al., 2012) leading to a heavy reliance on pesticides. The introduction of a UK-wide ban on the most common pesticide, and that of acetamiprid in 2024 exacerbates the need for alternative sapling protection strategies as part of integrated pest management. The aim of this study was to investigate the natural variation in resistance to *H. abietis* in a non-native conifer *P. sitchensis*, both within the UK breeding population, which has been bred within the UK for 70 years and in material from across the native range. This allowed us to develop a knowledge base to better understand resistance at both a population and molecular level and also to build several resources to be taken forward which could allow for the future development of a resistance breeding program.

No significant variation in resistance to *H. abietis* was detected in *P. sitchensis* from the UK Breeding population in a controlled environment

Many known conifer herbivory defences have shown large additive genetic variation (Westbrook et al., 2015) and work in a dose-dependent manner (Phillips & Croteau, 1999), suggesting they could be utilized to develop resistance breeding programs. Prior work saw significant additive variation in resistance to *H. abietis* in field trials in several conifer species, including *Picea abies* (Zas et al., 2017) *Pinus pinaster* (Zas et al., 2005, 2006) and *Pinus radiata* (Zas et al., 2008) but it had not been tested in *P. sitchensis*. Our objective was to determine if variation in resistance to *H. abietis* exists in the UK breeding population of *P. sitchensis.* The main finding of our experiment was that family-level variation within the UK breeding population had no significant impact on the damage area caused by *H. abietis* on the *P. sitchensis* saplings. This contradicted our initial working hypothesis.

This could be because of experimental design. We used a no-choice experiment; some other no-choice experiments also saw no significant difference between families in damage levels in pine species (*Pinus spp*) (Moreira et al., 2013; Sampedro et al., 2011b). Additionally, quantitatively measuring damaged areas rather than a qualitative measure, such as mortality or a damage score, increases the spread of the data, making it harder to detect differences. Several studies have seen a significant family effect on survival or damage class data but not when looking at damage area (Puentes et al., 2018; Zas et al., 2017).

The lack of variation in resistance between families could instead be because of a lack of genetic diversity within the UK breeding population of *P. sitchensis*. Most of the UK breeding population is derived from one area, the island of Haida Gwaii, due to the trees being well adapted to the climatic

conditions in the north of England and Scotland (Lee & Connolly, 2010; Samuel et al., 2007). *P. sitchensis* from Haida Gwaii are highly susceptible to another bark-eating insect *P. strobi*, but resistance does exist in other populations (Alfaro et al., 2013). The UK breeding population was selected based on growth and form traits and only contains 360 individuals (Lee, 2001; Lee & Connolly, 2010). The families used in this study were full-sib crosses from individuals of those 360 trees. Therefore, our families are effectively from one population, reducing the gene pool. This could be why no effect of family is detected. When studying resistance to *H. abietis* in half-sib families within geographically distinct populations of *P. pinaster,* no significant effect of family on damage area was detected, but there was a significant effect of population (López-Goldar et al., 2018). The levels of some key defensive terpenes are heritable within the UK breeding population but present at comparatively low levels (Tumas et al., 2021); however, the study had not challenged the trees with pest attacks to assess if resistance was impacted. Our results show that the current UK breeding population does not support the development of a resistance breeding program and thus, both studies indicate that the introduction of new germplasm may be necessary to develop one.

Significant variation in resistance to *H. abietis* in *P. sitchensis* from different provenances was detected in a field trial

Given the lack of variation in resistance to *H. abietis* seen within the UK breeding population (Chapter 2) we wanted to determine if more variation in resistance to *H. abietis* exists in *P. sitchensis* from across the native range. We found only one other study that had previously looked at variation in resistance to *H. abietis* in geographically distinct tree populations, which reported significant variation between *P. pinaster* populations (López-Goldar et al., 2018). Unlike *P. pinaster, Picea sitchensis* is a non-native conifer species in the UK and therefore did not coevolve with *H. abietis.* At the end of the field trial, we did see a large and significant variation in damage score and mortality between provenances, with mortality ranging from 37% to 78% which confirms our working hypothesis that there are varied levels of naturally occurring resistance to *H. abietis* across *P. sitchensis* populations. This finding is a very promising finding for pest management, as it would suggest that with the addition of new germplasm, a resistance breeding program could be developed. Our study also identifies provenances which showed lower mortality, including provenance 4895, which had the lowest mortality; this represents a large step forward for developing resistance *H. abietis* in *P. sitchensis*, as previously no material from across the native range had been tested for susceptibility. The provenance 4895 also had a negative correlation between height and mortality suggesting that it could remain susceptible for a shorter period. It could be interesting to know how many trees would survive a second growing season and if there are heritable levels of resistance within families in these more resistant populations.

Resistance to *Pissodes strobi* confers resistance to *H. abietis*

As *Picea sitchensis* is a non-native conifer species in the UK, it did not coevolve with *H. abietis*; therefore, the variation we detected in mortality in Chapter 3 is likely to be the result of natural selection on generalist resistance traits, not species-specific traits. A successful resistance breeding program to *Pissodes strobi* in *P. sitchensis* was developed in Canada (Alfaro et al., 2013). There are several instances of increased resistance to one pest conferring resistance to another, including in *P. pinaster* where some populations were seen to have increased resistance to both the fungus *Fusarium circinatum* and *H. abietis* (Elvira-Recuenco et al., 2014; López-Goldar et al., 2018). We hypothesised that resistance to *P. strobi* may confer resistance to *H. abietis* if they have shared resistance mechanisms. To determine if this was the case, we compared the mortality of provenances from three areas known to have higher resistance to *P. strobi.* These areas are Haney and the Big Qualicum River which both have very high incidences of *P. strobi* and the Skeena River an area of natural hybridization between Sitka and white spruce (King et al., 2004; Ying, 1991), to mortality from other areas. We saw significantly lower mortality in the resistant populations (Chapter 3) suggesting our hypothesis was correct and resistance to *P. strobi* confers resistance to *H. abietis.* This represents a large advance in our understanding of defence mechanisms against *H. abietis* because there are a number of known biomarkers for resistance to *P. strobi* markers such as resin ducts, oleoresin quality and composition (Whitehill & Bohlmann, 2019). These biomarkers can now be tested to see if they also increase resistance to *H. abietis* as there has not been much investigation into the resistance mechanisms for *H. abietis.* We did not detect consistent resistance to *P. strobi* and *H. abietis* in the exploratory study using F1 crosses but this could be due to the small number of families tested and the low number of replicates.

Large localised and smaller systemic transcriptional response to attack by H. abietis

Understanding the molecular response to attack can help to find resistance mechanisms. Previous RNA studies have uncovered many genes induced by biotic stress in conifers (Kovalchuk et al., 2015; Nantongo et al., 2022; Ralph et al., 2006). Some of these genes have since been linked to increased resistance such as 1-aminocyclopropane-1-carboxylate (ACC) synthase (an ethylene synthesis enzyme) and *Pgβglu-1.* ACC synthase is upregulated in response to wounding and regulates the formation of traumatic resin ducts and polyphenolics (Hudgins & Franceschi, 2004; Ralph et al., 2007). Transcriptome profiling of *Picea glauca* showed that *Pgβglu-1* was highly differentially expressed between trees that were that were resistant and susceptible to spruce budworm (Mageroy et al., 2015) resulting in the identification of hydroxyacetophenones as heritable resistance biomarkers (Méndez-Espinoza et al., 2018; Parent et al., 2018). Therefore, we wanted to assess the

transcriptional response to *H. abietis* exposure in *P. sitchensis* in the hope we could develop a list of genes of interest to act as a resource for future studies. We saw a large localised transcriptional response to exposure (497 DEGs in bark) with the largest response immediately after exposure. Genes linked to jasmonic acid response were significantly over-represented, suggesting that jasmonate signalling is heavily involved in the response. This observations was consistent with other reports and with exogenous methyl jasmonate exposure linked to increased resistance (Huynh et al., 2024). A putative lipoxygenase gene was the second most highly differentially expressed gene between exposed and control saplings. Lipoxygenases catalyse a step in the Jasmonic acid biosynthesis pathway (Singh et al., 2022).

Interestingly we also saw a smaller number of DEGS in the needles that clearly increased over time, potentially indicating the time required for the response to become systemic. This supports our hypothesis that *P. sitchensis* will respond systemically to *H. abietis* exposure but it contrasts with other studies which lacked a response to mechanical bark stripping in the needles (Nantongo et al., 2022). These observations suggest that herbivore-associated molecular patterns are required for the response to be systemic. In *Pices abies* the response to defence induction by exogenous methyl jasmonate was shown to not spread extensively tangentially (Erbilgin et al., 2006; Wilkinson et al., 2021). The systemic response observed here in *P. sitchensis* contained an overrepresentation of auxin transporters and proteins involved in the attenuation of jasmonate signalling cascade, suggesting the involvement of both auxins and jasmonate in a systemic response. To our knowledge, this is the first-time auxins have been suggested to be involved in systemic defence responses in conifers, though hormonal cross-talk has been shown to be important in the defence growth trade-off (Denancé et al., 2013).

Small but biologically insightful transcriptomic changes in primed trees

Individual insect attacks studied in isolation are not very representative of the stresses faced by saplings in field settings where trees face a range of biotic and abiotic stressors in combination. Therefore, studying multiple attacks is vital to increase our understanding of plant responses. Some stimuli can elicit acquired resistance (AR), which can enhance resistance (Wilkinson et al., 2019). AR can involve two non-exclusive mechanisms the prolonged upregulation of inducible defences, but this is costly to the plant, and/or defence priming. Plants primed by certain eliciting stimuli such as a previous attack, mechanical stimulation or methyl jasmonate have a higher level of defence activity when subsequently challenged with no, or relatively small growth trade-offs (Buswell et al., 2018; Fedderwitz et al., 2016; Lundborg et al., 2016; Puentes et al., 2021; Sampedro et al., 2011a). A previous study reported no significant impact on the damage level in a subsequent attack from *H. abietis* between *Pinus sylvestris,* which had been previously challenged, and those that had not

(Chen et al., 2021). I wanted to see if this was also the case in *P. sitchensis* and to see if there was a transcriptional response to priming using a previous attack similar to that seen when priming using methyl jasmonate (Mageroy, Christiansen, et al., 2020; Mageroy, Wilkinson, et al., 2020). Similarly to the *Pinus sylvestris* study, we observed no difference in damage area between putatively primed saplings that had been previously attacked and those which were naive before exposure. However, this study did identify a small number of differentially expressed genes which could play a role in defence priming in *P. sitchensis*. There were 20 transcripts differentially expressed in the bark and 27 in the needles. Including a few genes of interest such as lipoxygenase 1, azelaic acid induced, a SAGA complex subunit and a Jasmonate Zim Domain protein. The observation of a transcriptional response but no increased resistance suggests that although defences may be primed, as we hypothesised, the effect is not strong enough for reducing damage from *H. abietis*.

Knowledge base and resources developed

We built a knowledge base in the *H. abietis* X *P. sitchensis* system, which is both directly applicable here and could be utilised to advance knowledge in other systems. Variation in damage caused by *H. abietis* was significant when sampling across the native range but not within the UK breeding population. These observations suggest there was a lack of adaptive genetic diversity in the UK population due to its narrowly based geographic origin. They also highlight that studying genetic diversity more widely may open the doors to new opportunities for resistance breeding to maintain resilience in a range of other conifer and tree species. Taken together, our findings clearly suggests that a resistance program could be possible with the integration of new germplasm. Our results also allowed us to note that resistance to a different pest conferred residence to *H. abietis* which may allow for the use of a range of known biomarkers to test resistance and explore naturally-occurring resistance even further regarding population genetics and mechanisms. We also found that the *P. sitchensis* transcriptional response may be systemic and present subtle but meaningful differences between naive and putatively primed plants. We developed two resources which can be taken forward for the future development of a resistance breeding program. These are provenances which present more resistant individuals on average and a series of differentially expressed genes which could be mined for use in future studies.

Future research directions

This study suggested that there is a systemic response to *H. abietis* attack in *P. sitchensis* and highlighted the potential importance of both jasmonate and auxin in mediating this response. There has been a large amount of research which demonstrates the effectiveness of exogenous application of methyl jasmonate to increase resistance (Huynh et al., 2024). Complementation studies

demonstrate both auxin and jasmonate are required to activate some defence responses (Machado et al., 2016), the over-expression of some auxin biosynthesis genes can lead to increased resistance to other stressors (water deficit) (Kim et al., 2013), and both suggest the importance of auxins in mediating stress. There have been mixed results on the effects of auxin application on herbivory (Johnson et al., 2023), but some systems did see a reduction in damage (Gutiérrez et al., 2020). Future research could test if an exogenous application with auxin or its precursors could increase pest resistance by treating saplings and then subjecting them to attack by *H. abietis.* This could also be done in conjunction with exogenous methyl jasmonate application*,* if demonstrated to be effective, it would highlight the importance of hormonal cross talk and increase our knowledge of *P. sitchensis* physiology. There would also be potential to spray saplings with auxins in the nursery. Currently, synthetic auxins are some of the most effective herbicides in agriculture (Grossmann, 2010) so could potentially be repurposed, however the environmental effects would also have to be considered (Johnson et al., 2023). To further examine the systemic response, another RNA-seq study comparing the effect of attack, this time taking samples from more places up the stem and in the roots could shed more light on how the response varied spatially.

Having developed a list of genes of interest that could potentially be involved in priming, they could be tested for their role in priming in model organisms where knockouts could be created. In our study, the family of lipoxygenase genes were differentially expressed in the bark between primed and naïve trees and were highly differentially expressed in response to the attack. We also noted a high copy number of homologues in the *P. sitchensis* transcriptome. In *A. thaliana*, there are six LOX homologs (Umate, 2011), whereas in P. sitchensis, there are 49, 22 of them make up a small gene family, all of which have LOX1 as their putative annotation. Lipoxygenase genes catalyse a step in jasmonate biosynthesis (Singh et al., 2022) and silencing of a lipoxygenase gene increased susceptibility to a chewing herbivore (Zhou et al., 2009). Initially, it would be interesting to do a trans complementation study to see if transgenic expression of conifer versions of the genes could restore function in model organism knockouts (Feng et al., 2024). If they could restore function, this would suggest that the genes are homologues and have not evolved new functions. It would also be interesting to see if over-expression mutants differed in their response to herbivory.

Impacts and potential applications of the research

The lack of a significant effect of family on resistance to *H. abietis* in *P. sitchensis* in this study suggests that the UK breeding population would benefit from the addition of new individuals from a wider genetic background to support a resistance breeding program. This thesis identified provenances with lower *H. abietis* mortality, which could if incorporated into the breeding program

and in conjunction with other silvicultural practices, bring mortality to commercially acceptable levels without the need for pesticides.

The 4895 provenance had the lowest level of mortality and is from the Skeena River, which is an area of natural hybridization between *Picea sitchensis* and *Picea glauca* (Bennuah et al., 2004). *Picea glauca* is known to be much more resistant to *P. strobi*, but would not make an appropriate replacement for *P. sitchensis* in the UK as it does not perform as well in the British climate. However, the analysis of provenance trials of various species as potential replacements for *P. sitchensis* in the UK showed that *Picea x lutzii* , a hybrid between *P. sitchensis* and *P. glauca*, was the only spruce able to match the productivity of *P. sitchensis* (V. Stokes et al., 2018; V. J. Stokes et al., 2023). Coupled with our finding that germplasm from the introgression zone had increased *H. abietis* resistance, we strongly suggest forest managers should consider *Picea x lutzii* as well as *P. sitchensis*, which would increase forest diversity and increase resilience. A series of trials using both *Picea x lutzii* and *P. sitchensis* material across a range of geographic conditions, which tests for both resistance and growth and form traits, could shed light on whether *Picea x lutzii* has increased resistance, as we predict, as well as the potency of the resistance and climatic suitability. Any plus trees identified in these trials could then be added to the current UK breeding population. This could drastically reduce the current reliance on pesticides and allow forestry to remain commercially viable in their absence.

Our findings indicate the potential interest of testing the UK breeding population for variation to known resistance markers (for *P. strobi*) such as resin ducts, oleoresin quality and composition. Groups with high levels of these markers could be challenged to see if resistant individuals can be detected within the UK breeding population. For example, tree cores could be taken from adult trees to quantify axial resin ducts (Hood et al., 2020) ; this method would not impact adult tree mortality (Wunder et al., 2011). This would allow for fast identification of putatively resistant individuals whose progeny could be tested speeding up the selection processes for a breeding program to be developed.

These different options for resistance breeding could form part of an integrated pest management programme, which is a novel proposition for the UK. A major advantage of resistance breeding for a pest such as *H. abietis* is that the benefits may be assessed and realised in relatively short timeframes, i.e. in 3-5 years after planting. We also found that *H. abietis* weight has a significant impact on damage area (Chapter 2), suggesting that when considering options to control *H. abietis* as part of the integrated management plan, the effect of various measures on *H. abietis* weevil weight, as well as population density, should be measured. This may allow the identification of new management options, in addition to tree breeding.

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