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Understanding Sudden Larch Death – from epidemiology to host resistance

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By

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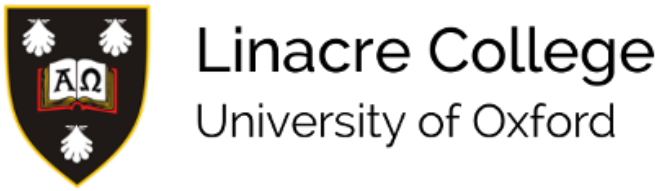
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Thesis Abstract

Phytophthora ramorum is responsible for disease outbreaks in a range of species, most prominently Sudden Oak Death in the USA and Sudden Larch Death in the UK. Our study focuses on the interactions between *P. ramorum* and two important forestry species in the UK, European larch (*Larix decidua* Mill.) (EL) and Japanese larch (*Larix kaempferi* (Lamb)Carr.) (JL). Larches are the third most important conifer species in the UK, with 126,000 hectares planted. By 2019 at least 20,700 hectares of larch had been killed due to *P. ramorum* infection or felled in efforts to contain the pathogen. Despite containment efforts, *P. ramorum* is expected to continue to spread and cause high levels of mortality in larch stands. Understanding the factors affecting spread, the response of larches to infection and the possibility of resistance to *P. ramorum* occurring within the larch population would help improve disease management.

This work aimed to increase our understanding of the epidemiology of *P. ramorum* through a study of the natural infection processes and disease progression of *P. ramorum* in JL in south-west Scotland over a multi-year period. The region had suffered an extensive epidemic in 2012 from a highly virulent new lineage (EU2) and a second period of increased spread in spring 2018, which allowed us to examine how *P. ramorum* infects individual trees and spreads across sites over a prolonged time. Using records from these two outbreaks, we considered how environmental conditions influence outbreaks and identified increased summer precipitation, cold temperatures in winter and early spring, spring storm winds and spring snowfall as important factors. Our field observations predict buds on fine shoots as sites of primary infection, with infection occurring between late autumn and flushing in the spring, followed by rapid lesion expansion during spring. Comparisons between the larch stands in our study area showed that infection and spread, and the response to that infection, varies within and between sites. In younger stands most trees died within 6-12 months of first infection but some still survived, although with severe branch dieback, more than three years later. Older stands with >95% mortality had occasional very healthy trees at least seven years after first reported infection.

EL was reported to be less severely affected by *P. ramorum* infection in field conditions than JL. We conducted inoculation experiments on potted JL and EL saplings and carried out RNA-seq analysis to study the RNA transcript accumulation profiles in response to inoculation with *P. ramorum.* Although similar in terms of lesion development, there was an interesting difference in expression of key defence pathways between the two species. EL had rapid induction of defence genes within 24 hours of infection followed by sustained or increasing expression until 25 days after inoculation. By comparison, results in JL were more varied with fewer key defence pathway genes clearly

responding to infection and the observed gene expression increases were both larger and more transient compared to EL.

The forest industry has great interest in retaining JL as a commercially viable timber species through the possibility of breeding for resistance to *P. ramorum.* This study is the first to investigate variation in resistance to *P. ramorum* in the JL population through inoculation of a clonal trial derived from putatively tolerant survivor trees and known susceptible trees. We found quantitative variation in susceptibility to *P. ramorum* within the JL population with some clones showing significantly shorter lesions than others. We propose that these clones, showing lower susceptibility, could be a useful starting point for further work investigating natural resistance in larch.

Declaration of Authorship

I declare that this thesis is entirely my own work, with the exception of section 3.2.4 which was carried out by TH Hung, who is part of the MacKay Lab at the University of Oxford. All information from external resources has been fully referenced within.

Heather Dun
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Chapter 1- Introduction

1.1 Project background

Since the 18th century, commercial forestry in the UK has relied on exotic conifer species (Forestry Commission, 1957), dominated by Sitka spruce which accounts for 51% of all conifer plantations and approximately 25% of all woodland in the UK. Another exotic forestry species of major importance in the UK is larch, with Japanese larch (JL) (*Larix kaempferi*) and European larch (EL) (*Larix decidua*), and their hybrids (HL), accounting for 10% of all conifer plantations and 4.7% of all woodland area (Forest Research, 2020a). This reliance on a small number of non-native species results in major consequences when one of these species is threatened by a novel pathogen. Increasing globalisation and the movement of species across continents and ecosystems, along with climate change creating more favourable epidemiological conditions, has led to increases in the introduction of new pests and pathogens around the world and into the UK (Potter & Urquhart, 2017; Sturrock et al., 2011). Phytophthoras are among the most successful plant pathogens and have benefitted from increasing global trade in live plants and subsequently many species have caused major disease problems around the world (Kurt Lamour, 2013) particularly in woody plants (Perez-Sierra & Jung, 2013). *Phytophthora infestans* causes problems in every country where potatoes (*Solanum tuberosum*) are grown (Fry et al., 2015) and *P. cinnamomi* causes mortality in almost 5,000 species including *Quercus* spp. in the Mediterranean, *Araucaria angustifolia* in Brazil, and globally throughout the countries that produce *Macadamia* spp., pineapples (*Ananas comosus*) and avocados (*Persea americana*), as well as 4000 native Australian plant species (Hardham & Blackman, 2018; Kurt Lamour, 2013). The accidental introduction of novel *Phytophthora* species into new environments often results in devastating disease outbreaks as the new hosts have not evolved resistance.

One of the most damaging and invasive tree-infecting *Phytophthora* species is *P. ramorum* which is highly aggressive and capable of host jumps. *Phytophthora ramorum* is responsible for two devastating disease epidemics caused by distinct, invasive clonal lineages. In 2002, what is now known as the NA1 lineage of *P. ramorum* was identified as the causative agent of Sudden Oak Death in California, USA (Rizzo et al., 2002). Sudden Oak Death is estimated to have killed 2.3 million coast live oaks (*Quercus agrifolia*) and 38.9 million tanoaks (*Lithocarpus densiflorus*) in California and Oregon (Cobb et al., 2020). In 2009, following a few years of sporadic outbreaks on various hosts, a different (EU1) lineage of *P. ramorum* was found to be causing extensive mortality of larches in south-west England (Brasier & Webber, 2010). Subsequently named Sudden Larch Death, outbreaks

have also occurred in Wales, Scotland and Northern Ireland. Research on *P. ramorum* in USA oaks has been extensive but there are gaps in the knowledge of the interactions between *P. ramorum* and larches. This study focuses on the interactions between *P. ramorum* and two larch host species, JL and EL. This relationship became significant after the EU1 lineage of *P. ramorum* was identified causing death of larch on the west coast of England, Wales and Scotland, with a different (EU2) clonal lineage subsequently found to be killing large numbers of larch in south-west Scotland in 2013 (Van Poucke et al., 2012). The host jump to larch by *P. ramorum* represented the first confirmed infection by this pathogen of a conifer species (Brasier & Webber, 2010), which has had large impacts in commercial forestry and prompted a search for mitigation measures.

The epidemiology of *P. ramorum* in larch is less well studied than on American oaks but evidence shows that its spread is variable across years. Climatic factors are thought to contribute to cycles of infection but these have not been conclusively investigated, largely due to the mandatory felling of infected trees. It has been suggested that EL is less susceptible than JL in the field (Harris & Webber, 2012) but the findings of different studies are inconsistent or inconclusive (Chastagner et al., 2013; Harris, 2014; Harris et al., 2021, 2014) and the molecular basis of the responses of EL and JL to infection are not well understood. Observations of survivor trees in heavily affected stands in south-west Scotland following the 2013 outbreak suggested that there may be naturally-occurring resistance in the JL population, allowing the potential for novel investigations and future resistance breeding. The key questions outlined here, aimed at providing a greater understanding of the potential to retain larch in the UK, form the basis of this thesis.

1.2 *Phytophthora ramorum*

1.2.1 Origin and appearance as a disease of prominence

Phytophthora ramorum was first described in 2001 as a pathogen of ornamental rhododendron species in Germany and the Netherlands, although retrospective analysis suggests that it might have been present in these areas since 1993 (Werres et al., 2001). In 2002, *P. ramorum* came into prominence as the causative agent of Sudden Oak Death in California (Rizzo et al., 2002). Sudden Oak Death was reported as killing 1 million live oak and tanoak trees over 1500 km of coastal forests (Webber et al., 2010) and this was estimated to have increased to 42.8 million trees by 2019 (Cobb et al., 2020). *Phytophthora ramorum* has also had a devastating effect on larches in Great Britain since its first identification as the cause of Sudden Larch Death in 2009 (Brasier & Webber, 2010), with at least 20,700 hectares of larch being killed or felled in efforts to contain it (R. Sketchley, 2021, personal communication). The mechanism behind the host jump of *P. ramorum*, from mostly broadleaved trees and shrubs into a conifer species, is unknown. Genetic adaptation is known to

occur after a host jump (Raffaele et al., 2010) but little is known about what may trigger a host jump. Oomycetes are known to have highly plastic effector genes (F. Martin & Kamoun, 2011), so it is likely that mutations in these genes allow infection of new host species. The consequences of this host jump are of more interest to this study than the process by which it occurred.

Morphologically, *P. ramorum* is characterized by abundant production of large chlamydospores and elongate, ellipsoid, semipapillate, deciduous sporangia with a short pedicel (Werres et al., 2001). The sporangia are caducous, enabling effective aerial spread. Analysis of the ITS1 and ITS2 sequences found that *P. ramorum* was most closely related to the also highly pathogenic *P. lateralis*, but differed by three and eight nucleotides, respectively, from the latter species (Werres et al., 2001). Phylogenetically, *P. ramorum* is found in clade 8c along with *P. lateralis, P. hibernalis and P. foliorum* (Jung et al., 2021; X. Yang et al., 2017).

1.2.2 Lineages of *Phytophthora ramorum*

Four clonal lineages of *P. ramorum* are currently known in the invasive ranges of North America and Europe; the North American lineages NA1 and NA2 and the European lineages EU1 and EU2. The use of microsatellite markers initially identified three lineages of *P. ramorum*: NA1, NA2 and EU1 (Ivors et al., 2006). Subsequent development of tests using polymerase chain reaction-restriction fragment-length polymorphism of the *Cox1* gene (Elliott et al., 2009) helped to distinguish between the lineages. These three lineages diverged between 165,000 to 500,000 years ago (Goss et al., 2009). This divergence supports the idea that the three lineages originated from different geographic locations that were isolated enough to allow independent evolution prior to their introduction to North America and Europe. It is thus probable that the emergence of *P. ramorum* in North America and Europe was the result of three independent migration events (Goss et al., 2009). In 2012 a fourth lineage, named EU2, was identified from isolates collected from common oak (*Quercus robur)*, bilberry (*Vaccinium myrtilus)*, common rhododendron (*Rhododendron ponticum*) in Northern Ireland, and JL in both Northern Ireland and western Scotland (Van Poucke et al., 2012). Although only identified in 2012, analysis of archived isolates showed that the earliest known occurrence of the EU2 lineage was in Northern Ireland in 2007 (Van Poucke et al., 2012). In-vitro characterisation of the four lineages found differences in growth rate at different temperatures with the EU2 lineage growing faster than the other lineages between 15-25^{o}C (O'Hanlon et al., 2017). O'Hanlon (2017) also found no significant differences between the linear growth rate or in-vitro aggressiveness of the EU1 and EU2 lineages on *Rhododendron* leaves, but the authors did note that aggressiveness might be different on other hosts.

In North America the invasive NA1 lineage is predominant in forests and found in nurseries across 21 states (Frankel & Palmieri, 2014), whereas the NA2 lineage is more scarce and mainly confined to nurseries on the west coast of the USA (Van Poucke et al., 2012), although occasional emergences occur in eastern states through the nursery trade (Press et al., 2020). The NA2 lineage was more common than the NA1 lineage in Canadian nurseries (Goss et al., 2010). The EU1 lineage is thought to have been introduced to North America from Europe and was detected in samples of *Viburnum* spp. and *Rhododendron* spp. collected from nurseries in Canada and Oregon between 2003-2008 (Goss et al., 2010), on *Viburnum tinus* and *Osmanthus heterophyllus* in a Californian nursery in 2006 (Grünwald, Goss, Larsen, et al., 2008) and on ornamental cherry laurel (*Prunus laurocerasus*) in Washington State in 2014 (Elliott et al., 2021). In 2015 the EU1 lineage was isolated from a tanoak in a wildland forest in Oregon (Grünwald et al., 2016) and subsequently observed causing shoot blight on grand fir (*Abies grandis*) and Douglas fir (*Pseudotsuga menziesii*) growing near infected tanoaks (LeBoldus et al., 2017). Similarities in microsatellite allele frequency suggested that the isolates collected in the forest were nearly identical to isolates previously found in a nearby plant nursery, suggesting that infection had originated in the nursery before infecting forest trees (Carleson et al., 2021).

The EU1 lineage is widespread across much of western and central Europe, and the European Union implemented emergency phytosanitary measures in 2002 (2002/757/EC). Subsequent amendments were made to expand the list of susceptible species and to include growing media and plant debris as well as live plants (Official Journal of the European Union, 2016). Surveillance and testing as part of this legislation has detected the EU1 lineage in ornamental nurseries across the majority of EU countries (Vercauteren et al., 2010). Infection has been found on 14 plant genera with 96 percent of findings being made on just three genera: *Rhododendron*, *Viburnum* and *Camellia* (Slawson et al., 2007). Sporulation occurred on rhododendron foliage in nurseries, gardens and parks (Harris et al., 2021). In the wider environment the EU1 lineage was occasionally found infecting understory vegetation (*Rhododendron* spp.) in the Normandy and Brittany regions of France (Schenck et al., 2018). In 2017 the EU1 lineage was found causing needle wilting and branch mortality in JL in two forests in Brittany (Schenck et al., 2018).

In the UK only the EU1 and EU2 lineages are found (Harris et al., 2021). The EU1 lineage is widespread along the west coast of Britain but not in Northern Ireland (King et al., 2015), whereas the EU2 lineage is limited in distribution to Northern Ireland and western Scotland (Everhart et al., 2014; Harris et al., 2021). These lineages show genetic variation as well as differences in growth and virulence. The EU2 lineage is particularly aggressive on larch (King et al., 2015) and was responsible for the 2012-13 larch epidemic in south-west Scotland.

The origin of *P. ramorum* was unknown initially but the divergence between the known lineages supported the theory of the lineages coming from geographically distinct areas, probably in Asia (Goss et al., 2009). In 2016 and 2017 *P. ramorum* was found on rhododendron leaves and in streams in Vietnam, including around Fansipan mountain close to the border with China (Jung et al., 2020). Jung et al. (2020) proposed that the presence of both A1 and A2 mating types and the apparent absence of overtly visible disease symptoms on potentially susceptible *Ericaceae*, *Fagaceae*, and *Lauraceae* genera in the sampled region was a consequence of long-term endemism and co-evolution. These authors also suggested that, as southern Yunnan, northern Laos, and the eastern Himalayas are within the same biogeographic area as the Fansipan region, mountain forests in these broader areas may also harbour endemic *P. ramorum* populations. Jung et al. (2021) subsequently found *P. ramorum* on Shikoku and Kyushu islands, in south-west Japan, and concluded from the high level of phenotypic and phylogenetic diversity in the sample populations, as well as the absence of overt host symptoms, that *P. ramorum* comprises many anciently divergent lineages native to the laurosilva forests between eastern Indochina and Japan. These isolates exhibited five slightly different ITS genotypes, including one genotype that was identical to an isolate of the EU1 Lineage collected in Germany. Sequencing of the *cox1* locus found that all but one of these isolates were identical and differed from the NA1, NA2, EU1 and EU2 lineages by 4, 1,5, and 5bp, respectively (Jung et al., 2020).

1.3 Epidemiology

Phytophthora ramorum has been identified infecting a wide range of species with more than 170 known hosts (Department for Environment Food & Rural Affairs, 2015). The symptoms of *P. ramorum* infection differ on different host species. Some hosts, like rhododendrons (*Ericaceae*) and bay laurel (*Laurus nobilis*), are sporulating hosts acting as key sources of inoculum, facilitating disease spread and causing disease in both sporulating and non-sporulating 'dead end' hosts (Davidson et al., 2005; Harris & Webber, 2016). Whilst these sporulating hosts may still suffer ill-heath and mortality after being infected, sporulation has also been found on asymptomatic rhododendron foliage (Denman et al., 2007).

In American oaks (*Fagaceae*), the most common symptom is bleeding bole cankers which are the defining feature of Sudden Oak Death (SOD) (Hansen et al., 2005). These cankers can be extensive and eventually girdle the trunk, destroying phloem and xylem tissues, and leading to death (Oßwald et al., 2014). Up to 80% mortality has been reported in tanoaks in California (Everhart et al., 2014) with inoculum originating from local sporulating hosts. In larch, however, *P. ramorum* infections are especially severe as the needles are infected, becoming sites of intense sporulation (Harris &

Webber, 2016). High inoculum loads originating from needles appear to result in rapid infection of bark on the branches and main stem of this same host, resulting in subsequent mortality as the lesions girdle the main stem.

1.3.1 Life cycle and dispersal mechanisms

Phytophthora ramorum is heterothallic, requiring interaction between opposite mating types (A1 and A2) for sexual recombination to produce oospores (Figure 1). Heterothallism implies the sympatric coexistence of both mating types in the organism's native range. Initial mating studies with *P. ramorum* determined that the A1 mating type was only found in Europe and the A2 mating type was limited to the United States (USA), with the exception of a single A2 EU1 isolate collected from an infected nursery plant in Belgium in 2002 (Werres & De Merlier, 2003). Two further isolates of EU1 A2 mating type were found in Belgium but there was no evidence of sexual reproduction between them (Vercauteren et al., 2010). In-vitro mating tests found that mating success was rare (O'Hanlon et al., 2017). Currently there is no evidence for sexual reproduction within any *P. ramorum* population and oospores have not been observed in naturally infected plant tissues (Jung et al., 2021). Both EU lineages are of the A1 mating type (Jung et al., 2021) which indicates that should the distributions of the EU1 and EU2 lineages overlap in forests, it is very unlikely that they will reproduce by sexual means (O'Hanlon et al., 2017).

All known reproduction of *P. ramorum* in field conditions is asexual. *Phytophthora ramorum*, like other Phytophthoras, produces sporangia on the surfaces of infected leaves and twigs of foliar hosts (Grünwald, Goss, & Press, 2008). The caducous nature of these sporangia enables effective aerial dispersal. In California, rain was found to splash inoculum up to 30 cm above the soil surface (Fichtner et al., 2009) and, when combined with wind, to move inoculum 5-10m (Davidson et al., 2005). In Scotland, *P. ramorum* inoculum was detected in spore traps up to 80m from the infected stand, presumably in windblown rain (Frederickson-Matika et al., 2019). Longer-distance dispersal, of up to 3 km in storm winds, has been reported for *P. ramorum* in California (Rizzo et al., 2005). The sporangia can then either germinate directly to produce mycelia and further growth (Davidson et al., 2005; Widmer, 2009) or each release 20–30 biflagellate motile zoospores (Figure 1) (Garbelotto et al., 2017). The zoospores are motile in water and can use chemotaxis to find new hosts (Garbelotto & Hayden, 2012). The numbers of sporangia produced vary by host, larches support the highest levels of sporulation; 22 times more sporangia are produced on JL needles compared to California bay laurel (*Umbellularia californica*) which is the sporulating host driving the epidemics of *P. ramorum* in the USA (Harris & Webber, 2016).

Inoculum can also be transported through contaminated soil on hiking boots (Tjosvold et al., 2006; Webber & Rose, 2007) and vehicle tyres (Davidson et al., 2005). Often, soil is contaminated with chlamydospores. These long-lived resting structures have also been found to exist on leaf litter in both infected oak forests in the USA and in larch forests in the UK (Davidson et al., 2005; Harris, 2014), and in the soil of infected Californian oak forests (Fichtner et al., 2007). Reports of reinfections from contaminated soil are common and can occur for a prolonged period, even after removal of living hosts, for example up to 49 weeks in Californian oak woods (Fichtner et al., 2009) and two years in UK larch forests (Harris, 2014).

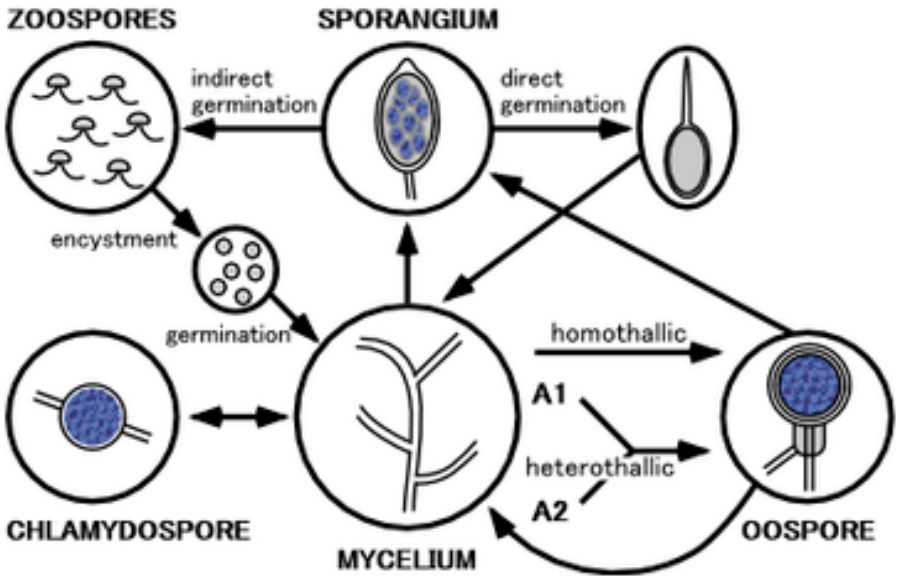


Figure 1: The life cycle of Phytophthoras. From Erwin & Ribeiro (1996)

In vitro the optimum temperature for growth of *P. ramorum* is 20 °C (O'Hanlon et al., 2017) although growth can continue between 2-30°C (Englander et al., 2006; Werres et al., 2001). However, temperatures of 37.5- 40°C kill hyphae within several hours, while temperatures of 42.5-50°C are lethal within minutes (Browning et al., 2008). Sporangia were produced in vitro at 10-30°C by USA isolates and 6-26°C by EU isolates, with optimal temperatures of 16-22°C and 22°C, respectively (Englander et al., 2006). Studies of *P. ramorum* on native oak species in America have identified the importance of high rainfall and humidity (Davidson et al., 2005) as well as warm temperatures between 10-20 °C (Davidson et al., 2005; Garbelotto et al., 2017) for inducing sporulation and spread. Garbelotto et al. (2017) found that sucessful coast live oak infection requires 250 mm of rain over 3-6 weeks and at least one week when the maximum daily temperature reaches 20°C. Hüberli et al. (2012) found that the highest levels of infection of California bay laurel occurred

in spring and concluded that infection took place in both warm-wet months and cool-wet months, with wetter months giving more successful infections than drier ones. Garbelotto et al. (2017) also found that wetter months gave more positive spore traps in Californian mixed oak forests than dry months. Hot and dry summer months in California limited the recovery of *P. ramorum* from soil and rainwater traps (Davidson et al., 2005). Observations of *P. ramorum* on rhododendron (*Rhododendron ponticum*) in the UK similarly suggested that high rainfall and humidity are required for sporulation and infection (Denman et al., 2007). However, the effect of climatic factors on spread of *P. ramorum* on larch are, as yet, not well understood, in particular the climatic conditions required for epidemic outbreaks. A better understanding of the climatic factors involved would be of interest to forest managers as predictive models could be made to forecast future outbreaks.

1.4 The role of forestry in the UK

The role of forestry is well-recognised as providing a broad spectrum of economic, environmental, societal and cultural benefits. Economically, the asset value of UK woodlands was estimated as £129.7 billion in 2017 (Office for National Statistics, 2020) with the sector employing 167,00 people (Parliamentary Office of Science and Technology, 2007). The environmental benefits of forests are coming to prominence with increased concerns about climate change. UK forests sequester 18.1 million tonnes of carbon dioxide annually, equivalent to 4% of total UK greenhouse gas emissions (Office for National Statistics, 2020). The value of locking up carbon dioxide emissions that might otherwise contribute to climate change has been calculated at around £16,000 per hectare (The Woodland Trust, 2017). Forests also provide other important ecosystem services including preventing soil erosion, water filtration, climate regulation, air quality regulation and flood mitigation (Harrison et al., 2010). The societal and cultural benefits of forests include recreation, with 475 million visits to UK woodlands recorded in 2017 (Office for National Statistics, 2020). Visiting forests is also linked to the reduction of stress levels and other mental health benefits (Nilsson et al., 2011; O'brien et al., 2020). During the COVID-19 pandemic, which led to increased isolation and lack of social contact, people whose homes had proximity to trees and urban green spaces reported higher levels of self-esteem, life satisfaction, and subjective happiness and lower levels of depression, anxiety, and loneliness (Soga et al., 2021). The NHS forest project promotes tree planting around hospitals to improve the recovery of patients (NHS Forest, 2021). The removal of air pollution, such as fine particulate matter, by UK woodlands has been equated to a saving of £938 million in health costs through fewer admissions to hospitals for respiratory and cardiovascular problems (Office for National Statistics, 2020). Forests and woodlands hold a key position in cultural heritage such as literature and art (Tabbush, 2010), shaping the landscape that people expect to see

in the countryside (Raum, 2020). All of these aspects have led to a recent shift towards increased tree planting in the UK. The UK Government's Net Zero greenhouse gas emissions target aims to increase the woodland area from 13% (3.2 million hectares) to reach at least 17% woodland cover by 2050, requiring the planting of around 30,000 hectares each year (Committee on Climate Change, 2020). As part of the Science and Innovation Strategy for Forestry in Great Britain, each devolved government has also set out plans for increased tree planting (Forestry Commission, 2020). In 2019-2020 13,700 hectares of new woodland were planted in the UK with conifers accounting for 57% of this area (Forest Research, 2020a).

1.5 *Phytophthora ramorum* on larch in the UK

In 2012 larch plantations in the UK covered an area of 126,000 hectares (Forest Research, 2020a; National Forest Inventory-Forestry Commission, 2014), making it the third most important commercial conifer in the UK after Sitka spruce (*Picea sitchensis*) and Scots pine (*Pinus sylvestris*). In Scotland, JL was the most widely grown species of larch with 55,034 hectares (included in this figure is a minor amount of HL) planted compared with 8,622 hectares of EL (Forest Research, 2001).

Larches are light-demanding trees, growing quickly when young and able to survive in high altitude areas including wet sites, although JL is thought to be better adapted to the higher rainfall areas of the west coast than EL (Harding, 1998). Larch provides high quality, durable timber due to its concentration of tannins (up to 10%) and resin content (about 2.6%) (Da Ronch et al., 2016). It is often used for fencing and cladding and is the preferred softwood timber for boat-building (Harding, 1998). The timber quality of JL and EL is comparable and is among the most dense of the British softwoods, with old growth larch averaging about 590 kg/m $^{-3}$ at 12% moisture and younger trees lighter at about 510-540 kg/m^{-3} (Harding, 1998). Larch forest has been deemed beneficial for rare native species including black grouse and red squirrel (Forestry and Land Scotland, 2017). Larches are deciduous conifers and the changing colours of the needles across the year provide seasonal interest compared to evergreen conifers, such as Sitka spruce and Scots pine, that make up the majority of commercial forestry (Dumfries and Galloway Council, 2014).

Japanese larch has a limited native range across scattered volcanic mountains in a 200 km square in the central island (Honshu) of Japan (between 35° and 36°N and 137° to 140°E) as part of subalpine forests at elevations between 1,200-2,400 m. In comparison, EL has a large continuous native range of 900 km across the Alps between 6°E and 16°E, with further scattered populations in the mountains of central Europe between 16°E and 26°E (Wagner et al., 2015). European larch is also

found across a wider range of elevations, from 180 m in lowland Poland to 2,500 m in the Alps (Da Ronch et al., 2016).

The genetic variation in the UK populations of EL and JL is unknown. European larch was introduced to the UK in 1629 and first grown on large scale, after 1750, by the Duke of Atholl at Dunkeld in Scotland. Later study of original Dunkeld plantings suggest that they are from the Alpine population of EL. These trees likely provided seed for much of the later planting of EL across Scotland (Lines, 1987). Current recommendations suggest planting stock from the Sudeten mountains in the Czech republic as it has the best combination of growth characteristics and resistance to canker (Lee, 2003). Japanese larch was introduced to the UK in the 1860s and large-scale planting started in 1885 (Lee, 2003). The first provenance trials of JL in the UK were planted in 1934 with seeds sourced from the Nagano Prefecture in Japan (Lines, 1987). A subsequent provenance trial, planted in 1956, included material from throughout the natural range of JL (Lines & Mitchell, 1989). From these trials Lines (1987) identified the Suwa region of Nagano Prefecture as the 'first choice' provenance for commercial planting in the UK. Two other provenances were identified as 'acceptable'; the Nikko region in Tochigi Prefecture and plantations on the island of Hokkaido. Lee (2003) states that for both EL and JL the best adapted seed sources are likely to come from British-registered seed orchards. It is therefore likely that the UK population of EL and JL have less genetic diversity than occurs in their native range, due to both the founder effect and subsequent selection for commercial characteristics. Clonal propagation is used in the multiplication of plus trees for seed orchards, but larch clonal material has not been deployed in commercial forests (Forest Research, 2021b). Larches are generally outcrossing with no evidence of selfing found in JL seed orchards (Chen et al., 2018) and experimental self-pollination of EL resulted in only 2% of seeds having normally developed embryo and nutritive tissue, compared to 18% in open-pollinated EL crosses (Slobodník, 2002). Hybrids between EL and JL can occur naturally or through controlled crosses and the subsequent offspring (*Larix x eurolepsis*) became popular as a commercial forestry species due to its vigour (Lee, 2003).

The low level of EL plantations in the UK (Forest Research, 2020c) and Europe (Yde-Andersen, 1979) is due to its susceptibility to a non-native fungal disease, larch canker (*Lachnellula willkommii*) (Kulej, 2006). Larch canker causes girdling cankers and stem distortion reducing the suitability for timber production and has led to a decline in the cultivation of EL in northern Europe since the 19th century (Yde-Andersen, 1979). Larch canker is thought to be native to Japan and it does not cause severe disease on JL (Kobayashi, 1970), which explains why JL is preferred in commercial forestry producing larger outputs of sawn timber compared to EL (Harding, 1998).

1.5.1 Range expansion of *P. ramorum* and detection on larch

Phytophthora ramorum was first detected in the UK in 2002 on *Viburnum tinus* from a garden centre in southern England (Lane et al., 2003) and was first detected on a tree species in the UK in 2004 when it was found on a southern red oak *(Quercus falcata*) in south-east England (Brasier et al., 2004). Between 2004-2009 fewer than 100 infections on tree species were reported in the UK. These included *Fagus sylvatica, Nothofagus obliqua, Quercus* spp. and *Castanea sativa*, growing near infected rhododendron (Webber, Mullett, et al., 2010; Webber, 2007). In 2009 the EU1 lineage of *P. ramorum* was found infecting and killing large numbers of JL trees in south-west England (Brasier & Webber, 2010). In 2010, *P. ramorum* infection was reported on JL in Wales, Northern Ireland and the Republic of Ireland, with half a million trees felled across 1,300 hectares in Wales alone (Everhart et al., 2014). In the same year, in Scotland, *P. ramorum* was found on a small 1.5 hectare stand of JL on the Craignish peninsula on the west coast and subsequently several more sites were detected in the same region (Scottish Government-Environment and Forestry Directorate, 2015).

Figure 2 illustrates the occurrence of *P. ramorum* outbreaks across the four nations of the UK between 2010 and 2019. The Forestry Commission issues statutory plant health notices (SPHNs) following confirmed *P. ramorum* infections (Figure 2a). Each SPHN requires the felling of all infected trees in the stand plus a 250 m buffer around them and so the extent of each outbreak is reflected in the area that is required to be felled under those SPHNs (Figure 2b). The area affected varies between years, however, two major increases in infection can be clearly identified in 2013 and 2018. The largest area affected in 2013 was in Wales where 4603 hectares were felled (Figure 2b). The 2013 data from Scotland reflect the extensive outbreak in the Galloway Forest region where larch accounted for 7% of all trees (Dumfries and Galloway Council, 2014) and 50% of the larch had been felled by 31st March 2016 (Sketchley, 2019). The extensive area of larch affected led to the policy decision, (*Plant Health (Forestry) (Phytophthora ramorum Management Zone) (Scotland) Order 2014),* to establish a 'Management Zone'(Figure 3), effectively allowing the phased removal of larch within the capacity of the forestry sector to harvest and process the timber, while focusing efforts on tackling new outbreaks elsewhere in Scotland (Scottish Forestry - *Phytophthora Ramorum* in Scotland, 2020). Within the Management Zone, SPHNs, which would usually require the felling of larch trees infected with *P. ramorum* plus any host species within a 250-metre buffer zone, were generally not issued (Forestry Commission Scotland, 2017) and so the 5,000-6,000 hectares of diseased larch in the Management Zone (Forestry Commission Scotland, 2017) are not included in the data shown in Figure 2. A second increase in infection occurred in 2018 (Figure 2) which was particularly prominent in Scotland outside of the Management Zone, resulting in SPHNs being issued and 1373 hectares felled. Further spread, although less extensive, occurred in 2019 (Figure 2) and

2020 (Figure 3). The environmental drivers of these outbreaks were unknown but the slower spread of *P. ramorum* in Scotland between 2013 and 2018 gives data points for comparison between epidemic and non-epidemic years.

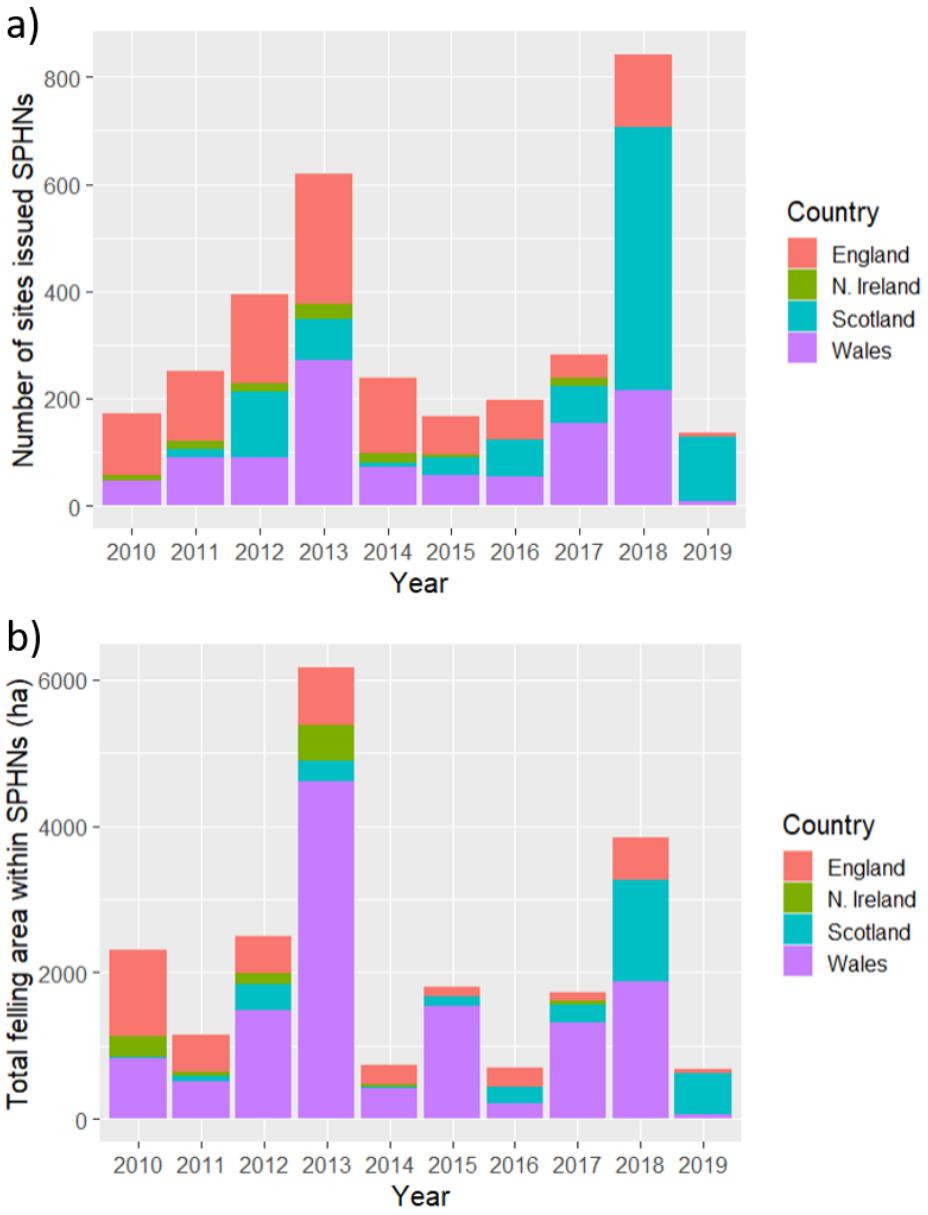


Figure 2: Extent of larch affected by P. ramorum by country in the United Kingdom between 2010 and 2019. a) number of statutory plant health notices (SPHNs) issued following confirmed P. ramorum on larch, b) area of larch required to be felled under those SPHNs. Data for Scotland since June 2014 do not include any suspect or confirmed infections within the Management Zone as SPHNs have not been issued in the zone since that date. The working presumption is that most of the 5,000-6,000 hectares of larch stands within the zone either have, or soon will have, infection present. Graphs made with data from R. Sketchley, 2021, personal communication.

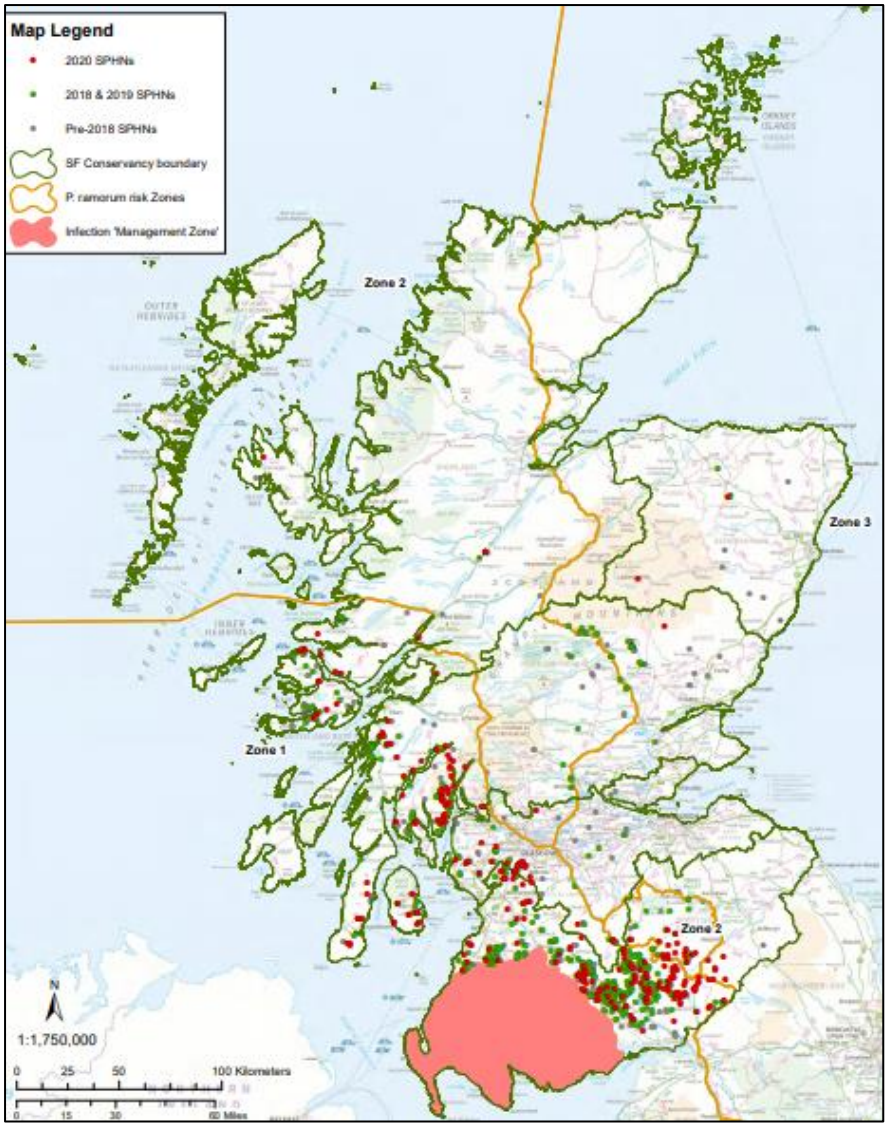


Figure 3: Map of Statutory Plant Health Notices (SPHNs) served for Phytophthora ramorum on Larch sites in woodland settings, adapted from (*Scottish Forestry - Phytophthora ramorum in Scotland*, 2020)*. The area coloured in pink indicates the extent of the P. ramorum Management Zone as set out in the Plant Health (Forestry) (Phytophthora ramorum Management Zone) (Scotland) Order 2014.*

1.5.2 The impact of *P. ramorum* on larch and the search for resistance to infection

By 2019 at least 20,700 hectares of larch had been killed or felled in efforts to contain *P. ramorum* (R. Sketchley, 2021, personal communication). The associated economic costs are considerable, including that of felling diseased trees, replanting with other species, and the economic loss from the unrealised potential of trees felled before full maturity as a timber crop (Scottish Government-Environment and Forestry Directorate, 2015). However, sawn timber from felled trees does not contribute to the spread of *P. ramorum* so it can enter the timber market as usual (Harris, 2014) as long as it is moved under the appropriate licence and processed at licensed facilities (Forest

Research, 2021a). The large quantity of larch timber, from mandatory felling, entering the timber market resulted in falling prices, leading to a financial loss to landowners and sawmills.

The continued spread of *P. ramorum* has resulted in larch no longer being recommended for planting (Webber, 2017). Similarly, susceptibility to Dothistroma needle blight resulted in the Forestry Commission suspending planting of Corsican pine (Dobson et al., 2013). There are concerns over suitable species being available for restocking felled areas. Some conifers such as firs (*Abies* spp.) are not recommended for replanting infected sites due to their known susceptibility to *P. ramorum*, although other conifer species can still be planted, including western red-cedar (*Thuja plicata*), Scots pine, and Sitka spruce (Webber, 2017). However, of these species only Sitka spruce is suited as a viable commercial replacement on the wet acid upland sites of the west-coast of the UK. Sitka spruce already accounts for 51% of all conifers planted in Great Britain, predominantly in Scotland which contains 507,000 hectares of the UK total of 665,000 hectares of Sitka spruce (Forest Research, 2020a). Replacing larch with more Sitka spruce will increase the fragility of a system that already predominantly relies on a single species. The need for an alternative to increasing the amount of Sitka spruce adds to the importance of retaining larches as a commercially viable timber species.

The interest in retaining larches for commercial timber production has led to the establishment of a clonal trial of young JL trees (ramets) grafted with scion material collected from survivor trees in the Galloway Forest which are thought to have putative resistance (Green et al., 2016). Survivor trees are often investigated as the first source of possible resistance for breeding programmes (Slavicek & Knight, 2012; Sniezko et al., 2020). However, survivor trees may miss infection by chance or due to their position within the stand and so inoculation trials must be carried out to test this putative resistance, as in previous studies across many tree species (Green et al., 2020; Hansen et al., 1989; Slavicek & Knight, 2012; Sniezko et al., 2020). This study includes the first inoculation trials of the scion material from JL survivor trees to investigate whether variation in resistance occurs within the population.

1.5.3 Differences in susceptibility or tolerance between the two larches

Early field observations in England suggested that EL was more resistant to stem infections caused by *P. ramorum* due to the low frequency of resinous bark lesions (Harris and Webber, 2012). However, the results of experimental inoculations do not support this hypothesis. Mycelial inoculations in controlled conditions found that the bark of EL was much more susceptible than bark of JL (Harris and Webber, 2012) but further work found larger lesions on JL than EL (although this

difference was not statistically significant) when inoculating with the EU1 and NA1 lineages (Harris, 2014) and with the EU2 lineage (Harris et al., 2021).

Contradictory results were also found when intact potted seedlings of JL and EL were inoculated with zoospores of the EU1 lineage, resulting in longer mean lesion lengths at 21 days post inoculation (dpi) on JL compared with EL. However, mortality of EL was higher than JL at 55 dpi (Chastagner et al., 2013). RNA-seq analysis of the response to infection with the EU2 lineage found more defence genes expressed in EL than JL and downregulation of some defence related pathways in JL (De La Mata Saez, 2015) thus suggesting that EL has a stronger immune response to *P. ramorum* infection than JL. However, De La Mata Saez (2015) used a small number of biological replicates and a short experimental time frame, studying host responses up to only seven days post inoculation. They also used the traditional mycelial inoculation method whereas inoculation using a sporangial and zoospore suspension would better mimic the proposed natural infection process. Therefore, further study using a different inoculation approach, more biological replicates and a longer timeframe for evaluation might enable further insights into the genetic responses of each species to infection. Time frame is particularly important as the susceptibility of the two species differs with different times of assessment after infection (Chastagner et al., 2013).

These previous observations suggest that differences in susceptibility or tolerance may depend on the criteria used to define them and also on experimental methodology. As there is considerable interest from the forest industry in the continued use of EL in plantation forestry it is necessary to thoroughly investigate the circumstantial evidence of differences in susceptibility between the two larch species. Further exploration could lead to insights in the constitutive defences, by comparing expression between the two species, and induced defences, by investigating the regulation of genes in response to infection. An understanding of the genetics behind the defence response could also inform the selection of trees for future resistance breeding.

1.6 Project rational

This project was made possible due to collaboration between Forest Research and the Forestry Commission (now Forestry and Land Scotland). The phased removal of larch within the Management Zone allowed the retention of trees for disease monitoring and research purposes, and it is due to these conditions that the field surveys detailed in Chapter 2 of this thesis could be carried out. It also facilitated the identification and grafting of survivor trees from heavily infected stands in the region, and therefore further investigation of their putative resistance, as covered in Chapter 4.

1.7 Project aims and objectives

The overall aim of this research is to enhance our understanding of *P. ramorum* on larch through a long-term field study and associated inoculation experiments. Currently, there is a lack of information on the epidemiology of *P. ramorum* on larch in the field, including the site and timing of primary infection, rate of spread within stands, and the influence of climatic conditions on disease severity. Although both EL and JL are important forestry species and are both susceptible to *P. ramorum,* it has been suggested that differences in the level of susceptibility exist between the two species with EL possibly less susceptible. Further investigations of the interactions of these two host species with *P. ramorum* were important as the findings of previous studies were inconsistent and there is industry interest in maintaining larches as viable forestry species. This interest in retaining larch in the forestry landscape also provided the rationale for investigating whether JL trees which survived the 2013 disease outbreak have greater natural resistance to the pathogen.

To answer these questions the following research objectives were identified:

Objective 1: Investigate the epidemiology of *P. ramorum* on JL in the field to identify the primary infection sites, timing of initial infection, and the temporal and spatial development of symptoms within stands.

Objective 2: To determine which climatic conditions might affect disease severity and spread.

Objective 3: Develop a methodology to allow inoculation using sporangial suspension and then use this method to investigate factors that might influence infection in JL and EL including wounding and different stages of bud flush.

Objective 3: To consider the differences in response to infection with *P. ramorum* between JL and EL by investigating RNA transcript accumulation profiles to identify the defence response pathways that are activated in response to infection.

Objective 4: To determine if variation in resistance to *P. ramorum* exists in the JL population by comparing the response to inoculation with *P. ramorum* in grafted scions from putatively resistant and known susceptible mature JL trees.

Chapter 2– Investigating the epidemiology of *P. ramorum* on Japanese larch in field conditions in south-west Scotland.

Abstract

We carried out a unique study of the natural infection processes and disease progression of *P. ramorum* in Japanese larch over a multi-year period. Field surveys in south-west Scotland, which suffered an extensive epidemic in 2012 from a highly virulent new lineage (EU2), allowed detailed examination of how *P. ramorum* infects individual trees and spreads across a site over a prolonged time. A period of increased spread in spring 2018 allowed us to consider how environmental conditions influence outbreaks, with summer precipitation as an important factor. Our field observations predict that buds on fine shoots are the primary infection sites with infection occurring between late autumn and spring.

There is variation in response to infection within the standing population of larches. In younger stands most trees die within 6-12 months of first infection but some were still surviving, with severe branch dieback, more than three years later. Older stands, with >95% mortality, have occasional very healthy trees at least 7 years after first reported infection. It is likely that site specific factors influence infection spread and subsequent mortality. We have found infection and spread is not consistent within or between sites.

2.1 Introduction

In 2013 there was a major outbreak of *P. ramorum* in the Galloway Forest in south-west Scotland which affected up to 6,000 of the 9,000 hectares of Japanese larch (JL) plantations (King et al., 2015). A new virulent lineage of *P. ramorum* (EU2) was found to be causing this outbreak. The EU2 lineage was first identified from samples collected between 2007-2011 in Northern Ireland, and had been detected on larch near the western edge of the Galloway forest in October 2011 (Van Poucke et al., 2012). This EU2 lineage was found to be more aggressive at colonising larch bark than EU1 but produced fewer sporangia on needles (Forestry Commission Scotland, 2017; Harris, 2014) and was responsible for causing the major new outbreak with the EU1 lineage only found at the southern edge of the region (King et al., 2015).

There was significant mortality in JL due to *P. ramorum* in 2013, with some stands suffering >95% loss. However, variation in mortality levels occurred across stands with some stands showing no symptoms of infection. Even in stands with >95% mortality, some trees survived while surrounding

trees quickly succumbed to infection. After the 2012-13 outbreak, the spread of *P. ramorum* in the Galloway forest seemed to slow and there was no further significant expansion of the disease between 2013 and 2016, when the current study was initiated. Little is known about the factors leading to the extensive outbreak of *P. ramorum* first observed in Galloway in April 2013. It was suggested that wet autumn conditions and a mild winter in 2011/12 contributed to the first smaller outbreak in the west of Galloway in 2012 (Forestry Commission Scotland, 2017). Subsequently, as reported by Forestry Commission Scotland (2017), "favourable weather conditions in late summer/early autumn 2012, aided by topography and the relatively contiguous distribution of larch in the area, gave rise to a major surge in disease expression in south-west Scotland" in spring 2013. However, this is anecdotal, and more detailed analysis is needed of the climatic factors associated with outbreaks of *P. ramorum* on larch in the UK in order to draw robust conclusions. In order to explain the spatial and temporal variability in disease occurrence in the field, a better understanding is needed of the epidemiological factors driving infection.

The disease cycle of *P. ramorum* on larch and the effect of climatic factors on spread are not well understood. Studies of *P. ramorum* on native oak species in America, coastal live oak (*Quercus agrifolia*) and tanoak (*Notholithocarpus densiflorus*), have demonstrated the importance of high rainfall, humidity and temperature for inducing sporulation and spread (Hansen et al., 2005). The presence of a film of water on the leaf surface was also found to be important for infection (Davidson & Shaw, 2003). Observations of *P. ramorum* on Rhododendron in the UK suggest that similar factors are required for sporulation and infection (Denman et al., 2007) however, this has not been fully explored. In particular the climatic conditions required for epidemic outbreaks are not known.

Previous reports of natural infection processes of *P. ramorum* on larch in the field have been limited in both scale and time course due to the compulsory felling of infected stands (Forest Research & FERA, 2010). Field observations have shown that needle symptoms associated with infection and sporulation by the pathogen only become visible toward the end of the growing season just before needle senescence (Harris & Webber, 2012). Sporulation occurs in the autumn but mortality of larch only becomes notable following the normal flushing period in the spring, giving an intervening period where the sequence of events leading to infection and the climatic drivers of these processes have not been investigated.

Phytophthora ramorum infection in larch leads to black or grey-purple discoloured needles, aborted bud flush, wilting of shoots and needle loss (Webber, Mullett, et al., 2010). Death can occur if the infection spreads to the branches and main stem, resulting in bole cankers that eventually girdle the

main stem in the same way as Sudden Oak Death (Oßwald et al., 2014). Investigations of infection and sporulation of *P. ramorum* on larch have been carried out in controlled environment conditions on excised material (single leaves, shoots or cut logs) (Harris, 2014) or in potted trees in greenhouses (Chastagner et al., 2013) rather than in the field. In larch, *P. ramorum* infection is especially severe as the needles are a site of intense sporulation. It was recorded at up to 22 times higher than on bay laurel which is the main sporulating host that facilitated epidemic spread on oaks in America (Harris & Webber, 2016). High inoculum loads appear to result in rapid infection of the main stem and subsequent mortality. Interestingly, inoculated needles on excised shoots of JL showed higher sporulation when inoculated in August compared to inoculations in October and May (Harris, 2014).

The timing of natural infection in the field is still unknown but inoculation experiments showed that pathogenicity is influenced by the time of year. Chastagner *et al.*(2012) investigated the susceptibility of various young, potted conifers including European and Japanese larch to *P. ramorum* zoospore inoculation when testing lineages NA1, NA2 and EU1. When zoospores were sprayed on intact foliage in spring, slightly larger lesions resulted in Japanese larch 21 days after inoculation, but lower mortality 55 days after inoculation, compared to European larch. However, trees inoculated using the same method in autumn developed minimal needle symptoms and no stem lesions. Using inoculated cut logs Harris (2014) found smaller lesions in summer compared to spring, which suggests that susceptibility declines as the growing season progresses.

The first point of infection by *P. ramorum* has been studied closely in rhododendron and native oak species in the USA. Infection was found to take place through the stomata and lenticels of oaks (Florance, 2005) and also through direct penetration of the leaf surface in rhododendron (Lewis and Parke, 2006). In coastal live oak, infection through the bark was observed and a positive correlation was found between bark thickness and development of *P. ramorum* cankers, which was suggested to be linked to the development of fissures in the bark (Swiecki & Bernhardt, 2006). In tanoak, *P. ramorum* colonises xylem vessels (Parke et al., 2007) and it was suggested that discrete lesions could be connected by infection through the xylem (Oßwald et al., 2014).

On a landscape scale there is a gap in the understanding of factors influencing spread of *P. ramorum* between individual larch trees and between stands, and the outcome of infection on individual trees within a stand. In 2015, survivor trees showing no symptoms of infection and being otherwise healthy, were found in stands that had suffered >95% mortality in the 2013 outbreak (S. Green, 2020, personal communication). These trees may have some level of resistance or tolerance to infection by *P. ramorum*. If this is the case, differences in the rate of disease spread within stands and individual trees might be observed over time, but this has not been investigated in larch. In

coast live oak differences have been reported in survival and infection rates between stands (Nagle et al., 2011) with susceptibility varying more strongly within populations rather than between populations (Dodd et al., 2004).

One approach to study variation within the population and spread of pathogen within a stand is to closely monitor individual trees over a long period of time as they are exposed to, and respond to, natural infection. To date, all comprehensive studies of *P. ramorum* infection in the field are focused on the Oak-North American lineage pathosystem (Davidson et al., 2005). The Galloway Forest *P. ramorum* outbreak is of particular interest as the EU2 lineage is the predominant lineage causing infections and it has not yet been studied in the field. The existence of the management zone in south-west Scotland, and resulting retention of diseased larch stands alongside asymptomatic stands, has allowed for a unique study of natural infection processes and disease progression in larch over a multi-year period.

The aims of this chapter were to investigate the epidemiology of *P. ramorum* on larch in the field, and to carry out artificial inoculation studies to test hypotheses of infection developed from field observations. In order to better mimic the proposed natural infection process in larch, we developed a methodology to allow inoculation using sporangial suspension since prior work had only looked at zoospore or mycelial infections (Chastagner et al., 2012; Harris, 2014). The specific objectives of the study were to (i) investigate how *P. ramorum* spreads between trees and within stands, (ii) consider what climatic conditions might affect spread, (iii) investigate the pathway of natural infection to identify the point of first infection in a tree and also the temporal development of symptoms, (iv) investigate the impact of tree phenology and the importance of wounding to infection by using artificial inoculations. Our hypotheses for the inoculation experiments were that (i) infection can take place through both wounded and unwounded bark during the dormant period, (ii) infection can take place though the fresh needles at the first stages of flush, (iii) both sporangia and zoospores would attach/encyst onto the needle bases prior to infection.

2.2 Methods

2.2.1 Observations of disease progression in the field

Galloway Forest Park in south-west Scotland was chosen for the location of the field plots (Figure 4 left). Commercial conifer planting in the area started in 1922, predating the establishment of the Galloway Forest Park in 1947, and some areas are on their second or third rotation of planting. The forest is mostly managed as commercial coniferous forestry with about 70% Sitka Spruce and contains significant plantations of Japanese and some European larch planted from the 1950s up until 2012, covering 8,000– 9,000 hectares in 2013 (King et al., 2015). This area accounts for ~12% of the approximately 66,000 hectares of larch in Scotland (Forestry Commission Scotland, 2017).

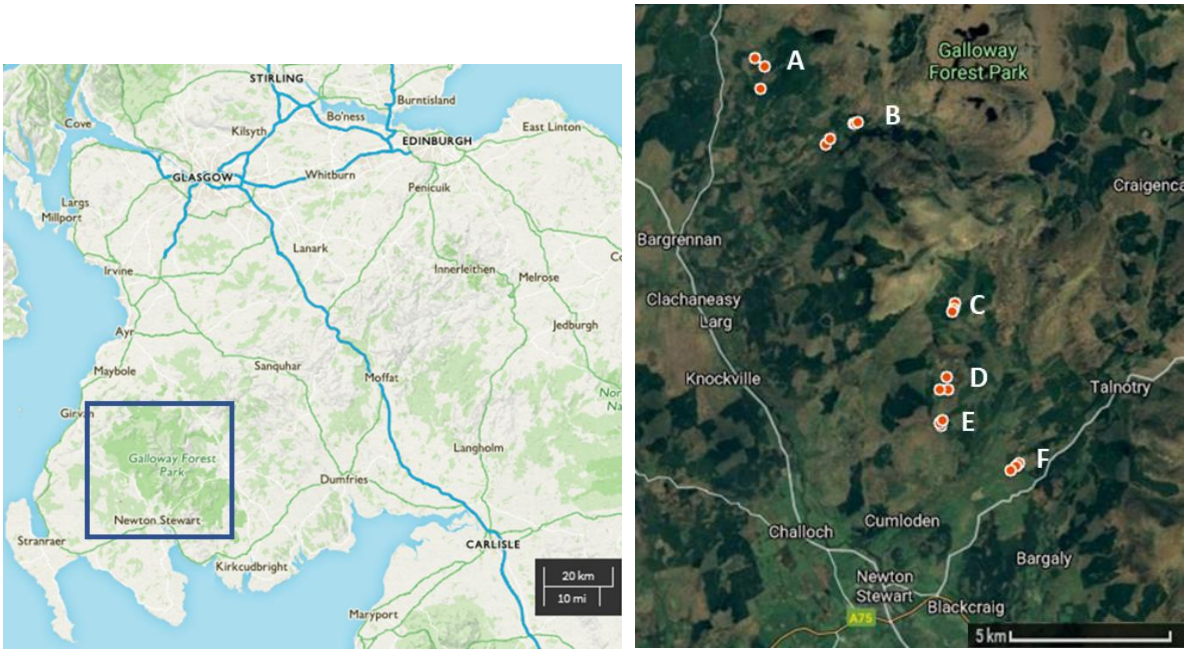


Figure 4: Location of the Galloway Forest Park in south-west Scotland (left) and location of our six field survey sites within the Forest Park (right); A) Palgowan, B) Glen Trool, C) Lamachan, D) Garlick Hill, E) Glenshalloch Hill, F) Loch of Lowes. Red points indicate locations of survey plots, three per site. Base map- Google maps.

Suitable site locations for observation of disease progression in thicket stage and mature stands of Japanese larch were identified from maps and Forestry Commission databases in the summer of 2016 (Figure 4). Thicket stage stands, between 4 and 10 years old, were chosen to allow observations and sampling of entire trees (Figure 4 right, sites A,C-F; Table 1) and a 60 year-old stand was included to investigate disease progression in a mature stand (Figure 4 right, site B; Table 1). The sites were visited in October 2016 and selected based on accessibility, size of trees and low-moderate prevalence of *P. ramorum* at the site to allow monitoring of disease progression. There were three study plots at each thicket stage site and five plots in the mature stage site (Table 2). At

the Palgowan, Lamachan and Garlick Hill sites, each plot was located in a separate, discrete stand of larch located in close proximity to each other (Figure 5a, c and d, respectively), whereas at the Loch of Lowes, Glenshalloch Hill and Glen Trool sites all plots were located within a single continuous stand of larch (Figure 5b and d and Figure 6, respectively). In May 2017 two more plots were established in the mature stand as felling was planned in the western end of the stand where the original plots were located (Table 3). Each plot covered an average of 1,300m^2 and the total area covered by all the plots was 27,000m^2. All stands contained Japanese larch alone except for Glenshalloch Hill which also contained Sitka spruce (*Picea sitchensis*).

In each plot where infection was present (Table 1), a centre tree was selected that showed symptoms consistent with *P. ramorum* infection, such as bleeding resinous cankers on the main stem with underlying dark brown lesions in the phloem when the outer bark was cut away. Samples of phloem tissue were taken from lesions on the centre tree of each plot for confirmation of the presence of *P. ramorum* as described in section 2.2.2. In the Loch of Lowes and Lamachan sites, where there was no *P. ramorum* infection during site selection, a centre tree was chosen away from the edge of the stand. Four transects were set out from each selected centre tree following the cardinal points of the compass and the first five trees lying directly on each transect were marked for survey. The distance between each tree on the transect was measured and the estimated height and diameter at breast height (DBH) was recorded (Table 2)

Table 1: Characteristics of the chosen sites.

Site	**Planting date**	**Number of stands within site**	***P. ramorum* symptom prevalence at start**
Palgowan	2006	3	Moderate
Loch of Lowes	2012	1	Absent
Glenshalloch Hill	2005	1	Low
Garlick Hill	2009	3	Low
Lamachan	2009	3	Absent
Glen Trool	1956	1	High

Table 2: Details of specific characteristics of each plot within the study sites.

Site	Plot	Elevation (m)	Aspect	Average DBH in 2016 (cm)
Palgowan	1	180	Flat	Data not obtained
Palgowan	2	170	West	Data not obtained
Palgowan	3	170	West	5.2
Loch of Lowes	4	150	South	2.4
Loch of Lowes	5	150	South	3.1
Loch of Lowes	6	157	North	2.8
Glenshalloch Hill	7	190	South-west	10.2
Glenshalloch Hill	14	181	South-west	8.6
Glenshalloch Hill	15	172	South-west	7.1
Garlick Hill	8	295	South-west	2.8
Garlick Hill	12	266	South-west	3.2
Garlick Hill	13	253	South-west	3.6
Lamachan	16	270	South-east	2.7
Lamachan	17	267	East	2.4
Lamachan	18	262	East	2.3
Glen Trool	9	85	South-east	24
Glen Trool	10	80	South-east	26
Glen Trool	11	90	South-east	28
Glen Trool	19	110	South-east	Data not obtained
Glen Trool	20	110	South-east	Data not obtained

Table 3: Number of plots surveyed at each time point during the study. Each plot contained 21 trees.

	September 2016	May 2017	September 2017	May 2018	September 2018	May 2019	September 2019
Mature plots	3	5	5	5	3	3	1
Thicket stage plots	15	15	15	15	15	15	15
Total number of trees	378	420	420	420	378	378	336



Figure 5: Maps showing locations of the three replicate plots within each of the five thicket stage study sites in Galloway Forest Park; a) Palgowan, b) Loch of Lowes, c) Lamachan, d) Garlick Hill (plots 8, 12, 12) and Glenshalloch Hill (plots 7, 45, 15). Scale is the same for all maps. Base map- Google maps



Figure 6: Location of the five mature plots within the Glen Trool stand. At the time the photograph was taken the west end of the stand (plots 9 and 10) had already been felled. Base map- Google maps

Infection in plot trees was monitored over a three-year period, from September 2016 until September 2019, through twice-yearly surveys conducted in mid to late May, approximately one month after the start of needle flush, and in early September, approximately two months before needle fall (Table 3). For investigation of mature plots, the level of crown dieback was visually assessed at 5 % increments. For investigation of thicket staged trees, disease symptoms were visually assessed separately on the following components i) needles, ii) fine shoots, iii) branch systems adjoining the main stem and iv) main stem (Figure 7).



Figure 7: Examples of symptoms scored in the thicket stage plots of Japanese larch. a) Healthy tree showing no signs of P. ramorum infection, b) needle whorls with central discolouration in September 2017, c) fine shoot dieback in May 2018, d) branch dieback in May 2018, e) main stem lesion in September 2016 showing white resin bleeds, the bark has been removed at the margins of the lesion to show the discoloration of the pholem characterisitc of P. ramorum infection. f) whole tree discoloruation caused by a lesion at the base of the main stem girdling the tree inn September 2018.

Mortality on each component was recorded as indicated in Table 4 whereby 0 % = asymptomatic and 100 % = dead. Thus, mortality was cumulative; a tree with 50 % main stem mortality would also have at least 50 % branch mortality and 50 % fine shoot mortality, due to girdling lesions causing death of all tree components above that lesion. The level of symptoms recorded in the field was converted into a 10-class system to allow statistical analysis and graphical depiction (Table 4). An average symptom severity score was also calculated; this was the mean symptom severity class score, for all the trees recorded, for each symptom, at each time point.

Table 4: Symptom severity class determination from observed symptom level. An observed symptom level of 0% indicates a healthy or asymptomatic tree whereas an observation of 100% would correspond to death.

Observed Symptom levels (%)	Symptom severity class
0	0
1-5	1
6-10	2
11-20	3
21-40	4
41-60	5
61-80	6
81-90	7
91-95	8
96-99	9
100	10

Notes on the general health of the entire stand were recorded, including prevalence of dead trees, deer damage and waterlogging/drainage. Shoots with grey/brown needles suspected to be infected with *P. ramorum* were sampled, brought to the lab and placed in sealed plastic bags, containing damp tissue to induce high humidity, and incubated for a few days at room temperature, in order to determine whether the pathogen was sporulating. Forty needles were then mounted on microscope slides, stained with lactophenol blue and the abaxial and adaxial surfaces examined under a light microscope to observe sporangial production. Needles were also surface sterilised with ethanol, washed with SDW and plated onto SMA to attempt *P. ramorum* isolation.

2.2.2 Isolation and identification of *P. ramorum* on symptomatic larch

Direct isolation and DNA extraction was attempted from lesions that were suspected to be caused by *P. ramorum* on trees in thicket staged plots. This was done to confirm the presence of *P. ramorum* in the plot and to subsequently determine the lineage of *P. ramorum* present. Where isolation was unsuccessful the lesion was resampled on the following visit.

For isolation from lesions on branches or the main stem the outer bark was removed with a scalpel. Lesions in the phloem and cambium caused by *P. ramorum* are characteristically dark brown in colour and sometimes contained resinous patches of a pink-red colour. The leading edge of the lesion was then identified and small sections (5x5mm^2 and as thin as possible) were cut from the cinnamon brown lesion edge. Tissue with pink-red colouration was avoided as isolation was very rarely successful from these areas. In the field, three tissue sections were plated onto modified synthetic mucor agar (SMA) *Phytophthora* selective medium in a Petri plate (see details below).

Following field isolations, the plates were double bagged and stored in a fridge box at 4°C until return to the lab. In the lab, plates were incubated in the dark at 16°C and checked every other day. Colonies that grew from the tissue sections were then sub-cultured onto carrot agar using aseptic single hyphal tip technique to ensure the resulting colony was a single genotype. All cultures were maintained between experiments on carrot agar plates at 16°C in the dark. To promote sporulation for identification colonies were incubated in continuous light for 21 days at room temperature. Isolates that were suspected to be *P. ramorum* were examined under a microscope for characteristic morphological features including abundant chlamydospores formed on short pedicels and semi-papillate, caducous sporangia (Werres et al., 2001).

Artificial growth medium

SMA was prepared according to Elliot et al. (1966) with the addition of antibiotics (Brasier et al., 2005) as follows: 10 g Sucrose, 1 g l-asparagine, 0.25 g magnesium sulphate, 0.001 g thiamine hydrochloride, 1 ml trace elements, 15 g technical agar, 0.5 g potassium dihydrogen orthophosphate, 0.5 ml 4% benomyl hydrochloride solution and 1000 ml distilled water, mixed in a one litre media bottle. The trace elements solution was made fresh on the day of SMA preparation as this was found to increase the later success of *Phytophthora* isolation. The pH was adjusted to 6.5 by adding 1 M sodium hydroxide. The bottles were then autoclaved at 121°C/15 lb psi for 15 minutes. After cooling 0.4 ml of a 2.5% suspension of pimaricin and 3 ml of a 1% w/v solution of rifamycin SV was added (Brasier et al., 2005). The SMA was then poured into 100 mm petri dishes and stored in the fridge. Plates were used within two weeks.

Cultures were grown and maintained on carrot agar (CA) plates for all experiments, which was made as follows: 200 g of carrots were washed and macerated in a blender with 500 ml of tap water. The carrot water mix was filtered through three layers of tissue and squeezed to extract all the juice. The resulting liquid was made up to one litre with additional tap water and poured into a one litre media bottle containing 15 g of agar (OXOID No.1, Fisher Scientific, UK). The bottle was gently shaken by hand to mix. The bottles were then autoclaved twice for 10 minutes at 121°C/15 lb psi.

Identification

Identification of cultures and the causal agent in lesion samples was carried out using a *P. ramorum*-specific qPCR assay (Tomlinson et al. 2005) (Table 5). Lineage was determined using lineage-specific qPCR assays developed by Feau et al. (2019) (Table 6).

Tissue samples were ground for DNA extraction by using two sterile steel 3mm ball bearings added to the tubes with each frozen sample and shaking at 25,000 revolutions in a ball mixer mill (Retsch, Germany) for up to 1 minute. The tubes were refrozen in liquid nitrogen and shaking was repeated

until a fine powder was obtained. For isolates in culture, sterile sand was added to mycelia scraped off the surface of one CA plate and placed in a tube with 400 µl of the lysis buffer from the Nacherey-Nagel Nucleo-spin Plant II kit (Fisher Scientific. UK). The sample was then macerated by hand using a polypropylene pellet pestle (Sigma Aldrich, USA). DNA was extracted using the Nacherey-Nagel Nucleo-spin Plant II kit (Fisher Scientific. UK) following the manufacturer's protocol. During the cell lysis step using Buffer PL1 the samples were incubated at 65 °C for 60 minutes, vortexing every 15 minutes. The extracted DNA was stored at -20°C.

Samples were first tested by real-time PCR amplification in order to confirm the presence of *P. ramorum*. The assay used TaqMan Environmental Mastermix 2.0 (Applied Biosystems, Foster City, CA, USA) in 20 µl reaction volumes containing 2 µl template DNA. qPCR was carried out in an ABI Prism 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) following the protocol of Tomlinson et al. (2005), with the addition of generic 18s primers (Table 5) to confirm the presence of amplifiable DNA and to check for PCR inhibition if the *P. ramorum* primers did not yield a product. Reactions were carried out under the following conditions: a pre-incubation period of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. All isolates identified as *P. ramorum* were subsequently tested to identify their linage using assays developed by Feau et al. (2019) (Table 6). Reactions were carried out under the following conditions: a pre-incubation period of 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

*Table 5: Details of primers and Taqman probes for the P. ramorum-*specific *qPCR assay (Tomlinson et al. 2005).*

Target lineage	Primer/probe name	[Reporter] Sequence 5'-3' [Quencher]
P. ramorum	Pram114-F	TCATGGCGAGCGCTTGA
	Pram1527-190R	AGTATATTCAGTATTTAGGAATGGGTTTAAAAAGT
	Pram1527-134-T	[FAM] TTCGGGTCTGAGCTAGTAG [TAMRA]
18s	18sF	GCAAGGCTCAAACTTAAAGGA
	18sR	CCACCACCCATAGAATCAAGA
	18sP	[JOE] ACGGAAGGGCACCACCAGGAGT [BHQ_1]

Table 6: Details of primers and Taqman probes for the EU1 and EU2 lineage specific qPCR assays (Feau et al. 2019).

Target lineage	Assay name	Primer/Probe name	[Reporter] Sequence 5'-3' [Quencher]
***P. ramorum* EU1**	PramEU1-C358	PramEU1-358-F	GTCGGCCTTAAGAAGTCGTC
		PramEU1-358-R	ATCCCGAATAGGGCTAGAGG
		PramEU1-358-P	[FAM]CTTGTGCACCACCACAAGAATCC[BHQ_1]

***P. ramorum* EU2**	PramEU2-C268	PramEU2-268-F	GCCACCACAAATACAAGCAC
		PramEU2-268-R	TGTGCTACTCGACTGGGTCT
		PramEU2-268-P	[FAM]ATTTGAGGCCGAGCCATTAGTGA [BHQ_1]

2.2.3 Analysis of climatic data

Climate records were extracted from Centre for Environmental Data Analysis' (CEDA) archives through the CEDA OGC Web Services and included the Met Office MIDAS (Met Office Integrated Data Archive System) records. The records for eight weather stations close to the field sites (Figure 8) were used as detailed in Table 7. The averages of multiple stations were used to cover gaps in individual station records.

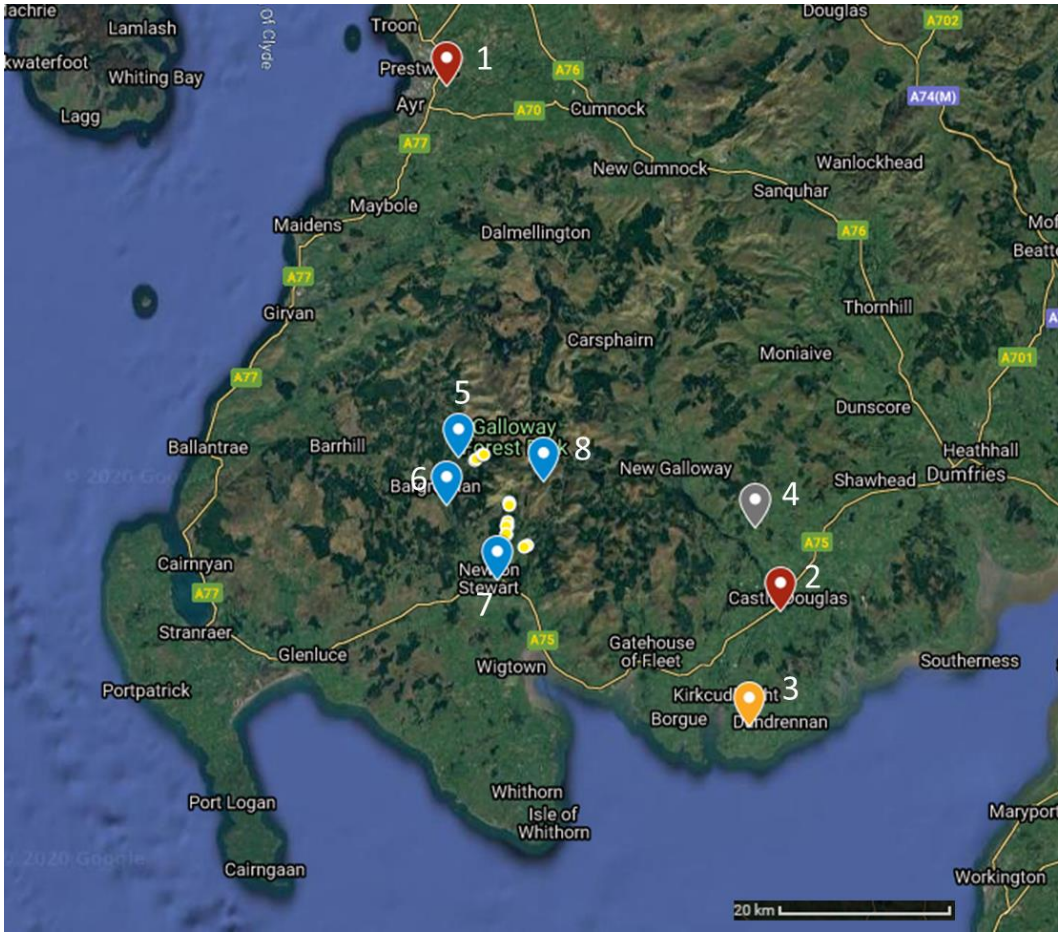


Figure 8: Locations of weather stations providing climate data in Dumfries and Galloway. Colour of markers show the climatic data set used from each station; Red-temperature, orange-wind, grey-snowfall, blue-precipitation. Yellow points show location of field survey sites.

Table 7: Weather stations data sets used to extract climate data.

Station name	Source Identifier	Met Domain	Data set used	Location on map
Auchincruive	1005	AWSDLY	Temperature	1
Threave	1035	AWSDLY	Temperature	2
Dundrennan	1033	HMC	Hourly Wind	3
Barwhillantry House	13298	DLY3208	Snow	4
Kirriedarroch Cottage	13312	WAMRAIN WADRAIN	Monthly precipitation Daily precipitation	5
Brigton	19017	WAMRAIN WADRAIN	Monthly precipitation Daily precipitation	6
Creebridge	56077	WAMRAIN WADRAIN	Monthly precipitation Daily precipitation	7
Upper Black Laggan	13253	WAMRAIN WADRAIN	Monthly precipitation Daily precipitation	8

2.2.4 Controlled inoculation experiments to investigate infection pathways

Two isolates of *P. ramorum* were selected with growth patterns and growth rates as described for EU2 isolates on carrot agar (O'Hanlon et al., 2017). Isolate 16.13a was from a branch lesion on a tree within plot 16 at Lamachan in May 2017 and isolate CT8 was from a main stem lesion on the centre tree of plot 8 at Garlick Hill in September 2016.

Sporangial inoculum was prepared according to Denman et al. (2005) except that in order to retain sporangia the inoculum was not filtered. Colonies were grown on carrot agar plates and incubated in continuous light for 21 days at room temperature. Production of sporangia was confirmed by examination under a dissecting microscope. Each plate was then flooded with 3 ml of sterile water and sporangia dislodged by rubbing the surface of the culture with a sterile glass rod. The resulting suspension from 15 plates was pooled and refrigerated at 7°C for 1 hour. After refrigeration the beaker was transferred to room temperature (~20°C) for 75 minutes to induce zoospore release. The concentration of sporangia and zoospores was determined using a haemocytometer. Three classes of sporangia were counted: full sporangia, those that had released their zoospores and those that had germinated directly. The suspension was adjusted to achieve a final concentration of 2–5 × 10^5 total sporangia mL^{-1}. Released zoospores were also present in the inoculum. Residual pieces of mycelium and chlamydospores in the suspension sank to the bottom of the beaker and were not observed in the suspension that was used in inoculation. Inoculations were conducted within 90 minutes of the inoculum being produced. For mycelial inoculations, 5 mm diameter plugs were cut from the growing margins of 21-day-old colonies on carrot agar.

Inoculum viability was tested on the same day that the experiment was set up by placing a 20 µL droplet of sporangial suspension, or a mycelial plug, onto healthy, surface sterilised Rhododendron leaves collected from a woodland close to Forest Research's Northern Research Station, Roslin, Scotland. The leaves were incubated in Ziplock bags containing damp cotton wool at 16 °C for 14 days and examined for development of lesions.

2.2.4.1 Preliminary experiment to compare sporangial and mycelial inoculation methods.

A preliminary experiment was carried out in December 2017 to test the effectiveness of sporangial inoculation methods in causing lesions on Japanese larch (JL) and European larch (EL) compared with mycelial plug inoculation method. Three-year-old JL and EL, approximately 20-40 cm in height, were sourced from a commercial forestry nursery and maintained outside in 15 cm diameter pots prior to the experiment. In preparation for inoculation, each plant was placed in a Ziplock bag with the bottom cut off, secured around the pot with an elastic band and supported by a garden cane to prevent contact with the tree. The bottom of the pot was not covered so that the soil could uptake water from the tray below.

The mycelial plug inoculations were carried out using isolate CT8. It was intended to use the same isolate for both types of inoculum but as CT8 failed to release any zoospore the backup isolate, 16.13.a, had to be used for sporangial inoculations. A suspension of sporangia and zoospores was desired as this would better mimic the natural infection system.

Four inoculation methods were tested in this experiment;

A) point inoculation by applying a 20 µL droplet of sporangial suspension onto intact bark
B) as in A) but onto wounded bark
C) 2 ml of sporangial suspension applied to each entire plant by misting
D) mycelial plug inoculation of wounded bark

For methods A and B, inoculum was pipetted onto three junctions per plant where a side branch joined the main stem in the upper, middle, and lower crown. In method B, bark was wounded immediately before inoculation by cutting a 2 mm T shape across the top of each branch junction using a scalpel. For method C, sporangial suspension was sprayed over the entire plant using a misting spray bottle. Following inoculation using methods A, B and C, plants were left for 20 minutes to allow the inoculum to dry, the Ziplock bags were then sealed to maintain humidity for 7 days, after which the bags were removed. Control inoculations were carried out for each of methods A, B and C as described above using sterile distilled water only. For inoculation method D a cork borer

was used to remove a 5 mm plug of bark from the main stem of each plant, 5 cm above soil level, and the mycelial plug placed, mycelial side down, into the wound. The inoculation point was covered in damp cotton wool, wrapped in Parafilm and covered in a layer of tinfoil. Control inoculations were carried out using sterile carrot agar. The plants were maintained on trays in a greenhouse at 16^{o}C with ambient lighting and watered twice weekly directly into the trays. At 5 and 8 weeks post-inoculation, the overall health of each plant was recorded and the length of lesions that formed in the phloem either side of each inoculation point was measured. For inoculation method C, where there was no defined inoculation point, the outer bark was removed from each entire plant to look for lesions in the phloem.

The experiment comprised 32 treatment combinations; two larch species (JL and EL), four inoculation methods (A, B, C, D), two inoculum types (*P. ramorum* and control) and two disease assessment timepoints (5 and 8 weeks). Three replicate plants were inoculated per treatment combination. The experimental design was a randomised complete block, with three blocks each containing one plant per treatment combination arranged randomly over four trays. The experiment was carried out once.

2.2.4.2 Experiment to investigate infection at different stages of bud flush.

Five-year-old potted JL (30-50 cm tall, average 39.8 cm) and EL (40-80 cm tall, average 71.5 cm) were maintained outside in ambient conditions up until December 2018. The potted trees were placed in a greenhouse at 20 ^{o} C with 16 hours of lighting per 24 hours for 25 days to promote flushing and then individuals were selected at each stage of flush (Figure 9). Trees still in a fully dormant state were selected from those that had remained outside.

Four stages of flush were inoculated in this experiment (Figure 9 and described below) plus a positive control in which bark was wounded and inoculated as described for method B in the previous experiment;

1. Fully dormant buds
2. Bud swelling, green of new needles just visible
3. First needle appearance, bud has closed spindle-shaped needles
4. Mid-flush, elongated needles starting to open into rosette

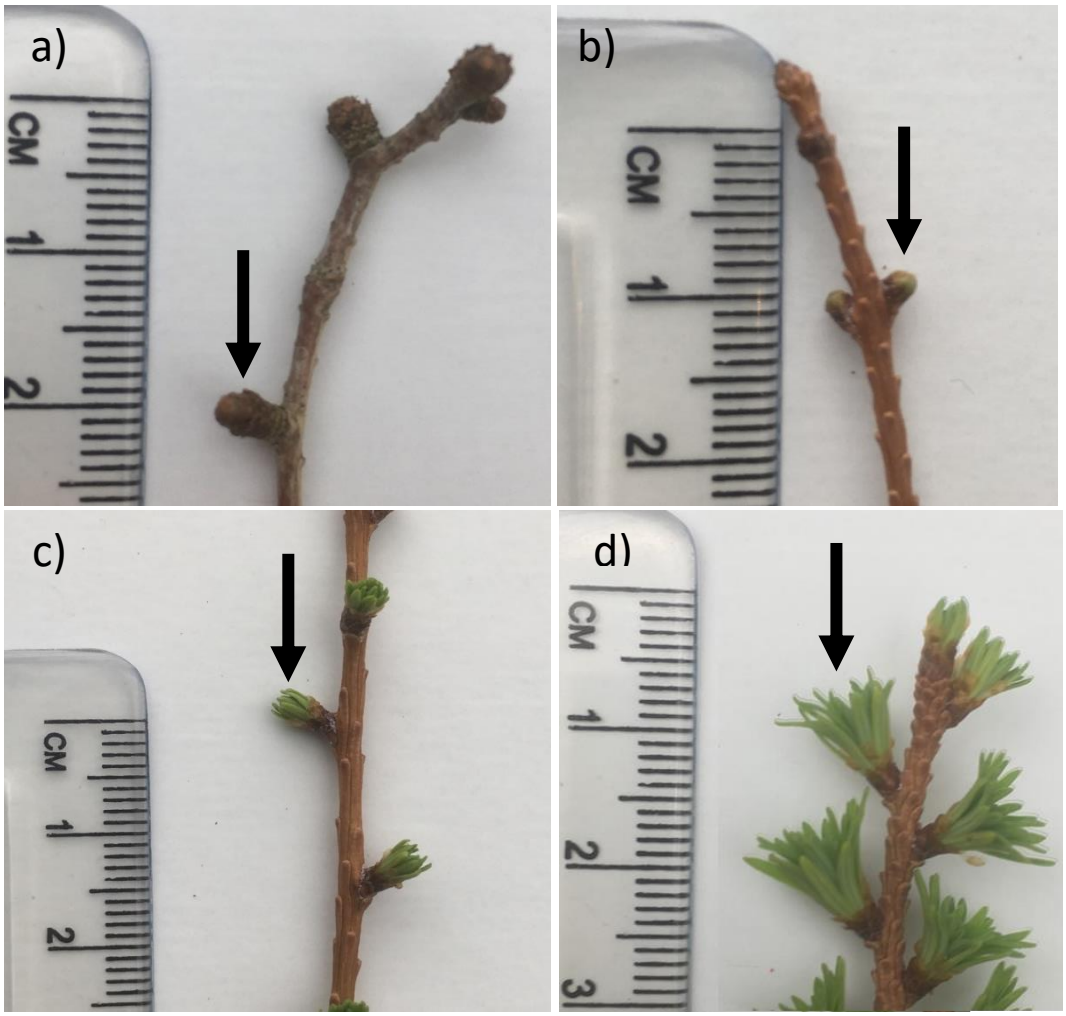


Figure 9: Different stages of flush in Japanese larch selected for inoculation. a) stage 1- full dormancy, b) stage 2- bud swelling with green colouration appearing, c) stage 3- first needle appearance, d) stage 4- mid flush. Similar stages were also selected in European larch.

The experiment comprised 16 treatment combinations; two larch species (JL and EL), five inoculation methods (stages 1,2,3,4 and a positive control of wounded bark), two inoculum types (*P. ramorum* and control) and one destructive disease assessment timepoint (3 weeks). Three replicate plants were inoculated per treatment combination. The experimental design was a randomised complete block, with three blocks each containing one plant per treatment combination arranged randomly over four trays (Figure 10e). The experiment was carried out twice, with the second replicate inoculated the day after the first and then running concurrently in separate greenhouses.

Sporangial suspension was prepared as described in section 2.2.4 using the EU2 lineage isolate 16.13a of *P. ramorum*. Three undamaged upward facing buds at each defined stage of flush were selected for inoculation and marked with electrical tape (Figure 10a).

For inoculation of stage 1 buds, 10 µl of inoculum was pipetted into each dormant bud where it soaked into the bud scales. For inoculation of buds at stages 2, 3 and 4 the needles were too hydrophobic to place the droplet on the bud using the pipette tip (Figure 10a), as the waxes on the needle surface prevented the droplet from wetting the needles. To inoculate these buds the droplet was placed onto the junction between the bud and the branch (Figure 10b) and then the droplet was drawn upwards onto the bud and around the base of the needles using a pipette tip. Control inoculations were carried out for each stage as described above except that sterile distilled water only was used. After inoculation, trees were enclosed in a plastic bag that was clipped shut around the main stem of the tree to maintain humidity (Figure 10d). These bags were removed after 7 days.

The trees were examined for *P. ramorum* symptoms after one week and then destructively sampled after three weeks (Figure 10c). Any discoloration in the bud base was recorded and the lengths of lesions which had developed in the phloem were measured.

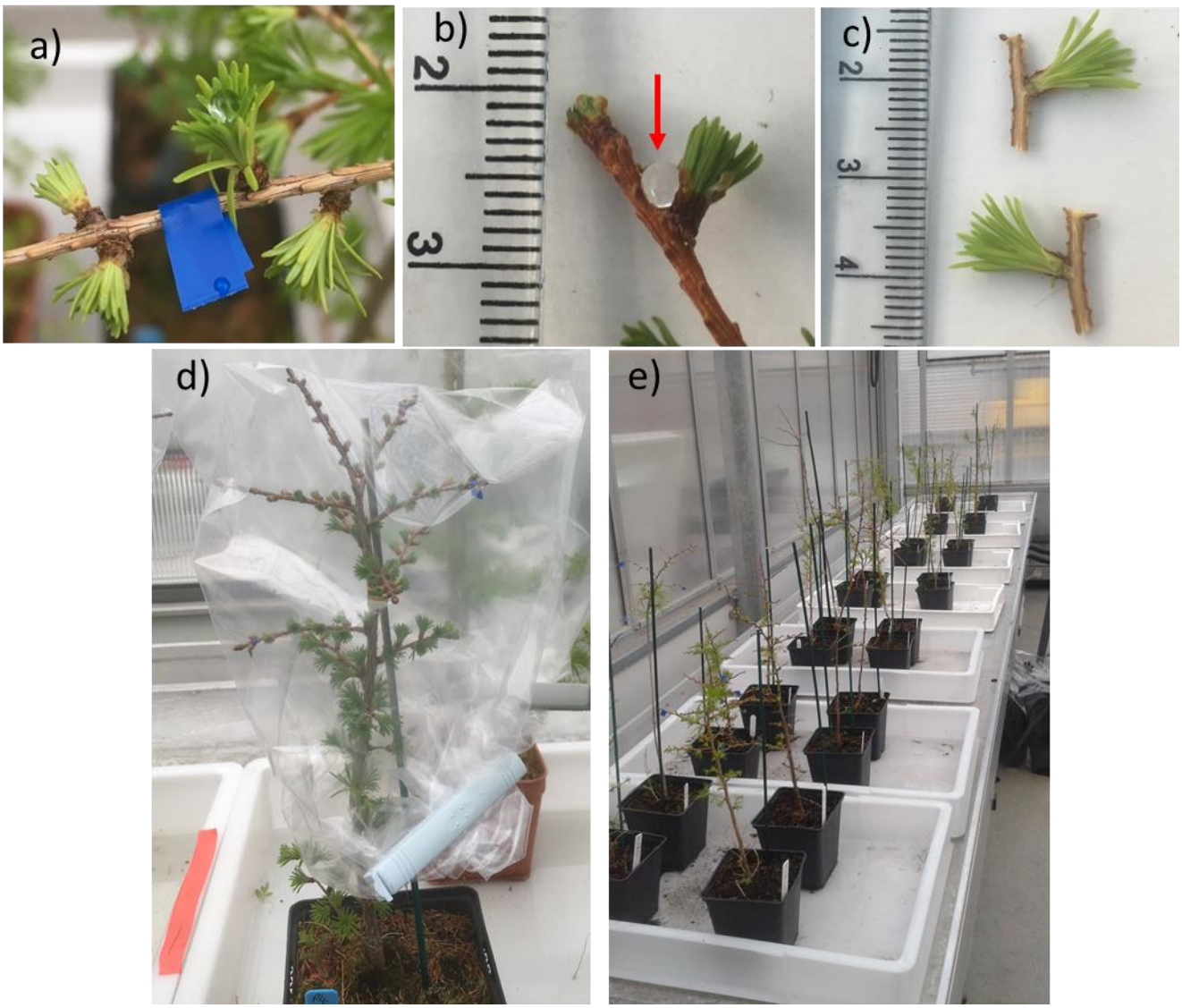


Figure 10: Photographs illustrating the experimental approaches used in the experiment to investigate infection at different stages of bud flush, a) mid- flush (stage 4) buds showing hydrophobic effect of the needle surface as a water droplet sits on the needles, b) Inoculation of stage 3 needle-showing buds. The red arrow shows the inoculation droplet placed at the bud collar, c) One inoculated flushing bud sectioned longitudinally for histological examination, d) Bagged inoculated trees, e) Trees arranged in random blocks.

2.2.4.3 Histological examination of inoculation points

In order to carry out histological examination of inoculation points, another set of inoculations was set up in one of the greenhouses used for the experiment in section 2.2.5. The two experiments were run concurrently but physically separated from each other. Only three stages of bud flush were selected for histological examination: fully dormant, bud swelling and mid-flush, (stages 1, 3 and 4,

respectively), as well as an additional positive control inoculation involving application of sporangial suspension to wounded bark as described in section 2.2.4.1.

The histology experiment comprised 12 treatment combinations; two larch species (JL and EL), three stages of flush (stages 1,3,4) and two inoculum types (*P. ramorum* and control). Three replicate plants were inoculated per treatment combination with up to six buds on each tree inoculated. The experimental design was a randomised complete block, with three blocks each containing one plant per treatment combination arranged randomly over four trays. The experiment was carried out twice.

Two buds from three trees per treatment combination were sampled at 24 and 72 hours post inoculation. For the wounded bark inoculations samples were taken at 24 and 72 hours and 7 days post inoculation. Each bud or stem section was dissected longitudinally (Figure 10c) and placed into 2ml Eppendorf tubes and fixed in formal acetic alcohol fixative (Atom Scientific Ltd, Manchester, UK).

The fixed samples were sent to the Easter Bush Pathology lab (The Royal (Dick) School of Veterinary Studies. Edinburgh, UK) where they were dehydrated in a graded series of ethanol, embedded in paraffin wax and sections 4 µm thick were cut and adhered to Poly-L-lysine coated glass microscope slides.

Slides were deparaffinised with Histoclear™ (Agar Scientific Ltd, Essex, UK), rehydrated in a graded series of ethanol and then stained with Toluidine blue, following the methods of Strable et al. (2020), or with Phloroglucinol, following the methods of Liljegren (2010). The stained slides were examined under a binocular Wild M10 microscope with attached DFC425C camera (Leica Microsystems, Wetzlar, Germany).

2.2.5 Statistical analysis

The data were analysed using R version 4.0.4 (R Core Team, 2020) in RStudio (RStudio Team, 2020). A linear mixed model fit by REML was fitted to the tree symptom data with plot and site treated as random effects. A linear mixed model was fitted to the plot mortality data with plot and site. A Kendall's τ coefficient test was used to analyse correlations between mortality at different time points. For each climate variable a Kruskal-Wallis test was used to investigate if that variable for each month differed significantly between years. A pairwise Wilcoxon rank sum test with Benjamini-Hochberg correction for multiple testing (Benjamini & Hochberg, 1995) was then used to identify which years were different.

2.3 Results

2.3.1 Observations of disease progression in thicket stage plots

2.3.1.1 Symptom levels

Plot trees were assessed over three years for symptom severity based on four different component categories; needles, fine shoots, branch systems adjoining the main stem and main stem. Data on progression of symptom severity for each component over time, pooled across all plots, is shown in Figure 11. Additionally, mean symptom severity scores were calculated for each symptom from the mean symptom severity score of all the trees assessed at each time point. Different progressions of symptoms in different tree parts over time were observed.

Levels of needle discolouration and needle mortality remained relatively low throughout the study period (Figure 11a,b). There was an increase in needle symptoms from May 2017 to September 2017 and the mean needle symptom severity score also increased from 1.1 to 2.4 during this period. Needle discolouration and mortality decreased in May 2018 since the discoloured needles were shed over winter, with fresh needle flush in the spring. The needle discolouration observed in September 2017 could have been due to early senescence as the needle discolouration was not the greying needles that are characteristic of *P. ramorum* infection (Figure 12g) but rather yellowing and browning (Figure 12a-f). Observations of needles examined under the microscope found no signs of *P. ramorum* sporulation at any timepoint.

Dieback of fine shoots on the upper crown or outer crown periphery (Figure 13c-e) was a very common symptom, particularly in the May assessments. In these cases, shoots had died at some point between the autumn assessment and spring assessment suggesting multiple simultaneous infections across the crown during the intervening time period. Shoot dieback across the plots increased significantly between May 2017 and September 2017 (Figure 11c), with mean disease severity scores of 2.3 and 2.8, respectively. Although statistically significant, this increase was not strikingly obvious during the field observations. A second significant increase in shoot dieback occurred between September 2017 and May 2018 (Figure 11c) with mean symptom severity scores of 2.8 and 4.1, respectively. This increase in shoot dieback was clearly obvious in the field and from aerial photographs of our plots (Figure 14), where lack of needles on the fine shoots was extensive enough that areas of the canopy looked brown. This coincided with a significant expansion of the epidemic on larch across south-west Scotland. There was no significant increase in shoot dieback severity over the summer of 2018 but between September 2018 and May 2019 another significant increase in shoot dieback occurred with an increase in mean severity score from 4.2 to 5.8.

Branch dieback was observed to occur when aerial lesions expanded in the phloem and cambium of fine shoots into the main branch systems, in many cases continuing down the branch to its junction with the main stem and extending into the main stem (Figure 13 and Figure 15). As observed for fine shoots, new branch infections appeared to occur over the winter period, between surveys, and became apparent by subsequent lack of flush and dead branches the following spring. The temporal progression of branch dieback followed a similar pattern to that of shoot dieback but over a slightly later time period (Figure 11d). Mean severity scores for branch dieback increased from 2.1 to 3.2 between September 2017 and May 2018 and again from 3.4 in September 2018 to 5.0 in May 2019. Some trees with significant branch dieback throughout the crown had fresh epicormic growth in May 2019 (Figure 15c), however, most of these epicormics were found to have died in September 2019 as the branch lesions extended into the main stem.

Lesions on the main stem caused by *P. ramorum* had characteristic resin bleeds from cracked outer bark (Figure 15a) and were associated with infection extending into the stem from a diseased branch. Removal of outer bark revealed dark brown lesions in the phloem and cambium extending both up and down and frequently girdling the main stem, causing death of all tissues above the lesion. In thicket stage larch lesions were also observed at the base of the main stem, extending from soil level upwards and sometimes girdling the stem base, causing death of the entire tree. These basal lesions were not connected to any aerial lesion and it is assumed that the initial infection point was at ground level. At the start of the survey in September 2016, nine trees had these basal lesions and were selected as centre trees when setting out survey plots (Figure 7e). Few new main stem lesions were observed across plots until September 2019 when main stem lesions were observed in 42 trees (19.7% of all live trees). These new main stem lesions were all connected to branch infections and not to an infection from a ground level infection point. Main stem dieback increased gradually in the first four surveys (Figure 11e) with mean severity scores increasing from 0.8 to 1.4. This was followed by a significant increase in main stem dieback between September 2018 and May 2019 with the mean severity score increasing from 1.6 to 3.4.

It was also clear as the study progressed that top dieback was a prevalent symptom and was particularly notable in May 2018 when 57 trees (20% of all live trees in the survey) were affected. Typically, the leading shoot had died over the winter with dieback extending down the crown to varying extents (Figure 13f and Figure 15b,d). Top die back varied in extent with some trees only having the top 20 cm dead and others with >1 m dieback, but in almost all cases the dieback was observed to progress down the main stem over subsequent surveys until the entire tree was killed. Many of the trees exhibiting top dieback in May 2018 had died by the following survey in September

2018. Frequent top dieback, as a symptom in itself, was recorded again in May 2019 with a total 94 trees affected across the plots.

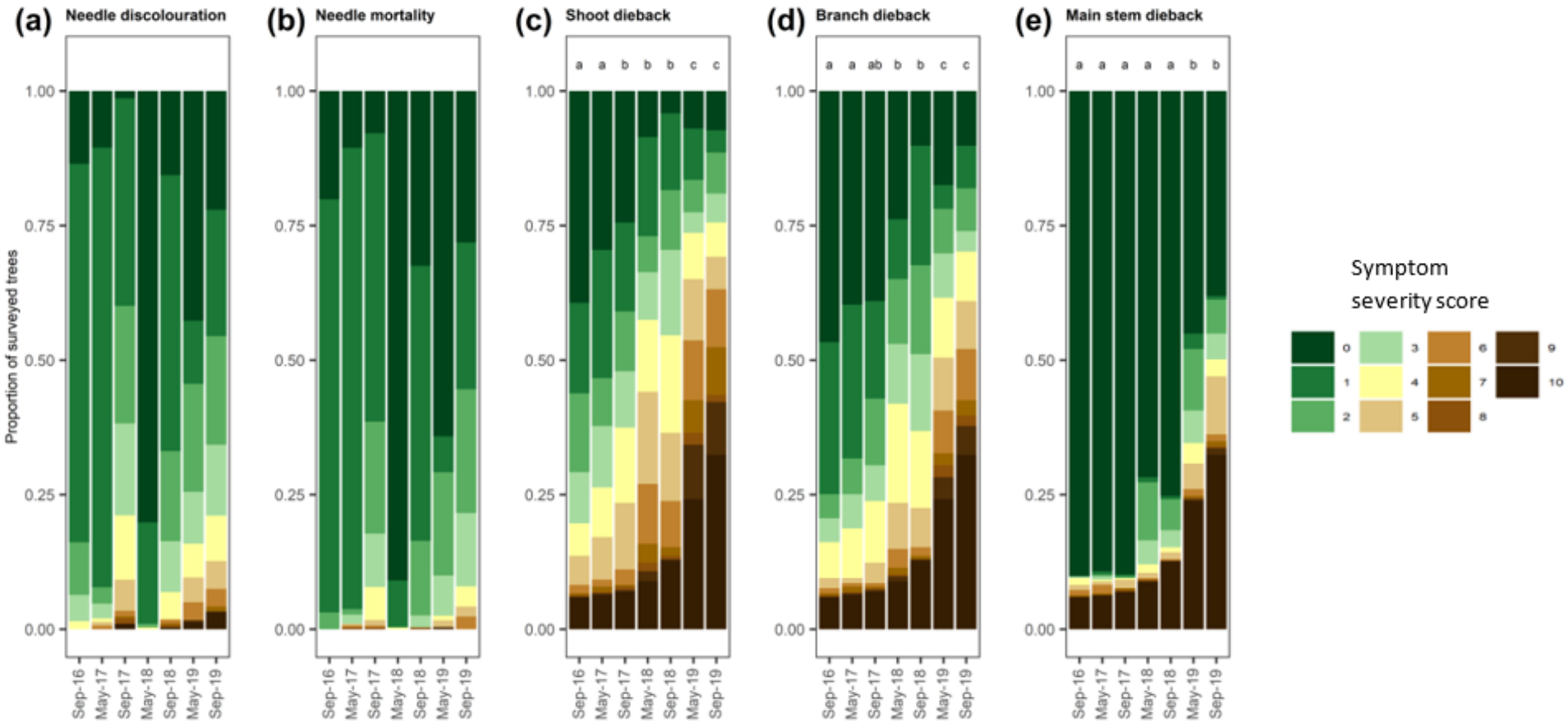


Figure 11: Temporal progression of P. ramorum symptoms on (a, b) needles, (c) fine shoots, (d) branches and (e) main stem, in thicket stage stands of Japanese larch assessed twice a year from September 2016 until September 2019. Data are pooled for all trees scored across fifteen plots (n=21 per plot, 315 total). Each bar represents a different disease assessment timepoint and is coloured to represent the proportion of trees with symptoms falling within each of ten symptom severity classes where by 0 is visibly healthy and 10 is dead. Different letters immediately above each bar indicate significant differences between time points determined through lmer and post-hoc analysis by Tukey test with Bonferroni corrections p<0.0083. Note: no statistical tests were carried out on needle symptoms as needles are shed each winter preventing comparisons between timepoints.



Figure 12: Needle symptoms observed during the plot surveys. a) upper left branch with healthy needles, lower right branch showing healthy but chlorotic needles in September 2016, b) Needle chlorosis, c) Needle discolouration from the tips of the needles to the centre of the needle whorls, occurring in May 2019, d) scattered needle discoloration and mortality across fine shoots, e) needles dying from the tips downwards, f) hanging clusters of dead needles in September 2017, g) freshly dead needles in September 2019, showing the greying colouration suggestive of P. ramorum infection.



Figure 13: Fine shoot and branch symptoms observed in thicket stage larch during the surveys. a) lesion on branch showing mortality of needle whorl, b) branch with bark removed to show fresh lesion which has gridled the branch and prevented flush in the outer portion of the branch, in September 2019, c) isolated branch dieback in May 2018, in this example the phloem is discoloured indicating the underlying lesion is tracking down the branch toward the main stem, d) needle absence in fine shoots observed in May 2018 where underlying lesions have resulted in failure to flush of needles on the tips of the shoots, e) isolated branch dieback in May 2018 in this example the phloem on the main branch is still healthy but the shoots are dead, f) top dieback in May 2018, with finger and knife showing the extent of the lesion observed when the bark was removed.



Figure 14: Aerial photography of the site at Glenshalloch Hill. Photos taken by Patrick Robertson (Forestry and Land Scotland) from a survey helicopter in a) July 2017 and b) July 2018. The increase in shoot and branch mortality that that was observed on the ground between autumn 2017 and spring 2018 can be seen in the brown areas where the canopy has died.



Figure 15: Main stem symptoms observed in thicket stage larch during the surveys. a) Cracked bark and white resin bleed of a main stem lesion that was connected to a lesion that had tracked down a side branch in September 2019, b) dieback of the main stem from the top downwards, c) epicormic growth in May 2019 on the main stem of a tree where all the branches had died the previous autumn, d) whole tree mortality in September 2019.

Pooling data across all five sites revealed the major trends of symptom development over time and the timing of significant increases in dieback. However, symptom severity and rate of symptom progression varied among sites (Figure 16), most notably, symptom prevalence remained low at Loch of Lowes throughout the course of the experiment, whereas prevalence of shoot, branch and main stem dieback increased markedly at all other sites during the course of the study (Figure 16). There were differences in the time of occurrence of significant increases in shoot, branch and main stem dieback among sites (Figure 16). For example, shoot dieback at Palgowan increased significantly in September 2017 (Figure 16a), whilst at Loch of Lowes (Figure 16b) and Glenshalloch Hill (Figure 16c) the first significant increases in shoot dieback occurred earlier, in May 2017. Similar trend variation among sites was observed for branch dieback, although it should be noted that all sites showed significant increases in both shoot and branch dieback between September 2017 and May 2018 (Figure 16)

Main stem dieback increased at all sites over the course of the study, but the extent and timing of the increase also varied among sites. Palgowan had the highest level of main stem dieback at the start of the survey (Figure 16a) with a severity score of 2 at this point, but Glenshalloch Hill and Garlick Hill had the greatest severity of main stem dieback at the end of the study period (Figure 16c,d) with mean severity scores in September 2019 of 8.3 and 6.1, respectively, compared with 3.8 for Palgowan. A more moderate increase from 0.5 to 3.9 was observed at Lamachan whilst Loch of Lowes maintained its low infection rate with an increase in average symptom severity score from 0 to 0.3 over the same time period.

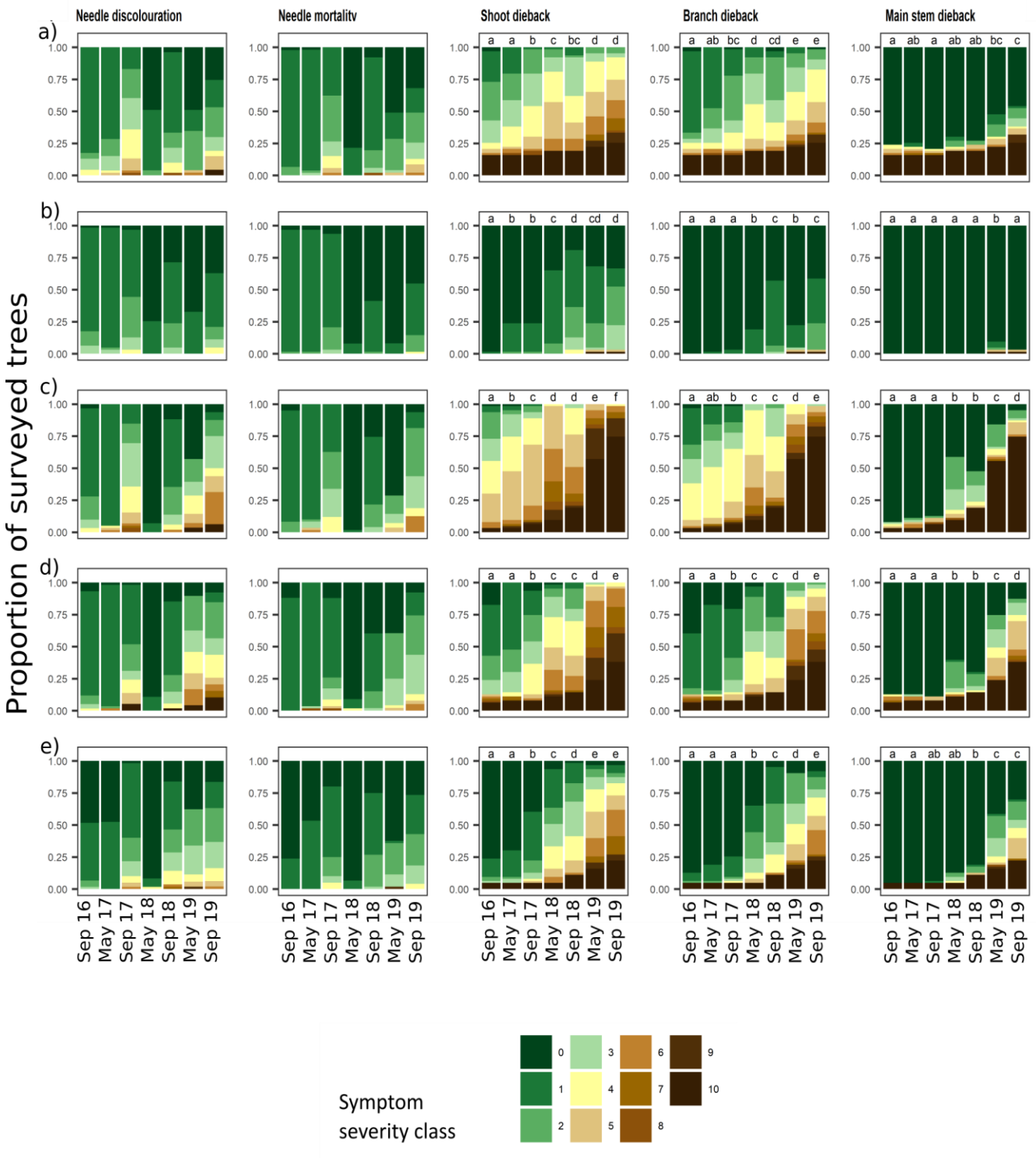


Figure 16: Temporal progression of symptoms by site. a) Palgowan, b) Loch of Lowes, c) Glenshalloch Hill, d) Garlick Hill, e) Lamachan. In each site n=63. Each bar represents a different disease assessment timepoint and is coloured to represent the proportion of trees with symptoms falling within each of ten symptom severity classes where 0 is visibly healthy and 10 is dead. Different letters immediately above each bar indicate significant differences between time points determined through lmer and post-hoc analysis by Tukey test with Bonferroni corrections p<0.0083. Note: no statistical tests were carried out on needle symptoms as needles are shed each winter preventing comparisons between timepoints.

2.3.1.2 Identification of the primary infection point

Observations of increased shoot and branch dieback in May 2018 across many of the plots also revealed a common pattern in terms of where infections appeared to originate. On many of the fine shoots that had been recently killed, a consistent pattern of bud deaths was observed whereby buds that formed the previous year did not flush in spring (Figure 17) and appeared to form the central point of lesions which were actively extending at the time of sampling in May 2018. These observations suggested that infection of branches had occurred through the buds over winter or during spring. Neighbouring fine shoots were affected at a later stage of lesion development, as they showed different stages of flush before they had died (Figure 17a,b). This indicated that the lesion was tracking down the fine shoot to the main branch and subsequently affecting neighbouring shoots (Figure 17c). These observations suggest that buds on fine shoots are the primary infection sites, with infection occurring between late autumn and spring, and lesions actively extending in the spring. This formed the hypothesis for the inoculation experiments in sections 2.3.4 and 2.3.5.

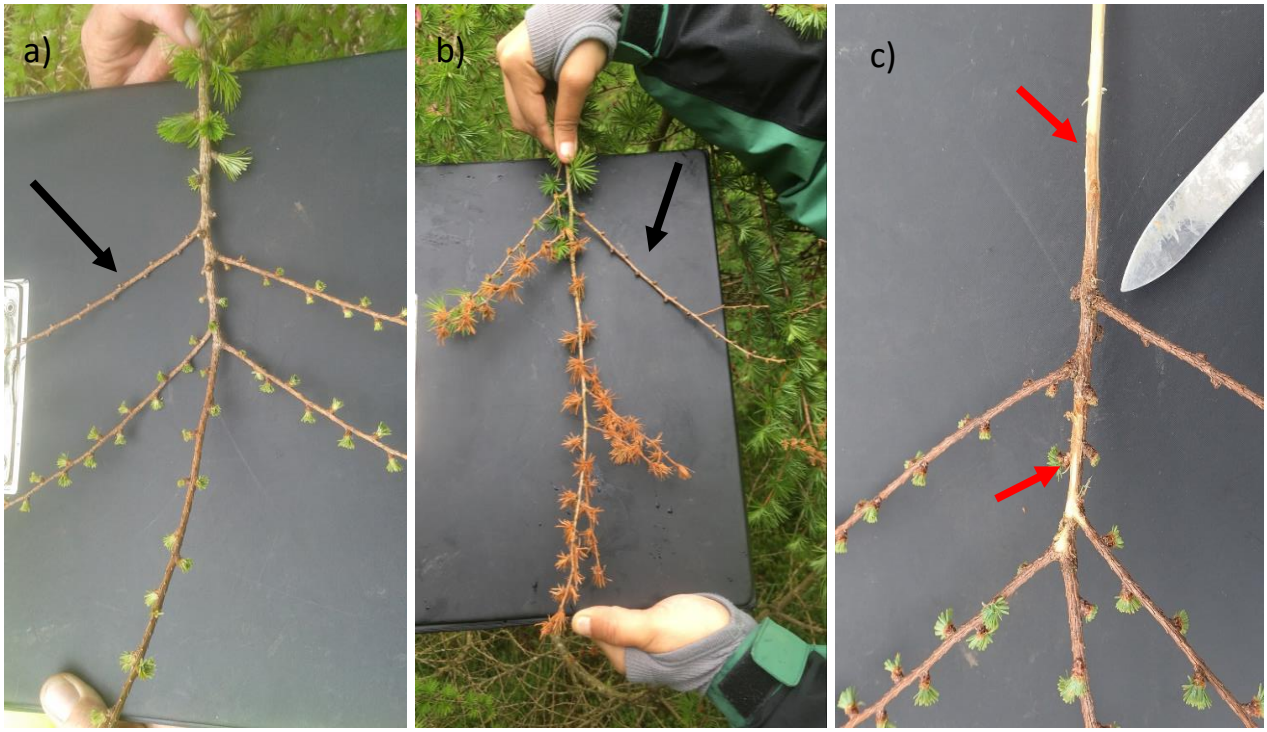


Figure 17: Bud deaths on fine shoots of Japanese larch in May 2018. a) and b) Black arrows indicate a side shoot that had died before flushing in the spring, either side of which lesions were actively extending. The lesion in each picture has extended to the point where buds have fully flushed on the main shoot and the lesion has girdled the shoot, killing and preventing further flush of the outer fine shoots, c) outer bark removed to show lesion extending down the main shoot. Red arrows indicate extent of the lesion which is centred around the dead shoot indicated by the knife blade.

2.3.1.3 Temporal variation in tree mortality in the thicket stage stands

Incidence of whole tree mortality increased significantly between September 2018 and May 2019 (Figure 18). Of the 315 trees surveyed across the five sites, less than 10% were dead at the beginning of the study. Mortality increased slowly at first, doubling in the first two years of the survey. However, mortality then doubled again over a single winter, between September 2018 and May 2019 (Figure 18).

The rate of increase in mortality over winter 2018-19 varied across sites. The mortality at Glenshalloch Hill increased highly, three sites (Palgowan, Garlick Hill and Lamachan) increased moderately and Loch of Lowes showed negligible increase (Figure 19). Variation in mortality was also observed among plots within a site, most notably at Garlick Hill whereas at Palgowan mortality progressed more similarly across plots (Figure 19). The level of mortality in a stand in the first survey in September 2016 was not a good predictor of the level of mortality in the stand at the end of the three-year study period in September 2019 (Figure 20). A Kendall's τ coefficient test showed that although a slight positive correlation of $R=0.27$ exists, this is not statistically significant ($p=0.19$). Mortality was not analysed by site as the small sample size (five sites) meant that any analysis would not have been robust enough to give reliable results.

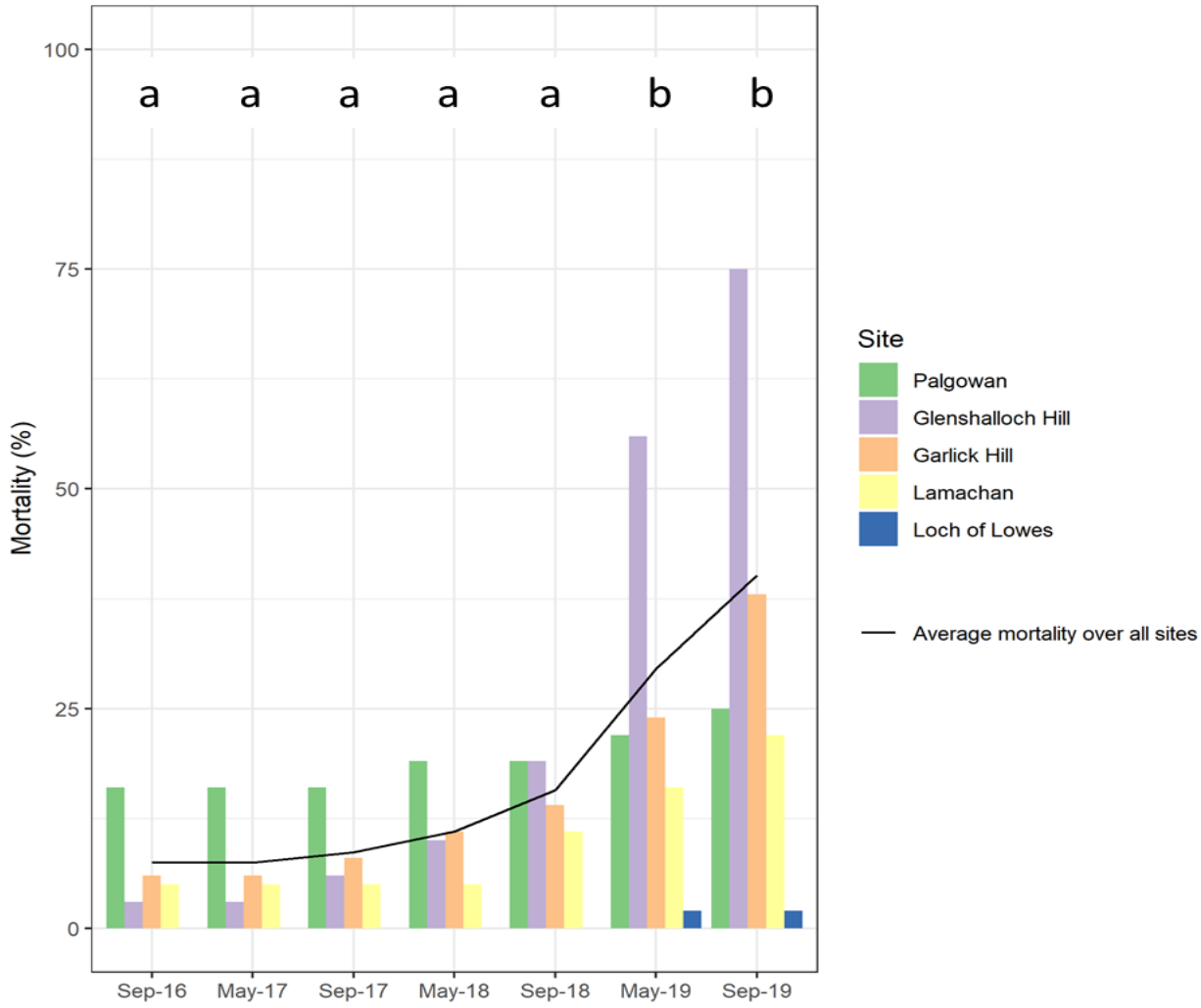


Figure 18: Mortality of Japanese larch trees within thicket stage stands infected with P. ramorum assessed twice a year from September 2016 until September 2019. Data are pooled for all trees scored across fifteen plots (n=63 per plot, 315 in total). Bars represent the different study sites each comprising three plots. Letters above each time point indicate significant differences between time points determined through lmer and post-hoc analysis by Tukey test p<0.05.

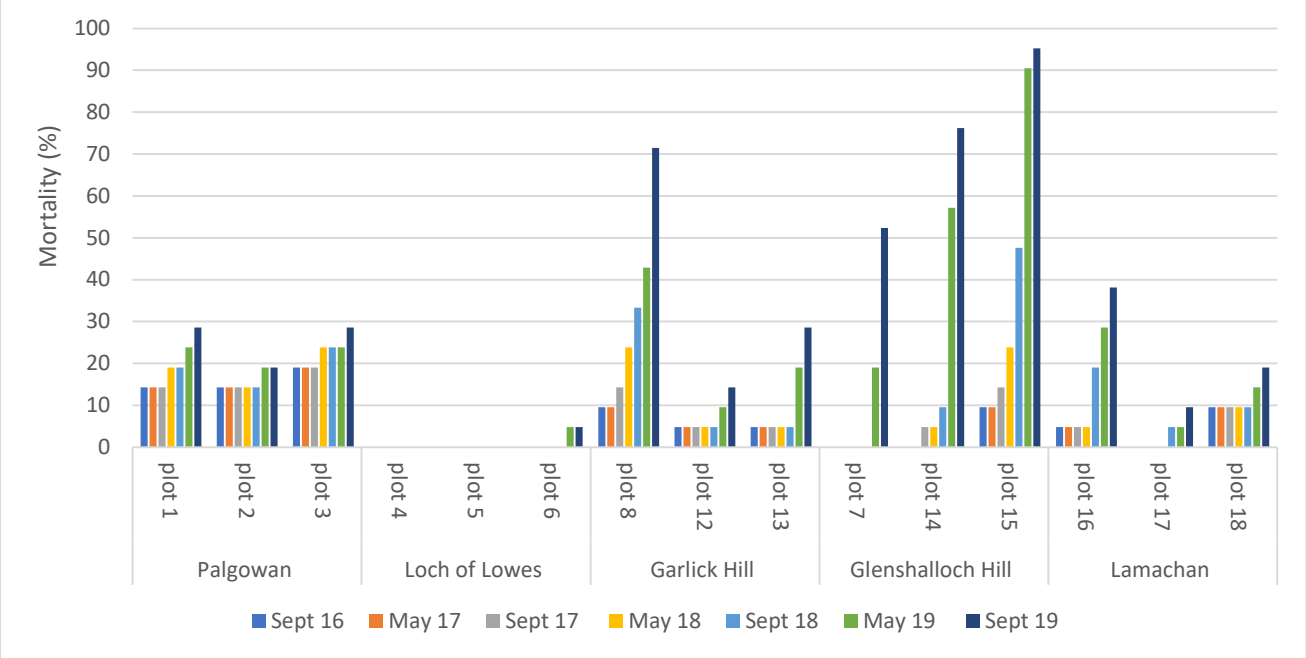


Figure 19: Mortality of Japanese larch trees in fifteen plots located within thicket stage stands assessed twice a year from September 2016 until September 2019. Each bar represents the mean of 21 trees assessed within each plot at each time point.



Figure 20: Relationship between mortality of Japanese larch trees in fifteen plots located within thicket stage stands in September 2016 and in September 2019. For each plot n=21. R and p values shown are from a Kendall's τ coefficient test of the relationship between the two sets of data.

2.3.1.4 Disease progression in post epidemic mature stands

A total of five plots were established in one mature stand of Japanese larch at Glen Trool with high levels of infection in 2016. Due to felling and wind throw it was not possible to record mortality and symptoms in all the plots across the time course of the experiment. Overall crown dieback increased only gradually over the course of the study and it was not significant (Figure 21). Many trees remained healthy at the end of the study. Mortality attributable to *P. ramorum* increased most notably in plots 9 and 10, which were felled after May 2018 (Figure 22). The increase in mortality in plot 19 in September 2019 (Figure 22) was due to wind throw of live trees in severe storms two days prior to the survey period; examination of these fallen trees found them to be generally healthy prior to uprooting.

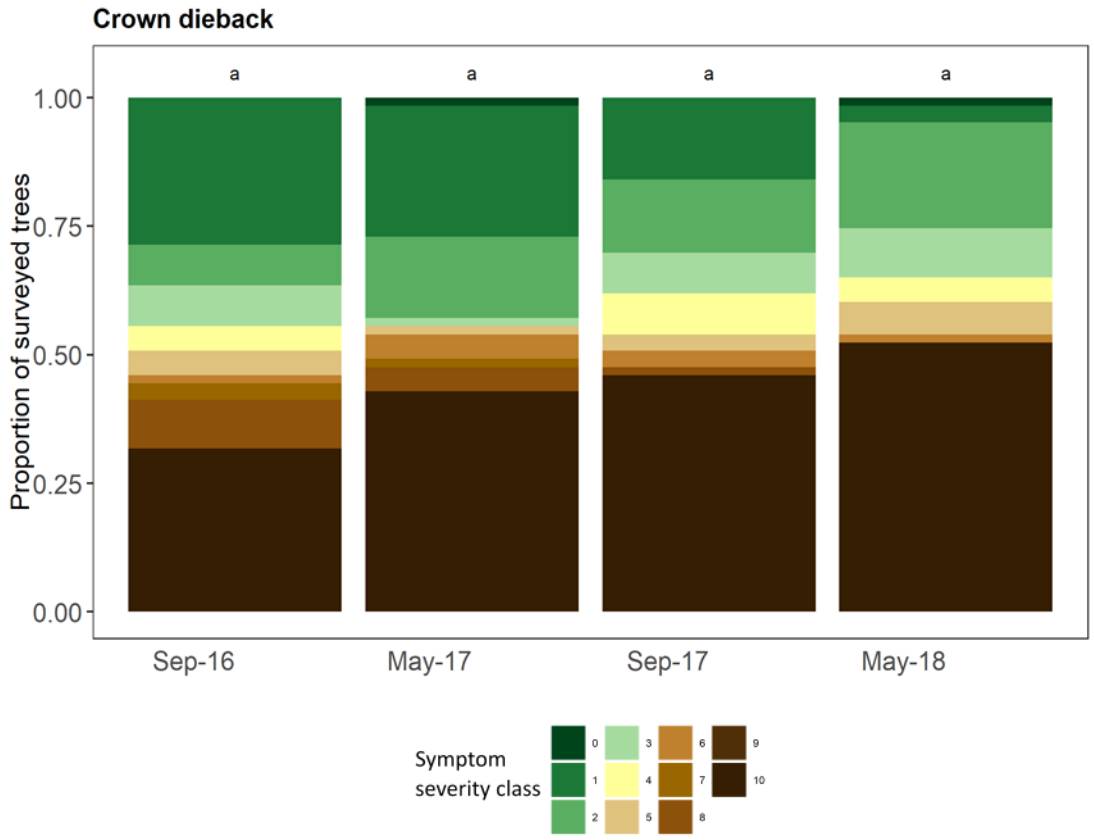


Figure 21: Temporal progression of crown dieback in mature stage stands of Japanese larch at Glen Trool, assessed twice a year from September 2016 to May 2018. Data are pooled for all trees scored across three plots within the site (n=63). Each bar represents a different disease assessment timepoint and is coloured to represent the proportion of trees with symptoms falling within each of ten symptom severity classes where by 0 is visibly healthy and 10 is dead. Same letters immediately above each bar indicate no significant differences between time points determined through lmer and post-hoc analysis by Tukey test with Bonferroni corrections p<0.0083.

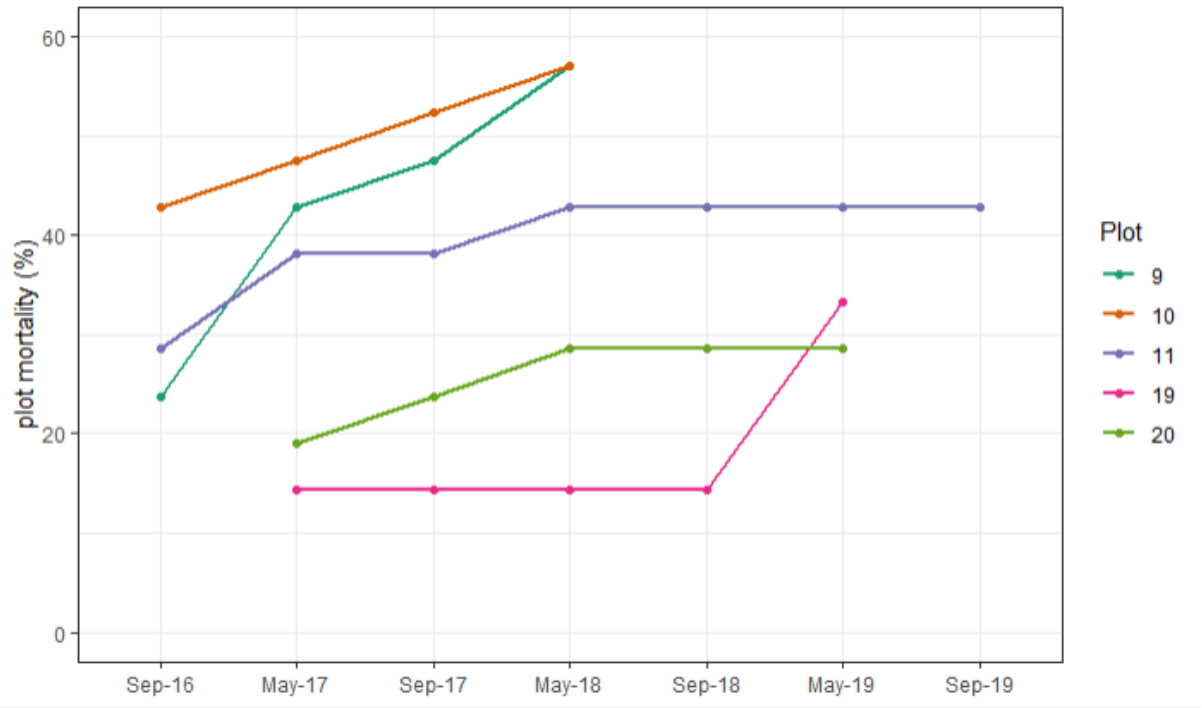


Figure 22: Mortality of Japanese larch trees infected with P. ramorum within five plots located in mature stands assessed twice a year from September 2016 until September 2019, n=21.

2.3.1.5 Isolation and identification of *P. ramorum*

A total of 32 isolates of *P. ramorum* were obtained from 16 of the 18 plots (Table 8). All successful isolations originated from phloem lesions. Attempted isolations from symptomatic needles were unsuccessful. All isolations positively identified as *P. ramorum* were subsequently put through lineage specific qPCR testing and all isolates were positively identified as being of the EU2 lineage. The plates included positive controls of known EU1 and EU2 isolates as well as standard negative controls. The EU1 positive control was the only positive result for the EU1 specific primers (Figure 23). No EU1 lineage positive results were found in the collected isolates. No successful isolations were made from plots 5 and 6. Repeated isolation attempts were carried out at each visit to confirm the presence of *P. ramorum* in each site. After infection was confirmed no further isolations were attempted.

In addition to the isolations, 24 tissue samples gave positive qPCR results for *P. ramorum* and were positively identified as being of the EU2 lineage. These included 12 samples from phloem lesions and 12 samples of symptomatic needles.

Table 8: Identification of P. ramorum in the study plots of thicket stage larch. Note that in some plots isolations were only successful after repeated attempts and that qPCR positives come from lesion DNA and so may predate successful isolations.

Plot	Site	Date of first qPCR positive	First successful isolation			Total number of isolates collected
			Date	Sampled Tree	Tissue	
1	Palgowan	Sep-16	May-17	Centre	Stem lesion	3
2	Palgowan	Sep-16	May-18	In plot	Aerial lesion	2
3	Palgowan	Sep-16	May-17	Centre	Stem lesion	1
4	Loch of Lowes	May-18	May-18	In plot	Aerial lesion	4
5	Loch of Lowes	No confirmed infection				
6	Loch of Lowes	No confirmed infection				
7	Glenshalloch Hill	Sep-16	Sep-16	In plot	Stem lesion	2
14	Glenshalloch Hill	Sep-17	May-18	In plot	Aerial lesion	1
15	Glenshalloch Hill	Sep-16	May-17	Centre	Stem lesion	2
8	Garlick Hill	Sep-16	Sep-16	Centre	Stem lesion	3
12	Garlick Hill	Sep-16	Sep-16	Centre	Stem lesion	1
13	Garlick Hill	Sep-16	May-17	In plot	Aerial lesion	4
16	Lamachan	May-17	May-17	On transect	Aerial lesion	2
17	Lamanchan	Sep-17	Sep-17	In plot	Basal lesion	3
18	Lamanchan	May-18	May-18	In plot	Aerial lesion	2

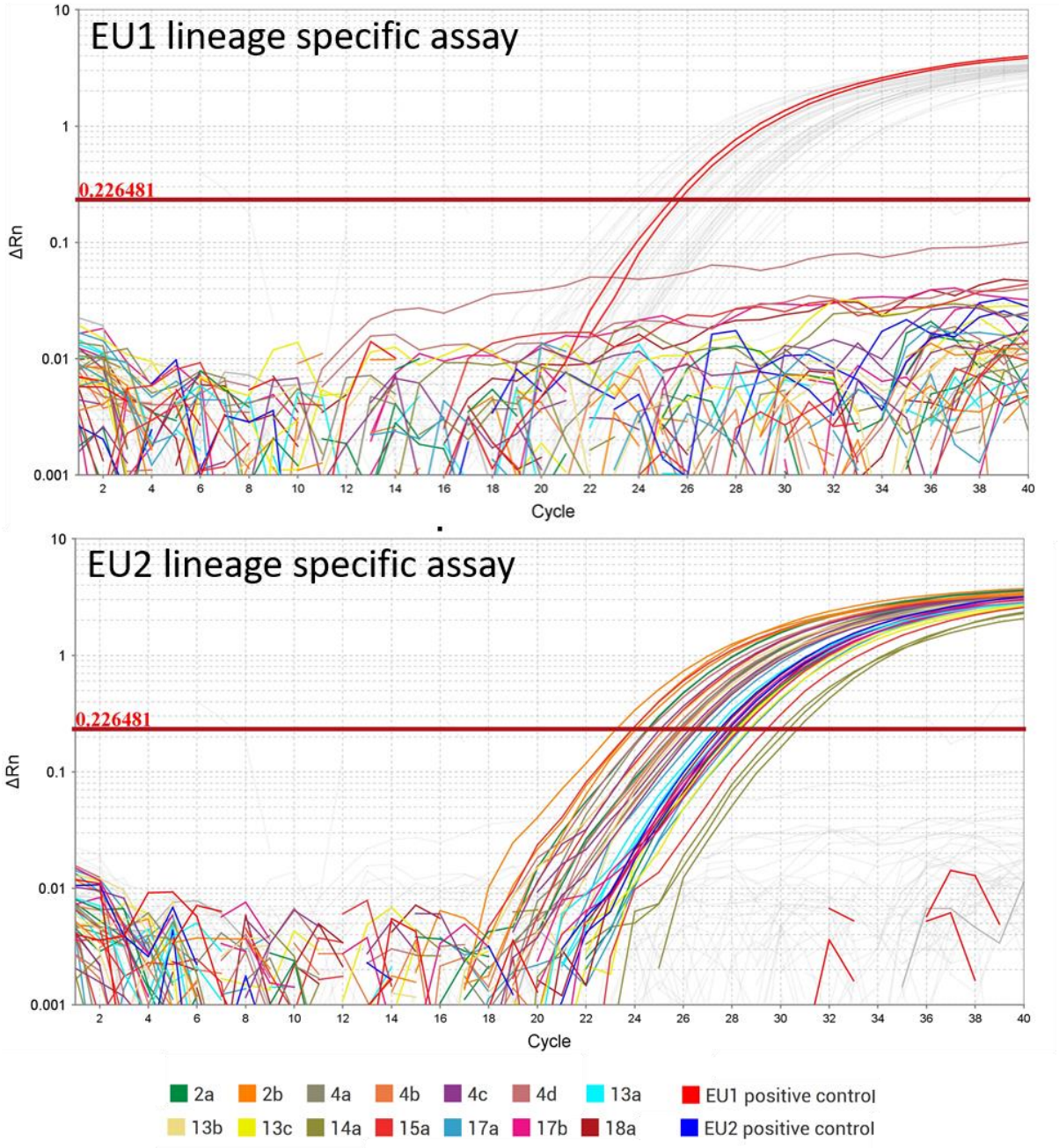


Figure 23: Amplification curves from lineage specific qPCR testing of a representative plate of known P. ramorum isolates collected from the Galloway forest. The numbering of the samples refers to the plot from which they were collected.

2.3.2 Temporal variation in climatic variables

A period of increased spread of *P. ramorum* infections observed both in our study plots and in the wider Galloway forest in spring 2018 allowed us to consider how climatic factors influence outbreaks as explanatory variables. By considering environmental conditions that might be similar around the observed disease expansions in 2013 and 2018 it might be possible to identify climatic factors associated with each major disease expansion event.

2.3.2.1 Precipitation

Increased summer precipitation was observed in the year preceding the 2013 and 2018 outbreaks. June-August 2012 had 60% more precipitation than the 19-year average and 2017 was 30% wetter than average (Figure 24). The Intervening years were 20% drier than average. A Kruskal-Wallis rank sum test with post-hoc Pairwise comparisons using a Wilcoxon rank sum test of the average daily rainfall shows that summer precipitation in 2012 and 2017 was significantly different from 4 other years within the decade (Table 9). The year of 2017 also had greater than average precipitation in autumn and winter.

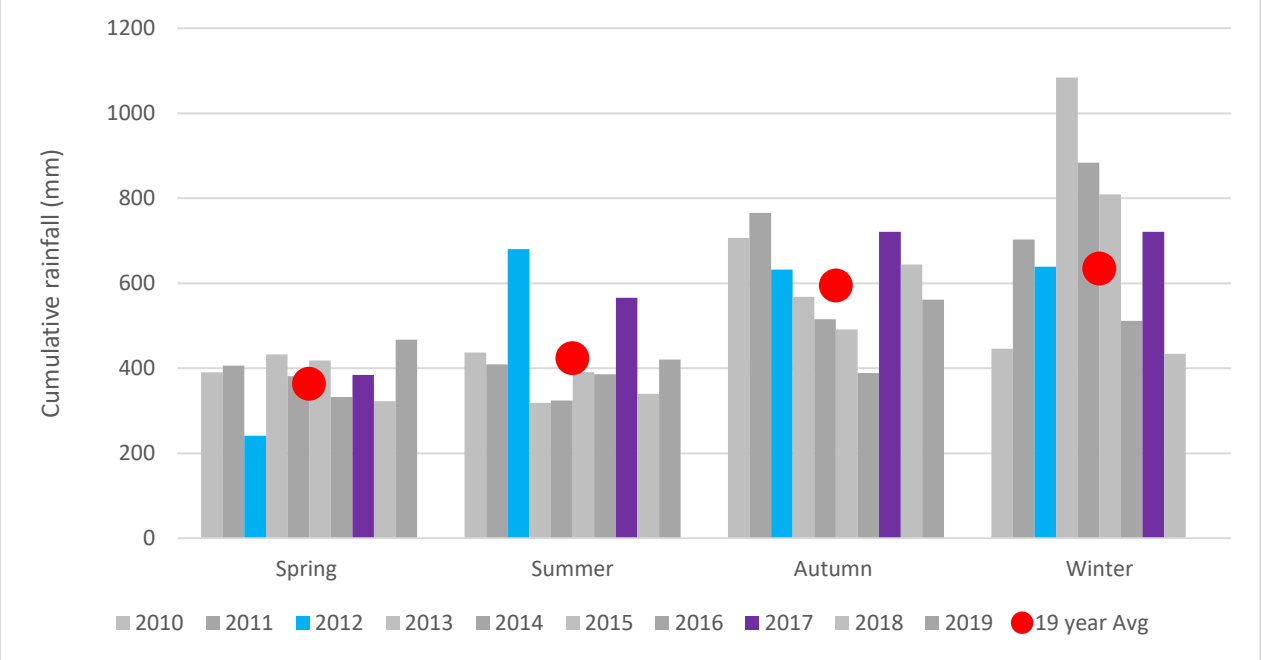


Figure 24: Seasonal precipitation in the region of the study site in the Galloway Forest area of south-west Scotland from 2010 to 2019. Each bar represents cumulative rainfall (mm) during each season per year with spring defined as 1st March to 31st May, summer as 1st June to 31st August, autumn as 1st September to 30th November, and winter as 1st December to last day of February. Cumulative rainfall was calculated from the average daily total recorded across four weather stations: Kirriedarroch Cottage, Brigton, Creebridge Upper Black Laggan (See Figure 1 for locations of stations). Red dots indicate the seasonal average over a 19-year period from 2000-2019 from the same weather stations.

Table 9: Differences in seasonal precipitation in the region of the study site in the Galloway Forest area of south-west Scotland from 2010 to 2019 using Kruskal-Wallis rank sum test with post-hoc pairwise comparisons using Wilcoxon rank sum test with Benjamini-Hochberg correction for multiple comparison. Years sharing the same letter are not significantly different from each other. p<0.05.

Year	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**
Significance indicators	ab	ab	a	bc	bc	ab	ab	a	c	bc

2.3.2.2 Wind direction and speed

The predominant wind direction recorded in the Galloway Forest Park is west to south-west from 210-250° (Forestry and Land Scotland, 2017). Occasional easterly storms occur. Analysis of wind direction from 2010 to 2019 showed that March 2013 and March 2018 differed significantly from all other years in that wind direction was predominantly from the east, with median wind directions of 70° and 80°, respectively (Figure 25). These differences in wind direction correspond to the easterly winds of the 'Beast from the East' storm in 2013 and 'Storm Emma' in 2018. Wind speed during each March across the ten years was less variable within the month and also across years. Although 2013 has the highest maximum wind speeds of the decade, and 2018 the third highest, there were no apparent correlations between wind speed and our years of interest (Figure 26).

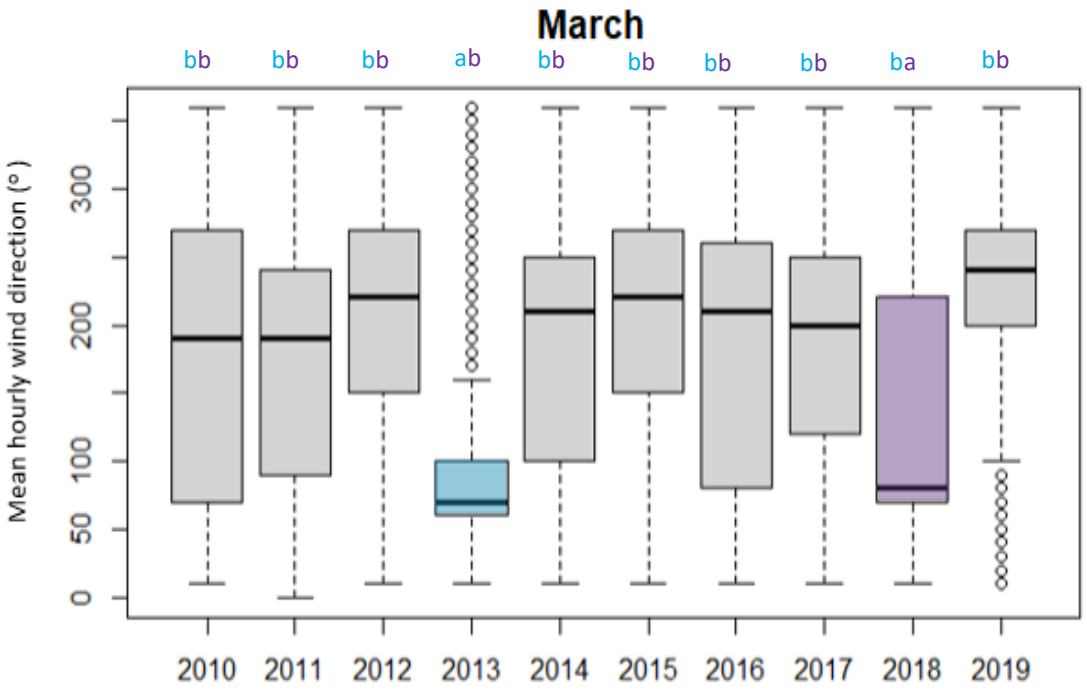


Figure 25: Wind direction in March for each of ten years 2010-2019, recorded as the mean direction each hour from 1am on the 1st to 12pm on the 31st of March. The box shows the interquartile range with the thick black line at the median. The whiskers show the top and bottom 25% of the data and the dots represent outliers that are outside 1.5 times the interquartile range above the upper quartile and below the lower quartile. n= 744 for each year. Kruskal-Wallis rank sum test with post-hoc pairwise comparisons using Wilcoxon rank sum test show 2013 and 2018 are significantly different from all other years and from each other p<0.05. The colour of the letters matches to years subject to the pairwise test.

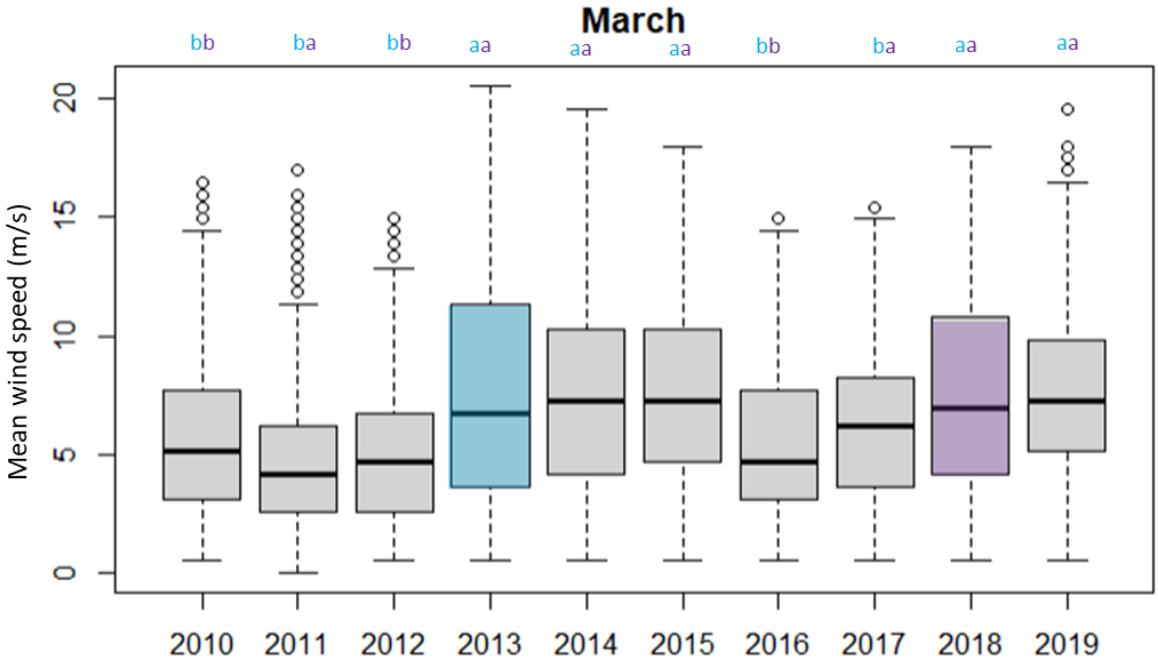


Figure 26: Wind speed in March for each of ten years 2010-2019, recorded as the mean direction each hour from 1am on the 1st to 12pm on the 31st of March. Different letters immediately above each box indicate significant differences between years determined through Kruskal-Wallis rank sum test with post-hoc pairwise comparisons using Wilcoxon rank sum test p<0.05. The colour of the letters matches to years subject to the pairwise test. n= 744 for each year.

2.3.2.3 Temperature

Minimum and maximum daily temperatures in winter showed differences in our years of interest (Figure 27). Kruskal-Wallis rank sum test with post-hoc pairwise comparisons using Wilcoxon rank sum test allowed identification of significant differences among years. December 2012 was significantly colder than usual with a lower daily temperature than five other years this decade and 2013 was colder than four other years (Figure 27). There were no significant differences in January temperatures across the decade. In February 2013 and 2018 minimum temperatures were colder than five other years. March 2013 was the coldest of the decade with significantly lower minimum and maximum daily temperatures than all other years. March 2018 was the second coldest of the decade and significantly colder than five other years (Figure 27)

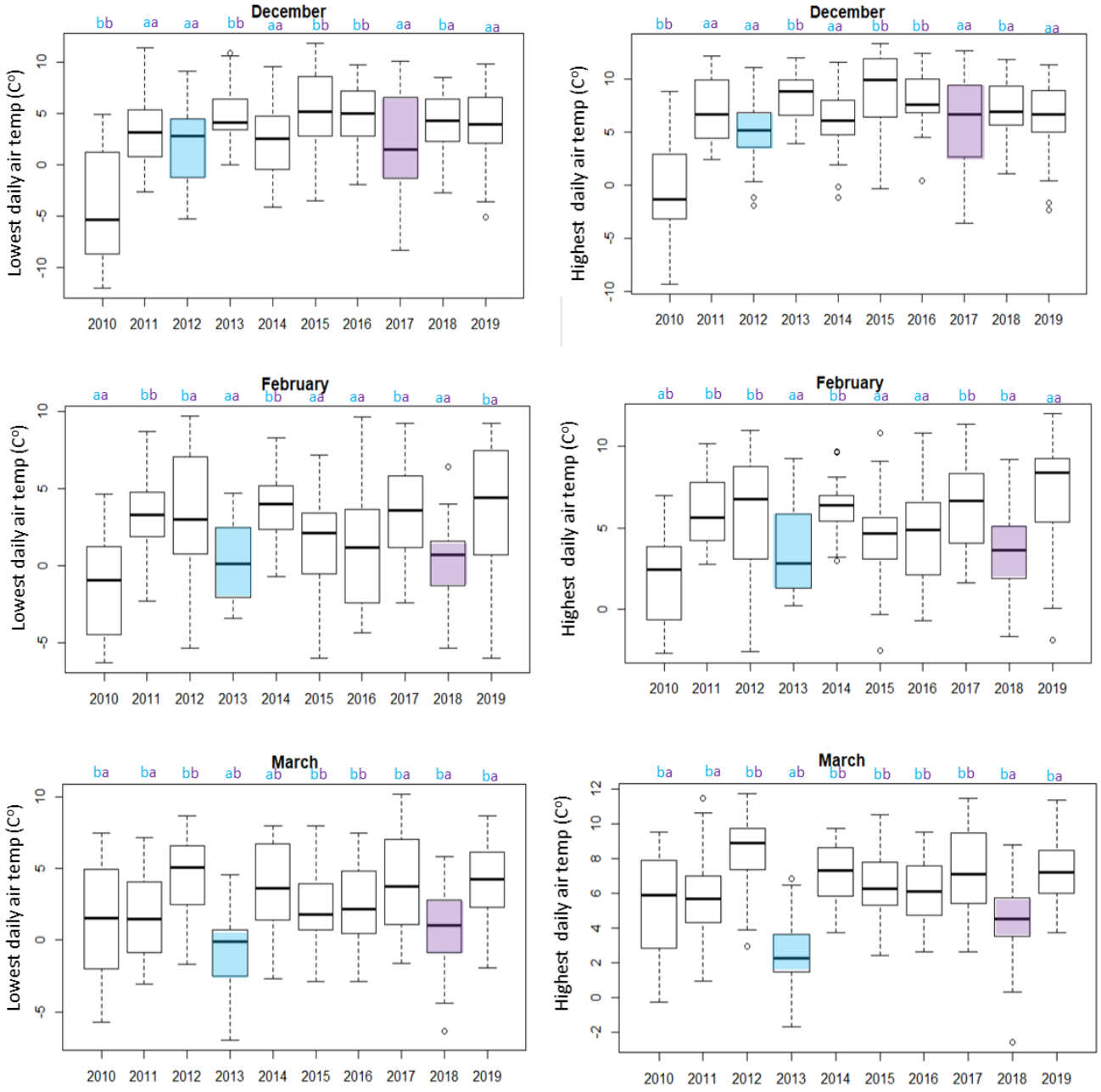


Figure 27: Minimum and maximum daily temperatures in 2010-2019 for months where our years of interest (marked by coloured boxes) are significantly different from other years. n=number of days for each month. Different letters immediately above each box indicate significant differences between years determined through Kruskal-Wallis rank sum test with post-hoc pairwise comparisons using Wilcoxon rank sum test p<0.05. The colour of the letters matches to years subject to the pairwise test

2.3.2.4 Snowfall

Winter snow cover varied substantially between years, both in terms of depth of snow and number of days with lying snow (Figure 28). The winters of 2012/2013 and 2017/2018 are of particular interest as they precede the springs which had increased *P. ramorum* spread. The winter of 2012/2013 had the most snowfall of the decade with 47 days of lying snow. Most of this was due to the so-called "Beast from the East" storm (official Met Office storm naming only came into force later that year), an anticyclone which brought cold air from Russia over the UK. The 2018 snowfall was due to Anticyclone Hartmut resulting in Storm Emma (26th February to 5th March). Of particular note is the timing of the snowfall during these two years. For most years of the decade the majority of snow fell in December and January, however in 2013 and 2018 the snowfall occurred later, in spring, from February to the start of April (Figure 28).

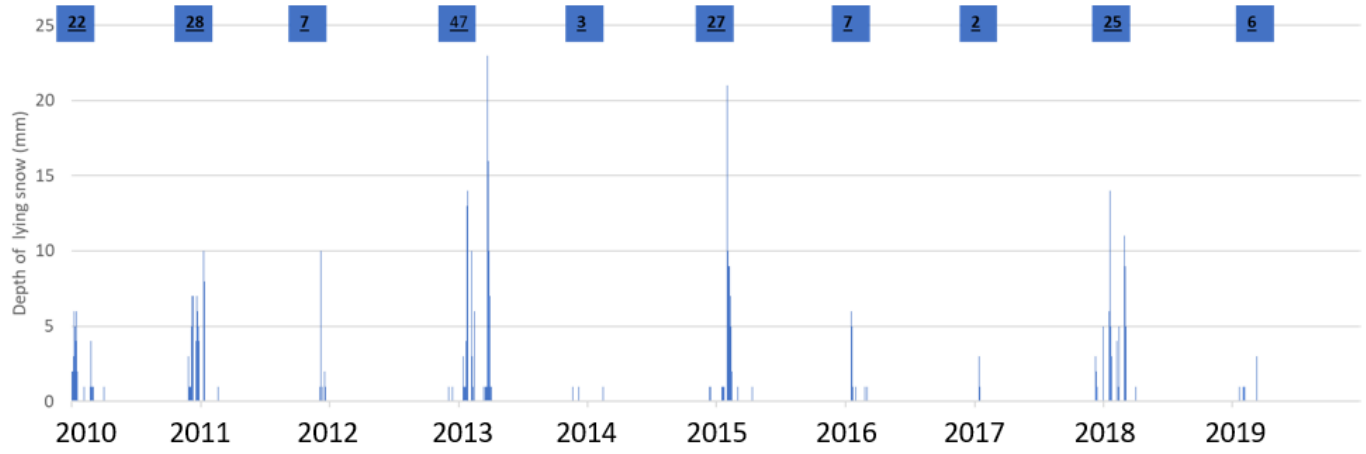


Figure 28: Temporal variation in annual snow cover at Barwhillantry House weather station between 2010 and 2019. Blue boxed numbers show number of days with lying snow each winter. Time period shown for each year is 1st of November to 31st of April. Each bar represents a single day with lying snow.

2.3.2.5 Climatic conditions of note

In summary, we identified various climatic conditions as potentially important factors linked to expansion *of P. ramorum* infections. Higher than average summer precipitation and lower than average winter temperature preceded expansion of *P. ramorum*. Of particular note are the climatic conditions in the month of March, both 2013 and 2018 had severe easterly storms which brought about changes in wind direction, with strong atypical easterly winds and unusually low temperature. Late-season snow cover is also a potentially important factor as both 2013 and 2018 had snow from February to the start of April rather than the usual snowfall in December and January.

2.3.3 Controlled inoculation experiments to investigate infection pathways

2.3.3.1 Preliminary comparison of sporangial and mycelial inoculation methods.

To test the effectiveness of inoculation methods lesion development was measured on EL and JL. Inoculation with sporangial suspension onto unwounded bark did not result in successful infection (Figure 29). However, inoculation with mycelial plugs and sporangial suspension onto wounded bark did result in successful infection (Figure 29). At each assessment timepoint the average lesion lengths from mycelial inoculation were smaller than for sporangial inoculation (Figure 30). Eight weeks after mycelial inoculation, average lesion lengths for EL and JL were 113 mm (SD=32.3) and 93.3 mm (SD=3.2), respectively. In comparison, sporangial inoculation of EL and JL resulted in mean lesion lengths of 258.3 mm (SD= 150.6) and 219 mm (SD= 40.3), respectively (Figure 30). Mycelial inoculation produced more consistent lesion lengths than sporangial inoculation. Across both inoculation methods lesion lengths were more constant in JL than EL.

Two sample T-tests showed that there was no significant difference in mean lesion lengths between EL and JL for mycelial inoculation at 5 weeks $t(5)=-1.19$, $p>0.05$ or 8 weeks $t(5)=-0.35$, $p>0.05$ post infection. There was also no significant difference in lesion length between EL and JL for sporangial inoculation at 5 weeks $t(5)=-1.09$, $p>0.05$ or 8 weeks $t(2)=-0.36$, $p>0.05$ post infection.

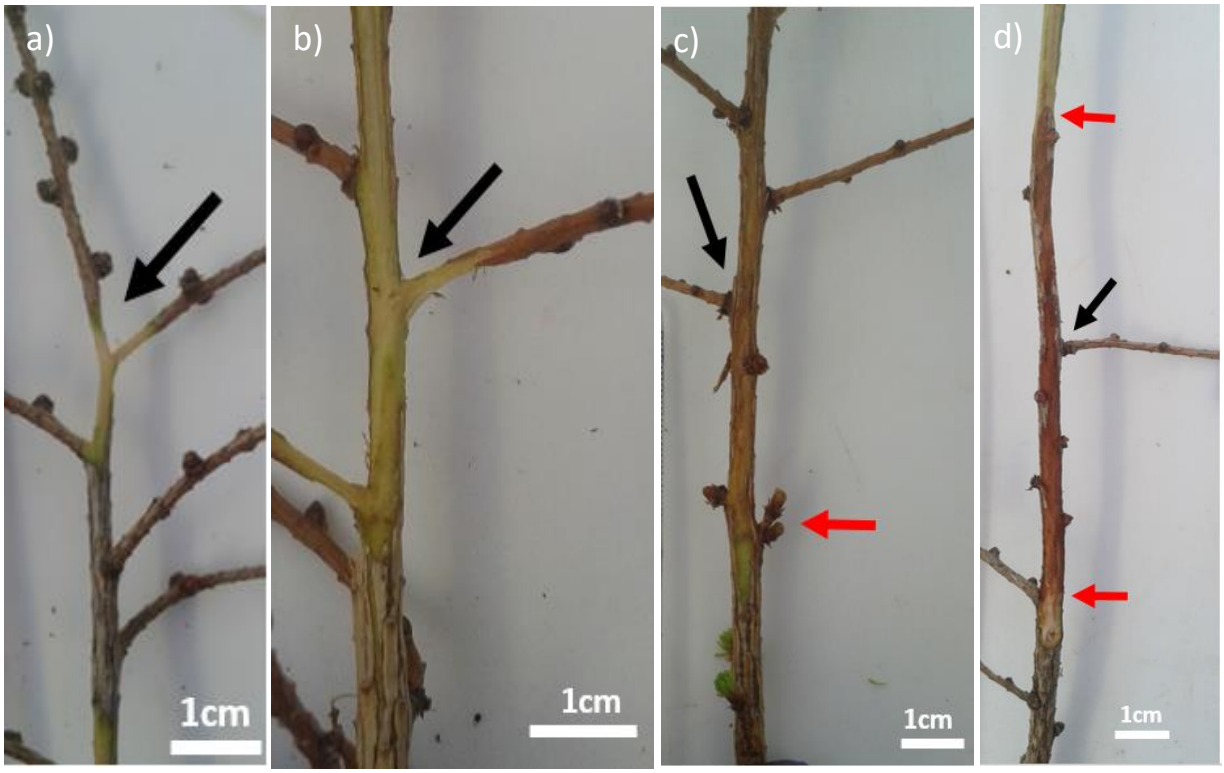


Figure 29: Photographs taken five weeks after inoculation with sporangial suspension of P. ramorum onto unwounded bark of a) Japanese larch and b) European larch (showing no lesion in either picture) and wounded bark of c) Japanese larch and d) European larch (showing lesions in both

pictures). The outer bark has been removed in all pictures. Black arrows indicate the inoculation point and the red arrows indicate the margins of lesions which developed in the wounded trees. In c) the upper edge of the lesion extends out of the picture.

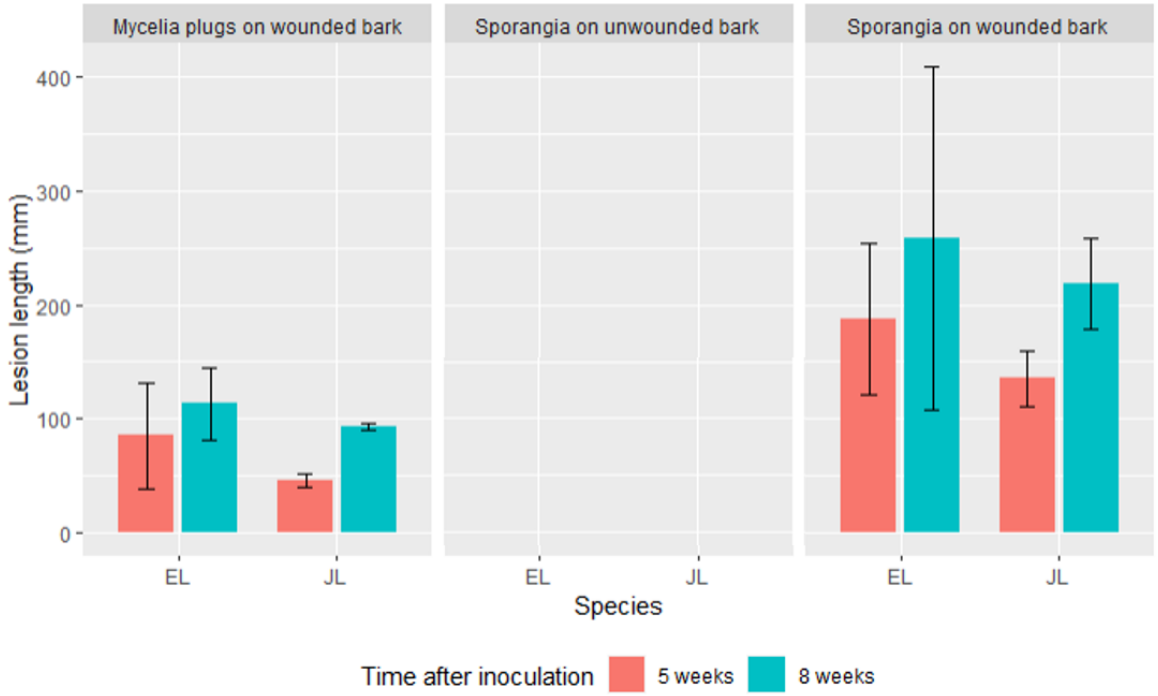


Figure 30: Lesion lengths on European larch (EL) and Japanese larch (JL) 5 weeks and 8 weeks after inoculation with P. ramorum using either mycelial plug or sporangial inoculation methods. Error bars indicate standard deviation of the mean. n=3 for each timepoint.

2.3.3.2 Investigation of infection at different stages of bud flush.

From the field observations detailed in section 2.3.1 we hypothesised that buds on fine shoots were the primary infection sites, with infection possibly occuring during bud flush. The bud infection pathways were investigated through greenhouse inoculations of buds at different stages of flush. Inoculation of buds did not result in infection, regardless of stage of bud flush. The positive control treatment in which bark was wounded and inoculated resulted in mean lesion lengths of 167.3 mm (SD=97.6) on JL and 27 mm (SD=11.9) on EL after 8 weeks. Lesions did not develop in the non-inoculated controls. Figure 31 illustrates selected treatments.



Figure 31: Photographs of inoculated trees taken eight weeks after inoculation with sporangial suspension of P. ramorum. Unsuccessful inoculations of stage two buds on a) Japanese larch and b) European larch, c) symptoms on Japanese larch in the wounded positive control treatment.

2.3.3.3 Experiment to use histological examinations to study the infection pathway

As shown above inoculation of buds did not result in infection, regardless of stage of bud flush, however inoculation of wounded bark gave well developed lesions. To investigate if this was due to a structural component of the buds preventing infection we used histology to examine the buds.

In dormant buds the needles are well protected by a covering of bud scales (Figure 32a), the larger scales are lignified and contain cinnamaldehydes as can be seen in the areas of bright pink staining when treated with phloroglucinol (Figure 32b). There were no abnormal cell structures (an indicator of necrosis) observed in the inoculated buds relative to the controls. Sporangia were observed caught in the curves of the bud scales or attached to their surface (Figure 32c). Sporangia were also found next to the surface of the bud base (Figure 32d). Although not obviously attached to the surface it is likely that they were prior to sectioning of the paraffinized samples and were disturbed during processing. None of the sporangia in the bud inoculations were found to have germinated. Direct germination of the sporangia was only observed in the inoculations of wounded bark (Figure 32e). There was not an obvious lignification response to wounding and cells around the inoculation points were not obviously different between control and inoculation treatments (Figure 32f-g)

despite the development of extensive lesions in the latter. Some slight pink staining can be seen in the upper cells of the inoculated wound and this could be related to lesion development but this was not consistent across replicates. There were swimming zoospores in the inoculum, however, no encysted zoospores were conclusively identified in any sample.

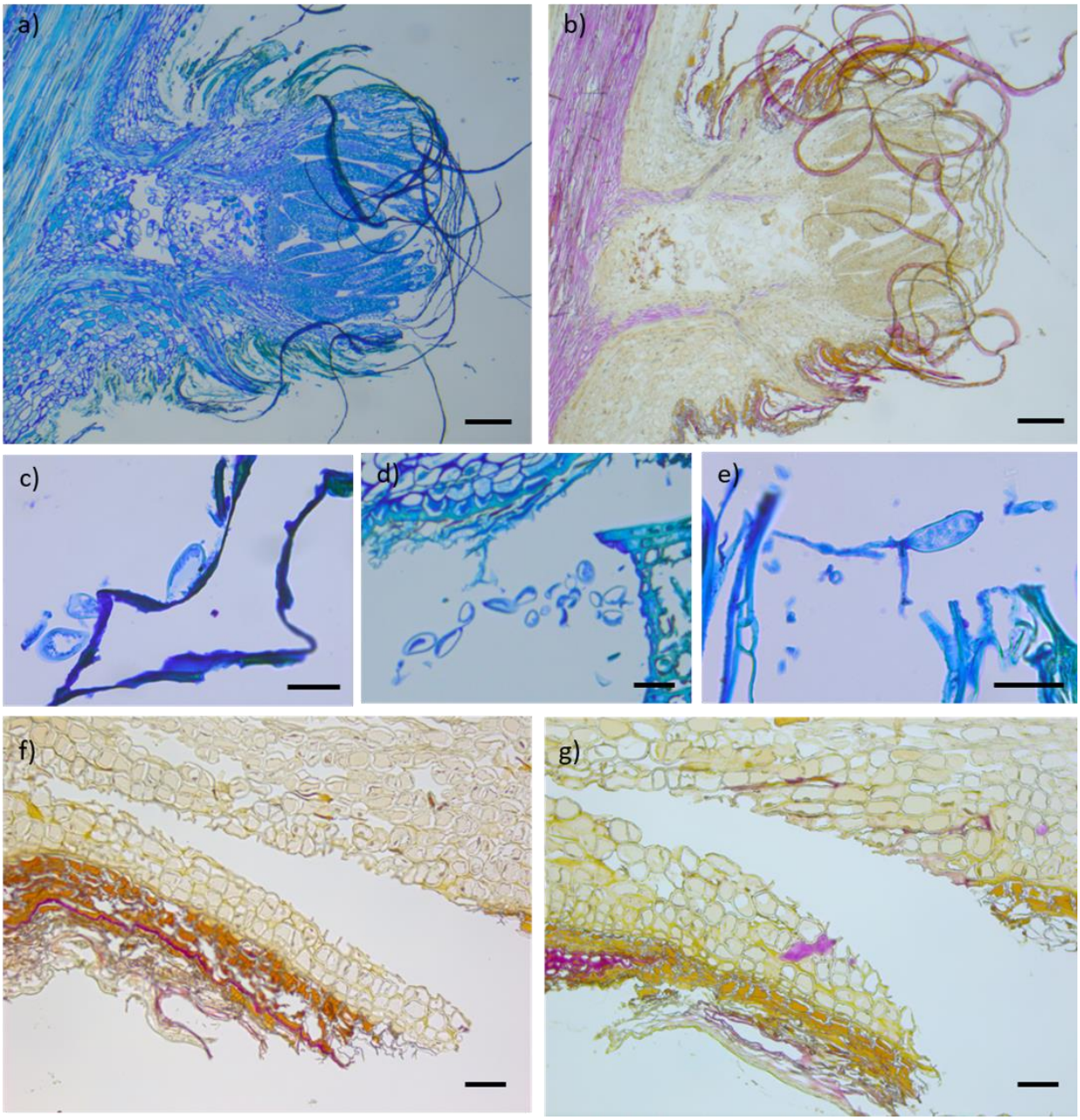


Figure 32: Histology. a) dormant bud, 72 hours after inoculation, b) dormant bud, 72 hours after inoculation, , c) ungerminated sporangia attached to needle scale from stage 3 bud 72 hours after inoculation, d) ungerminated sporangia near surface of bud base from stage 3 bud 72 hours after inoculation, e) germinated sporangia with hyphae growing to cell surface within inoculation point of wounded inoculation 24 hours after inoculation, f) control wounding treatment 7 days post inoculation, g) inoculated wounding treatment 7 days post inoculation. a,c-e stained with toluidine blue, b, f-g stained with phloroglucinol. a-c,e are Japanese larch, d,f,g are European larch. Scale bars indicate 200 µm for a-b and 100 µm for c-g.

2.4 Discussion

This is the first study of the natural infection processes of *P. ramorum* on larch in field conditions over an extended time period. Our observations span four growth seasons, cover a major increase in infection levels across multiple sites, and confirm that the EU2 lineage was present on all trees sampled. The findings represent novel information on the timing and location of primary infection on larch and how climatic variables might be linked to disease expansion events. Hypotheses derived from field observations were tested in a series of inoculation studies under controlled conditions, enabling us to surmise that the pathogen most likely enters woody tissue (young shoots) via infected needles during autumn, with lesions expanding rapidly during flushing in the spring.

2.4.1 Disease progression

This study showed that a significant increase in shoot and branch dieback occurred in spring 2018, leading to a subsequent increase in tree mortality. When all plots were considered together, the first significant increase in severity of shoot dieback occurred with increased needle discolouration in September 2017, followed by a large expansion of infections across the region in May 2018, similar to the disease expansion event that occurred in spring 2013. Subsequent progression of infection from fine shoots into larger branches and eventually into main stems, resulting in tree mortality, was shown by the significant increases in both branch and main stem dieback recorded in May 2019. This also led to the identification of buds on fine shoots as the primary infection sites.

The rate of infection development varied among sites and among trees within a site; many died within six months of first infection but some trees were still alive, although in various stages of decline, at the end of the study in September 2019. Fresh infections, including main stem lesions occurring in May 2018, expanded more rapidly than those observed to be present at the start of the survey. Observations over three consecutive years showed that the greatest increase in new *P. ramorum* infections occurred between September and May, with new infections becoming apparent by the subsequent lack of flush and dead shoots in the spring. This suggests that primary infection takes place at some point between autumn and spring, as discussed later.

This study overlapped with the second significant expansion of *P. ramorum* in the Galloway forest, and across the wider environment, in spring 2018 (Sketchley, 2019). New outbreaks of disease are often associated with changes in pathogen virulence caused by genetic changes. Phytophthora ramorum lineages have been found to have genotypic diversity, both between and within lineages, caused by mitotic recombination and leading to variation in pathogenicity-related genes which might influence virulence or infectiveness (Dale et al., 2019). In *P. ramorum* the major shift from infecting

nursery plants to widespread infection of larch plantations in 2009 has been associated with a change in the population structure of the EU1 lineage with the EU1MG1 multilocus genotype becoming dominant (Harris et al., 2018). The outbreak in the Galloway forest in 2013 was caused by the previously unidentified EU2 lineage which is more pathogenic on larch (Harris et al., 2021). However, isolates of *P. ramorum* are collected from new outbreaks and disease expansions, including the Galloway forest in 2018, by Forest Research and the Forestry Commission and undergo regular screening for new variants. No new variants have been reported related to the 2018 expansion in the Galloway Forest (S. Green, 2021, personal communication) and so it has to be considered that other factors are influencing the dynamics of *P. ramorum* expansion.

Considering both the 2018 expansion and the earlier expansion in spring 2013 allowed us to consider how climatic factors might influence outbreaks. Our analysis of the climatic conditions preceding the outbreaks in 2013 and 2018 identified higher than average summer (1st of June to 31st August) precipitation, lower winter temperature, easterly storms in March leading to changes in wind direction and late-season snow cover as potentially important factors linked to expansion of the epidemic. The sporulation period of *P. ramorum* on larch needles occurs during needle senescence in autumn, specifically from mid to late September to mid-October in the Galloway Forest (Frederickson-Matika et al., 2019; Green et al., 2019). Similar spore trapping in Cornwall found sporulation from early October to late November 2009 (Forest Research & FERA, 2010). Harris & Webber (2016) found, using inoculations of excised material, that the highest levels of sporulation occurred in August although the needles were asymptomatic at this time with needles becoming symptomatic just prior to senescence. It is not known how higher than average rainfall during June to August can stimulate greater inoculum production in autumn, enabling such a massive disease expansion event. Laboratory inoculations have shown that sporulation does occur on asymptomatic foliage (Harris & Webber, 2016) so it is likely that asymptomatic sporulation also occurs in field conditions during the high precipitation of July-August but spore trapping during this time period has not been carried out. Prior to sporulation the needles must become infected, but the source and timing of this infection is unknown. *Phytophthora ramorum* does not sporulate on the surface of bark but characteristically produces large numbers of chlamydospores on larch needles (Harris 2014). These long-lived resting structures have also been found to exist on leaf litter in infected oak forests in the USA and in larch forests in the UK (Davidson et al., 2005; Harris, 2014) and in oak forest soils (Fichtner et al., 2007). Reports of reinfections from contaminated soil are common and can occur for a prolonged period even after removal of living hosts, for example up to 49 weeks in Californian oak woods (Fichtner et al., 2009) and two years in UK larch forests (Harris, 2014). In California, rain was found to splash inoculum up to 30 cm above the soil surface (Fichtner et al.,

2009). Since summer precipitation was higher than the 19-year average in the year preceding each of the two outbreaks in south-west Scotland, heavy rain might have promoted sporulation of *P. ramorum* from infected needle litter and led to rain or wind driven infection of the needles on living trees which could then lead to subsequent sporulation on the needles. The autumns of 2012 and 2017 also had higher than average rainfall which would have provided the surface moisture required for further successful infection (Davidson & Shaw, 2003).

As *P. ramorum* has an optimum temperature for growth of ~20^{o}C ,and is very slow growing at 4^{o}C (O'Hanlon et al., 2017), it seems unlikely that the lower winter temperatures preceding the 2013 and 2018 outbreaks in the Galloway Forest would have directly affected pathogen growth rate within the host. It is also hard to understand how late-season snowfall could affect pathogen activity. It is possible that these conditions stressed the trees and affected their phenological traits, such as the timing of bud flush, which are highly heritable and so not completely plastic in response to environmental conditions (Dodd et al., 2008; Morgenstern, 1996). A colder than usual spring can lead to stress from the mismatch between the state of flush and the weather conditions later on in the season. This can compromise the trees' ability to produce an effective defence response and make them more susceptible to infection by necrotrophic and opportunistic pathogens (Ennos, 2015). This may explain why there was a lull in the wider spread of infection on larch in south-west Scotland, between the 2013 outbreak and the 2018 outbreak, as conditions are required that not only favour the pathogen but also place the trees at a physiological disadvantage. Another factor to consider is that late season snowfall would increase ground water availability as the snow melts, potentially leading to higher than normal levels of tissue moisture which might favour the rapid spread of the pathogen within the tree.

The influence of climatic factors on *P. ramorum* infection was studied in coast live oak by Dodd et al. (2008) who inoculated cuttings over the time course of an entire year. The authors observed two peaks in lesion development, the greatest one occurring after inoculations carried out in late April, linked to cambial activity, and a second after October inoculation, linked to lower temperatures and lower evapotranspiration after the hot dry Californian summer. They concluded that active cambial tissue is necessary for successful infection and also that external climatic factors had a great influence on the development of infection.

Dodd et al. (2008) also found that variation in cambial phenology among trees correlated well with variation in the timing of maximum lesion size among trees. Synchronicity between pathogen sporulation (determined by rainfall and temperature) and host physiological function (determined by phenological traits) was critical for infection. The mismatch between the two possibly explains

why some hosts that become physiologically active later in the spring could avoid infection. Florance (2005) found high water concentrations in *P. ramorum* cankers on coast live oak trees and found channels in the bark continuous with the medullary rays in the xylem, which conduct water radially though the stem, may serve as an avenue of infection for *P. ramorum.* Sap flow and water transport increase during flushing (di Francescantonio et al., 2018) which could promote movement of *P. ramorum* within the tree. Stem tissues with the highest amount of water are the most physiologically active ones; differentiating xylem cells, the cambium and differentiating phloem cells (De Schepper et al., 2012). Dodd et al. (2008) suggest that, for canker causing pathogens, the availability of newly differentiating vascular tissues in the host may be a prerequisite for disease progression, but it is likely that both moisture content and the newness of the cells are conducive to pathogen spread within the host.

This study considers the climatic conditions around the outbreaks in the Galloway forest in 2013 and 2018 and identified some correlating conditions. However, it is highly likely that future outbreaks and expansions will occur, both in the Galloway forest and in other areas. Further work could consider these outbreaks, along with analysis of additional historical outbreaks in other locations, such as the expansion in Wales in 2013, to establish the importance of the individual climatic conditions and how they interact to influence the timing and extent of *P. ramorum* outbreaks.

2.4.2 Variation in spatial progression of infection

Infection and spread of *P. ramorum* in larch was not consistent within thicket staged sites, or among sites, and the initial infection level in a site in September 2016 did not predict mortality levels at the end of the study period. Survey sites ranged from asymptomatic to moderate symptom prevalence in 2016, and by the end of the study some sites showed more rapid and severe increases in infection than others. The biggest contrast in final infection levels was between Glenshalloch, in which some plots were almost entirely killed, and Loch of Lowes, which had very low mortality even at the end of the study period.

Different rates of symptom development were observed among and within sites including among individual trees within a plot. This variability suggests that factors affecting pathogen dispersal and disease severity are complex and may involve local climatic variation and microsite differences, as well as differences in disease responses at the individual tree level. The study sites were within 15 km of each other and subject to broadly the same climatic conditions, but studies have suggested the importance of abiotic factors, such as microclimate conditions (Rizzo et al., 2005) or soil type (Fichtner et al., 2007), on infection by *P. ramorum* but these were not assessed as factors in this

study. Other variable factors such as tree provenance could not be assessed as planting records did not record provenance. There was also some variation in the age of stands as the youngest stand located at Loch of Lowes was also the least severely affected.

If inoculum from prior infections within the stand was the main source of subsequent infections in neighbouring trees, we would have expected stands with a higher starting inoculum load to continue to be the most severely affected. This was not the case and infection levels within the stand did not seem to be sufficient to drive infection in the stand. Whilst we cannot account for all trees in the stand, outside of those covered by our transects, we only observed one clear case of spread from an infected tree to its direct neighbour. It is likely that inoculum comes from outside the stand in order to drive infection. It was beyond the scope of this study to assess other inoculum sources given the large amount of infected larch in the area, and the fact that the pathogen can also sporulate on *Rhododendron* and *Vaccinium* (FERA, 2012; Harris & Webber, 2012; Herrero et al., 2011), both species being ubiquitous in the region.

Most of the transmission of *P. ramorum* inoculum occurs within a few metres (Rizzo et al., 2005) to within a couple of hundred metres of an infected tree (Fichtner et al., 2007, 2009; Frederickson-Matika et al., 2019; Hansen et al., 2008) but has been found to spread up to 1 km in south-west England (Forest Research & FERA, 2010) and in forest ecosystems in Oregon dispersal can occur over a few kilometres (Hansen et al., 2008; Rizzo et al., 2005). Further study of the distance of aerial dispersal of *P. ramorum* on larch, particularly thicket stage stands that are of a lower height than the mature stands studied to date, would be of interest.

2.4.3 Infection pathway

Our field observations identified fine shoots as the primary infection sites with lesions actively expanding in the spring either side of killed, unflushed buds. This suggests that primary infection occurred between autumn, after the surveys in early September, and flushing in spring.

We used greenhouse inoculations to gather evidence in support of our hypothesis on the infection pathway. We found that inoculation with sporangial suspension during dormancy produced shoot lesions only on artificially wounded bark. These results indicate that intact bark provides a good barrier to infection and suggest that in the absence of a wound in the bark *P. ramorum* needs a conduit into the inner tissues, at least in small trees. Observations in May 2018 indicated that buds might offer a conduit to infection sometime between dormancy and flushing in the spring. This hypothesis was tested by inoculating buds at different stages of flush; full dormancy, bud swelling, first needle appearance and full flush. None of these bud inoculations resulted in infections but lesions did develop on inoculated wounded bark used as a positive control. Therefore, we conclude

that primary infection is unlikely to take place through otherwise healthy dormant or flushing buds over winter or in the spring. Work by Forest Research & FERA (2010) unsuccessfully attempted to isolate *P. ramorum* from dormant buds of infected larch trees and suggested that it doesn't overwinter within the buds but that needle infections enter in the brachyblasts beneath the needle rosettes in autumn before infecting the new needles the following spring.

Chastagner et al. (2012) observed lesions and shoot dieback after spraying a zoospore suspension of *P. ramorum* onto unwounded, fully flushed larch saplings in spring but reported minimal symptoms and no lesion development with autumn inoculation, carried out in early October. The authors did not propose any explanation for this variation and the experiment was of a small size and was not repeated. Combining the results of these greenhouse inoculations suggests that the pathway of infection is through the needles and that susceptibility declines as the needles mature. A similar decline in susceptibility over the course of the growing period was found in cut log inoculations (Harris, 2014).

The lower infection levels following autumn inoculations in the experiments of Chastanger et al. (2012) runs counter to our field observations which suggested that infection occurs between late autumn and spring. Neither our experiments nor the experiments of Chastagner et al. (2012) involved inoculation of whole plants in late autumn with needles in the process of senescing. Abundant sporulation of *P. ramorum* on larch in the Galloway Forest has been observed from late September until mid-October in 2018 and 2019 (Frederickson-Matika et al., 2019; Green et al., 2019). These observations suggest that initial shoot infection takes place in autumn, either by extending from the needles into the shoot via infected needle traces around buds, or by inoculum entering otherwise healthy shoots via needle traces around buds at the time of needle fall. This interpretation is supported by data from a spore trapping study, which showed a dramatic reduction in *P. ramorum* DNA captured after the needles had been shed in early November (Frederickson-Matika et al., 2019; Green et al., 2019), indicating that inoculum dispersal will be minimal after this time. However, in late November and December of the same study, localised necrosis was observed within buds on shoots containing infected needles (S. Green, 2020, personal communication), suggesting that the pathogen infected the bud from infected needles in late autumn. Cohen (1967) observed that hyphae of the larch needle blight fungus *(Hypodermella laricis)* were able to grow within the mesophyll cells of larch needles but were not able to cross the abscission layer, suggesting that these thickened cells form a protective layer. *Hypodermella laricis* is a needle pathogen that does not infect the shoots or other woody parts of the tree, unlike *P. ramorum* which may have a mechanism of penetrating the abscission layer in the same way that the xylem can be penetrated (Florance, 2005). The scars caused by needle drop may provide a conduit to the inner shoot tissues,

even if for a brief time period, in a similar way to the bark fissures in coastal live oak suggested by Swiecki & Bernhardt (2006). Further inoculations of whole plants in autumn, around the time of needle senescence, would allow testing of the hypothesis of infection at this timepoint.

Inoculation of buds at different stages of flushing did not result in successful infections. As the inoculum contained both sporangia and zoospores it was expected that both propagules would be involved in infection. Sporangia were observed on the surfaces of the buds and bud scales, and within the wounded inoculations. Direct germination of sporangia was only observed in the wounded inoculations and not in any of the bud inoculations. Directly germinated *P. ramorum* sporangia cause a lower level of necrosis on *Rhododendron* compared to a similar number of zoospores (Widmer, 2009) but direct germination was only observed in our wounded samples where there were well developed lesions. *Phytophthora* zoospores exhibit chemotactic behaviour and move towards plant exudates (Bassani et al., 2020; Suo et al., 2016) in particular those released by wounding (Adorada et al., 2000; Armstrong, 2018). It is possible that zoospores also played a role in the infection of the wound but were not conclusively identifiable in the samples as flagella are lost after encystment (Judelson & Blanco, 2005) and so identification becomes more difficult.

Wound response in conifers includes production of secondary metabolites and callus formation by modification of cells surrounding the wound through lignification and subsequent suberisation (Hudgins et al., 2004; Oven & Torelli, 1999). These changes would have been obvious through phloroglucinol staining, which stains lignified and suberised tissues red/pink, but there were no indications of this in either inoculated or control samples. However, these responses take time to occur, with callus formation in European larch taking 35 days after wounding (Oven & Torelli, 1999), so it is likely that they would not be apparent 7 days post inoculation at the time of sampling. Other wound responses that occur more rapidly, including the production of polyphenolics and synthesis of pathogenesis-related proteins (Franceschi et al., 2005) would not have been visible with our staining methods. Overall, we found no evidence for a structural host response to inoculation in the cells of wounded plants, even within the lesion.

The inoculation methodology used in this study involved a sporangial/zoospore suspension in order to better mimic natural infection pathways. Whilst zoospores are important agents of transmission the caducous nature of *P. ramorum* sporangia and their ability to germinate directly suggest that they are also important in the infection process. Mycelial plug inoculation gave consistent lesion development but it is not representative of the natural infection pathway and cannot be quantified in the same way as a sporangial suspension. We found that a sporangial suspension induced good lesion development after inoculation of wounded bark. The quantifiable nature of sporangial or

zoospore inoculation is important for any investigations into resistance or tolerance to infection as, in field conditions, even "non-host" species have been found to be infected when inoculum loads are high enough (Forest Research, 2020b).

2.4.4 Variation in tolerance to infection

Our survey results showed that the infection trajectory at the individual tree level varied between young and mature stands. In thicket stage stands most trees which became infected died within 6-12 months but some survived with severe branch dieback more than three years after initial infection. Mature stands planted in the Galloway Forest in the 1950s had greater than 95% mortality following *P. ramorum* infection but contained occasional very healthy trees at least seven years after first reported infection (personal observation). The mature larch stands in Glen Trool already had 20-40% mortality at the start of our survey in September 2016. These stands demonstrated what happens in a post epidemic situation, when most susceptible trees, or those exposed to higher levels of inoculum, will already have died. Many of the surviving trees were infected and continued to slowly decline over the course of our observations. However, at the final plot assessments in May 2018 (for plots that were subsequently felled) and May 2019 (for retained plots) there were still trees with full crowns that looked healthy, even after an expansion of the epidemic across south-west Scotland in spring 2018. The presence of these large survivor trees may be explained by genetic resistance to *P. ramorum* infection, 'disease escape' where trees, often on the edge of a stand, don't get infected, or just by chance. Other factors affecting disease spread, such as wind direction, might contribute to lack of infection in these survivor trees. All these factors might act alone or in combination to explain the occurrence of survivor trees. A collection of grafted scions from the mature survivor trees in Glen Trool are tested for resistance to *P. ramorum* infection in Chapter 4.

2.4.5 Conclusions

We investigated the epidemiology of *P. ramorum* through a detailed examination of infection in individual trees and within stands over three and a half years. We observed first infections in the fine shoots that led to branch mortality, often followed by whole tree mortality through girdling of the main stem. However, there is variation in response to infection within the larch population in the Galloway Forest. In younger stands most trees die within 6-12 months of first infection but some survive with severe branch dieback more than three years later. Observations of older stands found >95% mortality but also found occasional asymptomatic trees at least 7 years after first reported infection. Through observations of a period of increased spread in spring 2018 and retrospective consideration of the outbreak in 2012-13 we investigated how environmental conditions might

influence outbreaks, with high summer precipitation, low winter temperature, late season snowfall and storm winds from an atypical direction as important factors. Our field observations predict that buds on fine shoots are the primary infection sites with infection occurring between late autumn and spring. Through artificial inoculations we determined that intact bark provides a good barrier to infection, and that buds are not infected during flushing but more likely infection occurs in autumn during periods of high sporulation around needle senescence, with the new infections becoming apparent in the failure to flush during the subsequent the spring.

Chapter 3- Investigating the differences in response to infection with *P. ramorum* between European and Japanese larch through analysis of transcript accumulation profiles identified from RNA-seq.

Abstract

Both European larch (EL) and Japanese larch (JL) are known to be severely affected by *P. ramorum* often resulting in high levels of mortality. However, there is evidence of variation in susceptibility between the two species, with EL thought to be less severely affected in the field, but the results of experimental trials are inconsistent and not all are statistically significant. We conducted inoculation experiments on potted saplings and carried out RNA-seq analysis to study the RNA transcript accumulation profiles in response to inoculation. Although not significantly different in terms of lesion development, there was an interesting difference in expression of key defence pathways between the two species. European larch had rapid induction of defence genes within 24 hours of infection followed by sustained or increasing expression until 25 days after inoculation. By comparison, results in JL were more varied with fewer key defence pathways clearly responding to infection and the observed gene expression increases were both larger and more transient compared to EL.

This variation in response to infection between the two species is of interest as it could help explain differences seen in the field and should be considered in the selection of trees for planting and future breeding

3.1 Introduction

Early field observations suggested that European larch (EL) (*Larix decidua*) may be less susceptible than Japanese Larch (JL) (*Larix kaempferi*) to infection by *P. ramorum* based on the frequency of resinous bark lesions (Harris and Webber, 2012). Between 2013 and 2020 there were 1247 Statutory Plant Health Notices (SPHNs) issued for *P. ramorum* on Forestry Commission land in England. Of the 217 SPHNs which identified the larch species concerned, only 6 SPHNs related to EL, with 17 issued for hybrid larch (HL) (*Larix × marschlinsii Syn. L. × eurolepis*), a naturally occurring cross between JL and EL, and 194 issued for JL (A. Mistry, 2021, personal communication) suggesting that a greater volume of JL is affected by the disease. Such observations raised the question as to whether EL could be retained as a viable timber species in the UK. However, no clear differences became apparent between EL and JL in laboratory inoculations and other methods to investigate differences

between the two species are required. RNA-seq analysis (Martin & Wang, 2011) allows the use of high throughput sequencing to investigate the host responses to infection in fine detail at the level of gene expression changes which indicate the signalling, regulatory and metabolic pathways involved.

Larch accounts for 10% of all conifer plantations and 4.7% of all woodland area in the UK, covering 126,000 hectares in 2012 (Forest Research, 2020a), making it the third most important commercial conifer species in the UK, after Sitka spruce (*Picea sitchensis*) and Scots pine (*Pinus sylvestris*). These numbers include EL and JL as well as HL. In Scotland, records from 2001 show that JL was the most widely grown species of larch with 55,034 hectares (included in this figure is a minor amount of HL) planted compared with 8,622 hectares of EL (Forest Research, 2001). This preference for JL over EL is largely due to the greater susceptibility of EL to larch canker (Kulej, 2006; Yde-Andersen, 1979), caused by the non-native fungal pathogen *Lachnellula willkommii,* which is thought to be native to Japan and this may be why JL is more resistant to larch canker (Kobayashi, 1970).

Japanese larch was thought to be relatively disease free until the arrival of *P. ramorum* in south-west England in 2010 (Webber, Mullett, et al., 2010). This was also the first record of *P. ramorum* causing widespread and lethal damage to any conifer species (Brasier & Webber, 2010). It is unclear whether the greater volume of diseased JL over EL is because JL is more susceptible to *P. ramorum* than EL, or due to the more extensive availability of JL. The records for Forestry Commission land in England do not always identify the species of larch but in 2009, EL accounted for 14.6% of the total larch plantations and JL for 58.4%, with the remainder unclassified at species level. However, by 2021 only 19% of the EL had been felled, compared to 30.8% of the JL, with JL accounting for 75.5% of all the larch felled over this period (A. Mistry, 2021, personal communication). Although a number of studies (as discussed below) have compared the responses of the two species to *P. ramorum*, results are variable depending on the inoculation method used, tissue type studied, lineage of *P. ramorum* tested, and the metric used to record the results.

Harris and Webber (2012) initially reported that EL had fewer resinous bark lesions caused by *P. ramorum* in field conditions but formed larger bark lesions following inoculation in the laboratory compared to JL. Further studies using mycelial inoculations with different lineages onto the bark of logs cut from mature trees reported either larger lesions on JL than EL, although differences were not statistically significant (Harris, 2014; Harris et al., 2021), or that JL had more susceptible bark than EL (Harris et al., 2014). Zoospore inoculation using the EU1 lineage on potted intact seedlings resulted in longer lesion lengths at 21 days post inoculation (dpi) on JL compared with EL (80 mm and 61 mm, respectively), however, mortality of EL was higher than JL at 55 dpi (46.7% and 33.3%,

respectively) (Chastagner et al., 2013). RNA-seq analysis of the response to infection with the EU2 lineage found more defence genes expressed in EL than JL and downregulation of some defence related pathways in JL (De La Mata Saez, 2015) thus suggesting that EL is more resistant to *P. ramorum* infection than JL. Further analysis of the genes expressed in response to infection could help clarify these complicated and often contradictory observations and help elucidate the changes in gene expression that underpin the defence response.

The general strategy of plant resistance to disease is summarised as a zig-zag in stages of immunity and susceptibility (Jones & Dangl, 2006). The zig-zag model forms the background understanding of much of the current ideas on plant immune response (Gouveia et al., 2017; Kushalappa et al., 2016) and details the interaction between host plant and pathogen. The plant has transmembrane pattern recognition receptors that may recognise conserved pathogen-associated molecular patterns (PAMPs). This leads to the plant activating primary defence responses including callose deposition, cell wall remodelling and accumulation of defence-related proteins (Oßwald et al., 2014) which is known as PAMP-triggered-immunity (PTI). In the second stage, the pathogen develops effector molecules to suppress or manipulate PTI and the host evolves resistance genes (*R* genes) which can recognise and combat the effectors. This results in an arms race of co-evolution between host and pathogen as each is forced to evolve defence and counter-defence to overcome the other. This arms race has been well studied in Phytophthoras that infect crop plants, in particular, the soybean (*Glycine max*) and *P. sojae* (Ma et al., 2017) and between the potato (*Solanum tuberosum*) and *P. infestans* (Fry, 2008; Martin & Kamoun, 2011).

Whilst the PAMP-triggered-immunity is an example of a specific inducible immunity pathway, there is also evidence of resistance to pathogens from variation in host expression of secondary metabolites. These variations in expression can be constitutive, formed during normal development regardless of outside influences, or inducible, with levels of expression varying in response to infection. Secondary metabolites can be part of the defence response interacting with the pathogen directly (Bennett & Wallsgrove, 1994), such as gallic acid and tyrosol which have strong inhibitory effects on growth in vitro bioassays against various *Phytophthora* species including *P. ramorum* (Ockels et al., 2007) or indirectly, such as the salicylates and jasmonic acid, which act as signalling molecules to induce further defence responses (Bennett & Wallsgrove, 1994).

Variations in the expression of secondary metabolites have been linked to variation in the susceptibility to *P. ramorum* in Californian coast live oak (*Quercus agrifolia*) (Nagle et al., 2011; Ockels et al., 2007), with higher constitutive levels of the tyrosol derivative ellagic acid present in putatively resistant trees in comparison to their susceptible neighbours (Nagle et al., 2011). Also,

induced accumulation of gallic and ellagic acid occurred in coast live oak phloem infected by *P. ramorum* and levels of tyrosol and ellagic acid increased in healthy phloem 60 cm away from the lesion margin (Ockels et al., 2007). Whilst these studies have been carried out using chemical analysis of the secondary metabolite content of the trees, the underlying changes in gene expression responsible for the differences in secondary metabolite production have not been studied.

Investigations of the interactions between *P. ramorum* and JL and EL through analysis of RNA expression could identify candidate host defences and immune responses. The use of RNA-seq allows the study of the RNA transcript accumulation profiles in response to inoculation. These RNA profiles could be indicative of molecular interactions between host and pathogen and may identify where differences in response between EL and JL occur. This could then help answer the question of possible greater resistance in EL to *P. ramorum* infection.

The objectives of this study are (i) investigate RNA transcript accumulation profiles in the response of JL and EL to inoculation with *P. ramorum* (ii) identify the defence response pathways that are activated in response to infection (iii) consider the differences in response between JL and EL.

The bioinformatic analysis in section 3.2.4 was carried out by TH Hung, who is part of the MacKay Lab at the University of Oxford.

3.2 Methods

3.2.1 Greenhouse inoculations.

Young JL and EL were sourced from a commercial forestry nursery and maintained outside in ambient conditions in 15 cm diameter pots. In November 2017 the dormant trees were placed in a greenhouse at 20 °C with ambient lighting for 63 days to promote flushing. The greenhouse was in the licensed quarantine facility at Science & Advice for Scottish Agriculture (Edinburgh, UK). At the time of inoculation, the four-year-old JL were 20-45 cm tall (average 32.2 cm) and EL were 40-65 cm tall (average 48.7 cm).

The experiment comprised four treatment combinations; two larch species (JL and EL) and two inoculum types (*P. ramorum* and control) with forty replicate plants per treatment combination. Plants were assessed for disease and destructively sampled at 1, 3, 10 and 25 days post inoculation (dpi). The experimental design was a randomised complete block, with 8 blocks each containing four plants per treatment combination arranged randomly over 3 trays (Figure 33). The experiment was carried out once with inoculations taking place in mid-February 2018. The greenhouse ventilation was shut off and the temperature was maintained at 16°C with ambient lighting (between 9 and 10

hours of daylight over the course of the experiment). The trees were watered twice a week with tap water directly into the tray.

Sporangial suspension was prepared as described in section 2.2.4 using the *P. ramorum* EU2 lineage isolate 16.13a. Two branch junctions in each tree were selected for inoculation; one in the upper crown and one in the lower crown, so as to be physically distant. These branch junctions were marked with electrical tape.

The bark on the upper side of each branch junction was wounded immediately before inoculation by cutting a 2 mm T shape across the top of each branch junction using a scalpel. A 15 µL droplet of sporangial suspension (inoculated) or sterile distilled water (control) was pipetted into the wound. After inoculation, each tree was enclosed in a plastic bag that was taped shut around the top of the pot to maintain humidity (Figure 33a). These bags were removed after 7 days. Inoculum viability was tested on the same day that the experiment was set up by placing a 15 µL droplet of sporangial suspension onto healthy, surface sterilised rhododendron leaves collected from a woodland close to Forest Research's Northern Research Station, Roslin, Scotland. The leaves were incubated on the greenhouse bench in Ziplock bags containing damp cotton wool to simulate conditions on the inoculated trees, and examined for development of lesions after 14 days.

The larches were examined for *P. ramorum* symptoms and bark samples were collected from the main stem of each tree for RNA sequencing at 1, 3, 10 and 25 dpi. For the earlier time points (1 and 3 dpi) the branches were removed and the main stem was cut 10 mm above and below each inoculation point. The inner and outer bark of the section was slit with a scalpel, removed from the stem and the length of any lesions which had developed in the phloem was measured (Figure 33b). Each bark sample was then placed in a separate 2 ml Eppendorf tube and flash frozen by submergence in liquid nitrogen. For the later time points (10 and 25 dpi) the bark was removed in the same way but slit all the way down the tree and peeled from the stem. The extent of the lesion was then measured based on the length of discolouration in the phloem (Figure 33c). A 10 mm bark section was cut either side of the live-dead junction at the extending margin of each lesion, placed in separate 2 ml Eppendorf tubes and flash frozen by submergence in liquid nitrogen. All samples were transported in dry ice and stored at -80°C and all manipulations were in compliance with the DEFRA letter of authority (Licence to import, move and keep prohibited plant pathogens Licence No. 52591/197437/5).

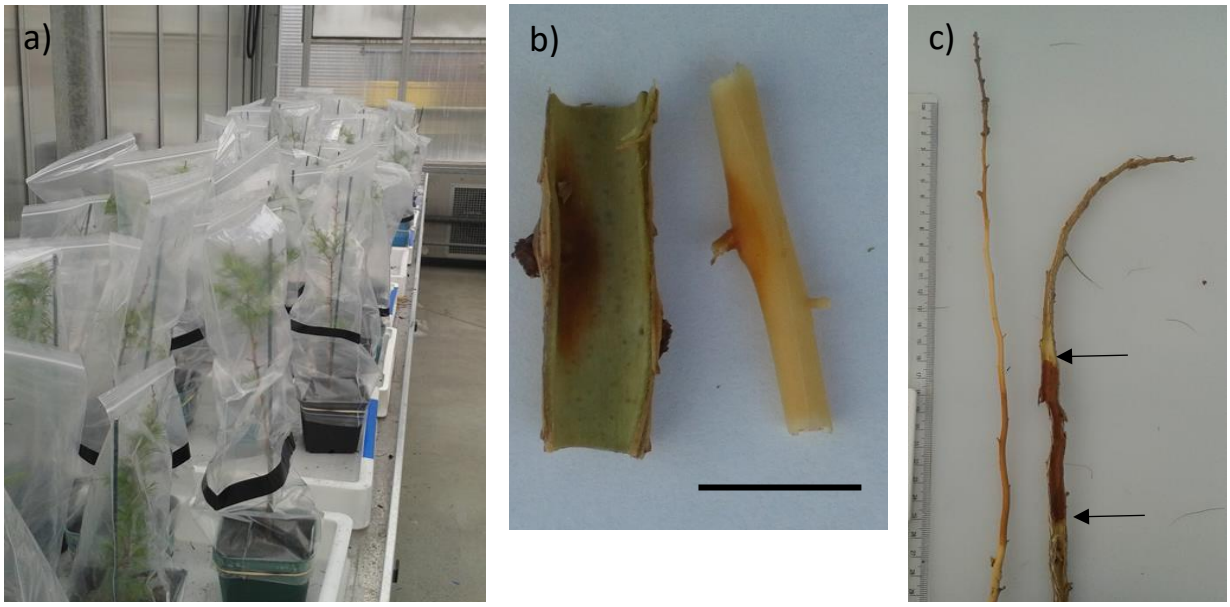


Figure 33: Inoculation methodology and examples of resulting lesions. a) Larch trees sealed inside bags to maintain humidity post inoculation, b) discoloration of phloem (left) and xylem (right) of inoculated Japanese larch 3 days post inoculation, scale bar is 10mm, c) excised bark of entire Japanese larch tree with the extent of the lesion shown by discoloration in the phloem (right) and xylem (left) 25 days post inoculation.

3.2.3 RNA extraction and sequencing

The bark tissue samples were ground by shaking each frozen sample at 25,000 revolutions in a tube with two sterile steel 3 mm ball bearings using a ball mixer mill (Retsch, Germany) for up to 1 minute. The tubes were refrozen in liquid nitrogen and shaking was repeated until a fine powder was obtained.

RNA was extracted using the Monarch Total RNA Miniprep kit (New England Biolabs, Massachusetts, United States) following the manufacturer's protocol for plant samples. The samples were homogenised in 800 µl of protection reagent at the start of the protocol. Residual gDNA was removed through enzymatic DNase I treatment. The RNA was eluted into 100 µl of nuclease-free water. Preliminary analysis of RNA concentration and contaminates was carried out using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (ThermoFisher Scientific, Massachusetts, United States). The quality and integrity of the RNA samples was then determined using an Agilent 2100 Bioanalyzer with RNA 6000 Nano kit (Agilent, California, United States). To ensure samples were suitable for sequencing only those with a RIN score of >8.5 were selected. Four individual trees per treatment combination were selected for the first three timepoints. At 25 dpi, there was high levels of intraspecific variation in lesion lengths in both JL and EL and so 8 JL and 7 EL samples were selected to ensure both long and short lesion individuals were included (Table 10), eventual analysis

of gene expression showed no obvious difference between long and short lesion individuals and so they were analysed together. The concentration was measured using a Qubit™ RNA BR Assay Kit (ThermoFisher Scientific, Massachusetts, United States) and adjusted to 25 ng/µl and 30 µl transferred to a 96 well plate for sequencing.

Table 10: Number of samples sent for sequencing.

		1 dpi	3 dpi	10 dpi	25 dpi	Lesion length at 25 dpi
Japanese larch	Inoculated	4	4	4	8	4 long 4 short
	Control	4	4	4	4	N/A
European larch	Inoculated	4	4	4	7	4 long 3 short
	Control	4	4	4	4	N/A

The RNA sequencing was carried out externally by the Oxford Genomics Centre (Wellcome Centre for Human Genetics, University of Oxford, UK). Samples underwent PolyA enrichment sequencing on the NovaSeq6000 system (Illumina, California, United States).

3.2.4 Bioinformatic analysis

The quality of the raw reads was assessed using FastQC 0.11.8 (Andrews, 2018). The raw reads were pseudoaligned onto the reference transcriptome of Larix laricina (NCBI GenBank GGJA00000000.1)(Van Ghelder et al., 2019) for each species using kallisto 0.45.1 (Bray et al., 2016) with 1,000 bootstraps. Principal component analysis (PCA) of the overall set of gene expression data was carried out using the 'prcomp' function in the 'stats' package in R (R Core Team, 2020) and the PCA graphs were drawn using the 'autoplot' function of the 'ggfortify' package (Tang et al., 2016). The differential expressed gene (DEG) analysis was carried out using R package 'DESeq2' (Love et al., 2014). The 'DESeq' function generates log2fold changes with adjusted p-values for each infected vs control, with timepoints in a linear scale. This resulted in respective gene lists that were differentially expressed by time, treatment, and their interaction under likelihood ratio test (LRT) with an FDR threshold of 0.05. Hierarchical clustering was also carried out on the differentially expressed genes under time-by-treatment interaction.

3.2.5 Analysis of differentially expressed genes

Venn diagrams of over- and under-expressed transcripts for each species were created by using R package VennDiagram (Hanbo, 2018). The lists of differentially expressed genes (DEG) for each species were compared to the annotated reference transcriptome of *Larix laricina* in order to

identify the corresponding TAIR annotations. The TAIR identifiers were then used to look for enrichment of Gene Ontology (GO) terms through The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (Huang et al., 2009a, 2009b). Each gene list was compared to the background *Larix laricina* transcriptome and GO terms were defined as significantly enriched if they had a Benjamini Hochberg corrected p- value of <0.05 (Benjamini & Hochberg, 1995). This allowed identification of the main biological functions of each DEG list. Enrichment of KEGG pathways was also studied, using the same criteria, in DAVID.

The subset of DEG for the enriched GO terms were compared to the hierarchical clusters to look for similarities in expression profile between the genes included in each GO category. These subsets were also analysed using the KEGG Automatic Annotation Server (Kanehisa & Goto, 2000) to identify the location of genes in the KEGG pathways. The graphs were created using the 'ggplot2' package in R (Wickham, 2016).

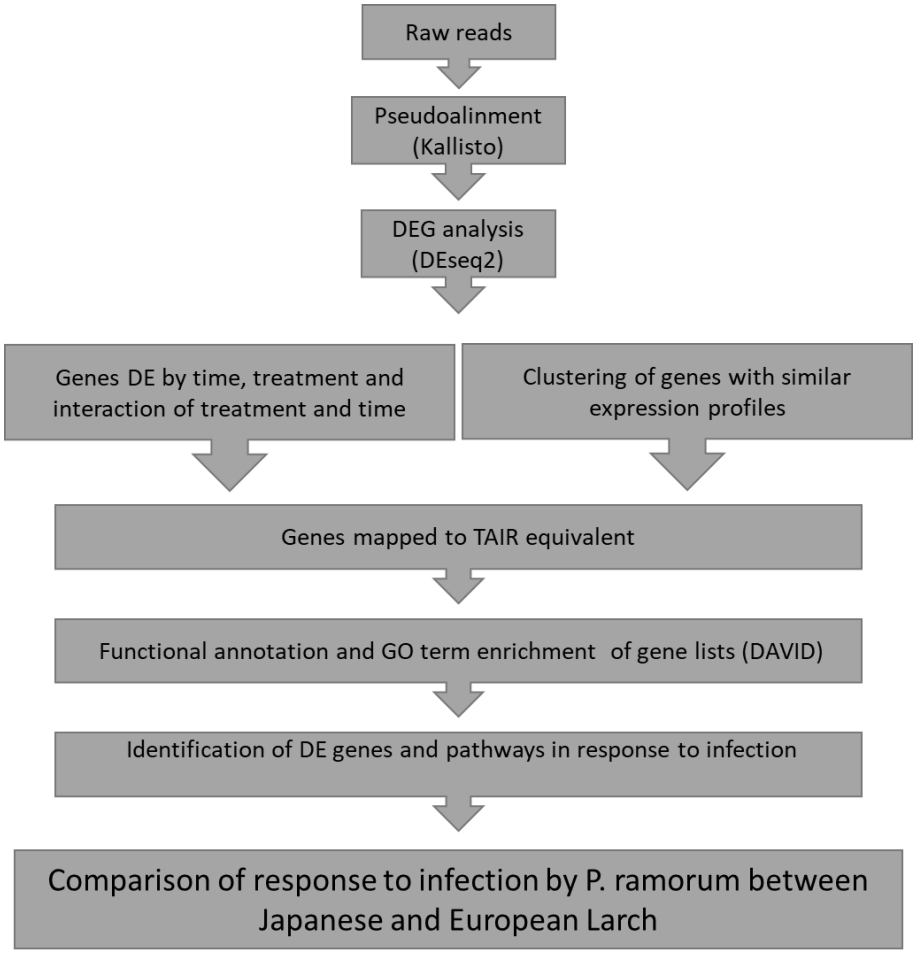


Figure 34: Pathway for analysing the RNA-seq data

3.3 Results

3.3.1 Lesion development

To investigate if there was a difference in lesion development between in EL and JL we examined lesion length at each sampling timepoint after inoculation. There was no lesion development on the control trees and no lesions were observed at 1 dpi in inoculated trees (Figure 35). Small lesions (2-11 mm) were observed on four trees at 3 dpi, and by 10 dpi all inoculated trees had well developed lesions (20-110 mm). In three trees the lesions from the two inoculation points had extended so that the two lesions had merged into one. To take this into account the lesion length for all trees, regardless of whether lesions had merged or not, was recorded as a single value; the combined total length of the two lesions on the tree. At 10 dpi the average total length for the two lesions on each tree was 69.6 mm (SD=42.3) and 81.6 mm (SD=44.0), for EL and JL, respectively. At 25 dpi the average combined lesion length was 178.1 mm (SD=68.7), and 168.3 mm (SD=69.8) for EL and JL, respectively (Figure 3). Two sample T-tests showed that there was no significant difference in mean lesion length between EL and JL at 10 dpi ($t(13)= -0.53423$, $p>0.05$) or 25 dpi ($t(14)= 0.28067$, $p>0.05$).

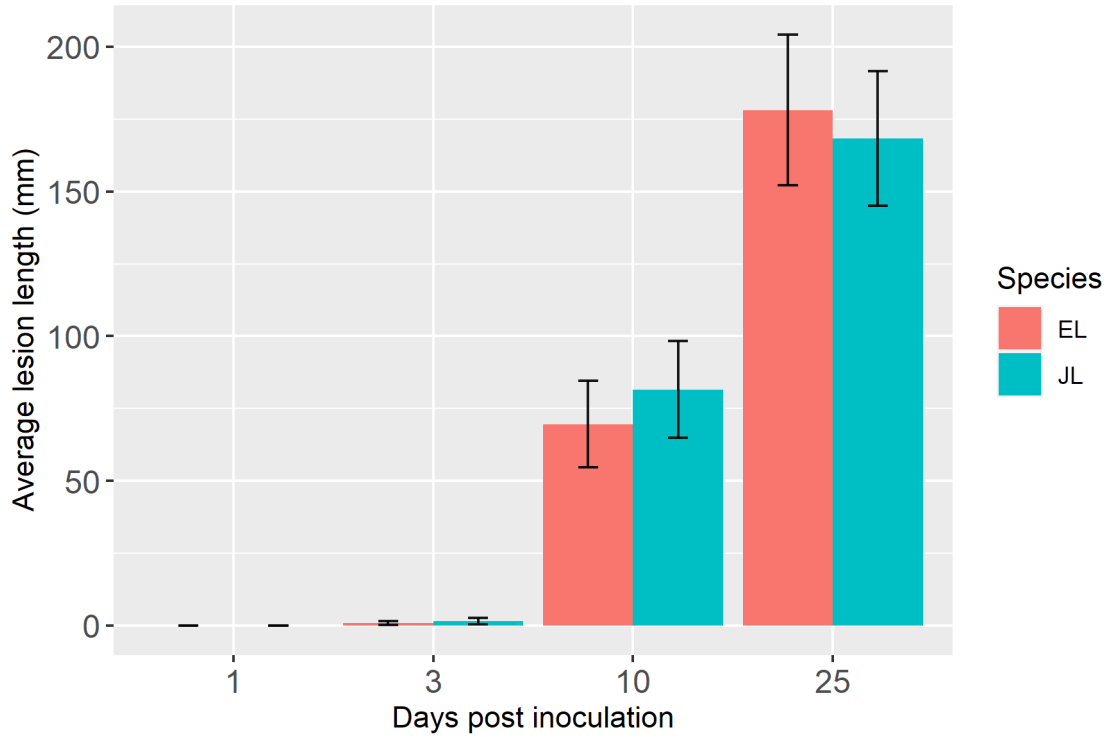


Figure 35: Lesion lengths on European larch (EL) and Japanese larch (JL) at 1,3,10 and 25 days post inoculation with P. ramorum. Error bars indicate standard error. n=8-10 for each timepoint.

3.3.2 RNA Sequencing

The output of the Illumina Hiseq2000 resulted in an average of 24 million raw reads per sample. After processing, the proportion of reads mapped to the *Larix laricina* reference transcriptome (Van Ghelder et al., 2019) were 67.6% and 64.9% for EL and JL respectively, which was statistically significant (two sample T-test between EL and JL, $t(69)=-2.76$, $p>0.05$).

A total of 50,658 transcripts were identified across all the samples, of these 26,757 (53%) were matched to annotated genes in the TAIR database.

3.3.2.1 Differentiation of treatments and time points

A PCA of the overall set of gene expression data indicated a clear differentiation of control and infected trees, except for the 1dpi samples from inoculated trees which clustered with the controls, at this time there were no visible lesions (Figure 36). The PC1 and PC2 accounted for 22.89% and 6.17% of the variance respectively. Time (as dpi) is partially separated along the y axis with more of the 25dpi gathered towards the top of the graph. When labelled with species identifiers, the same PCA shows a slight but non-diagnostic separation between EL and JL from top to bottom (Figure 37). Considering each species separately there is overlap between the control and inoculated trees at 1dpi and clear separation between control and infected trees at subsequent time points (Figure 38). Earlier time points also cluster more closely together, with the 25dpi time point showing greater spread.

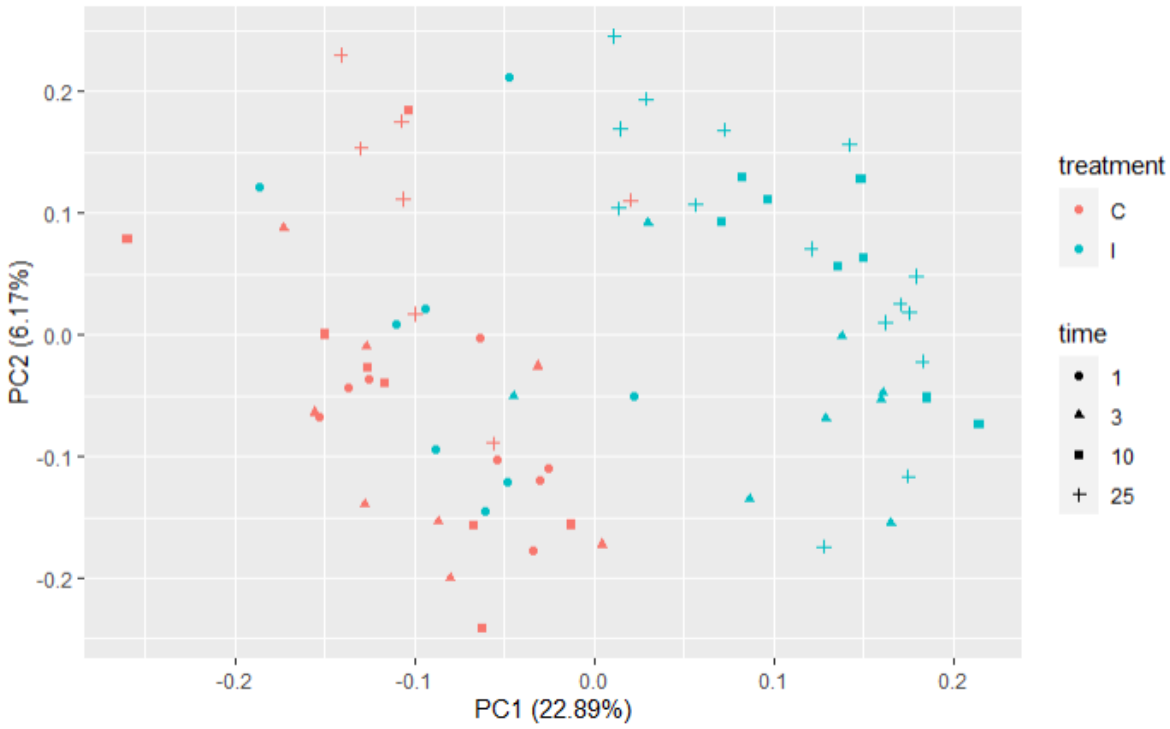


Figure 36: Principal component analysis of the overall set of gene expression indicating the sampling time of the samples. For treatment C=control and I= inoculated, Time is days post inoculation.

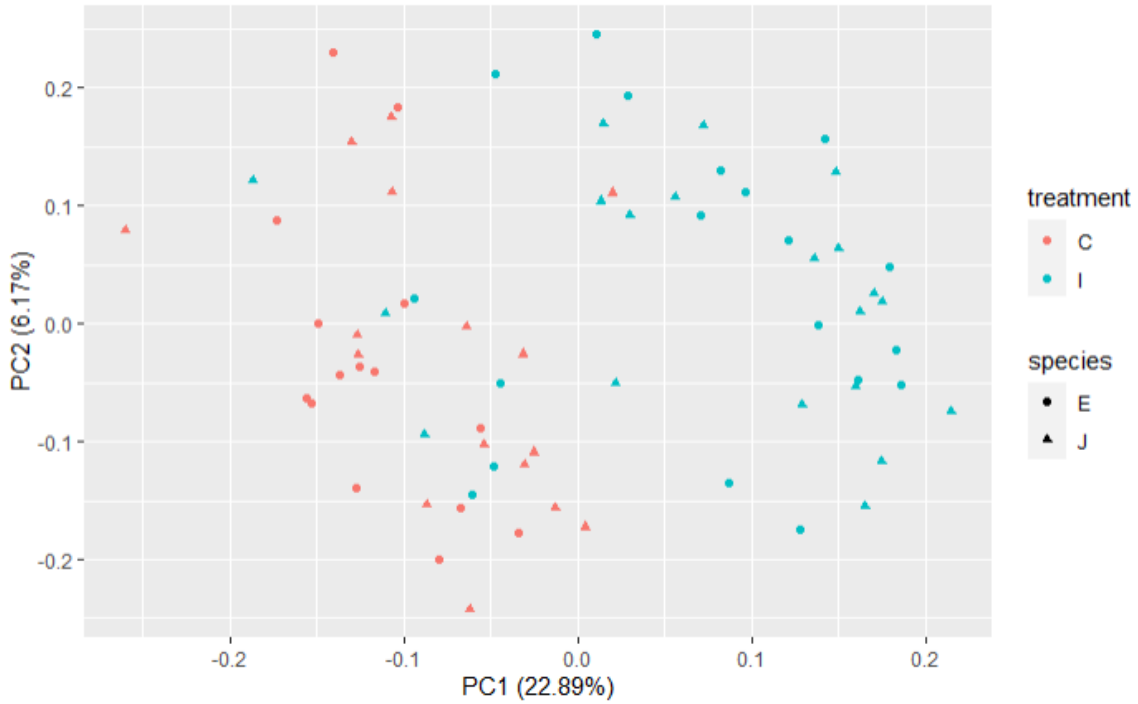


Figure 37: Principal component analysis of the overall set of gene expression indicating the species of the samples. For treatment C=control and I= inoculated. For species E= European larch, J=Japanese larch.

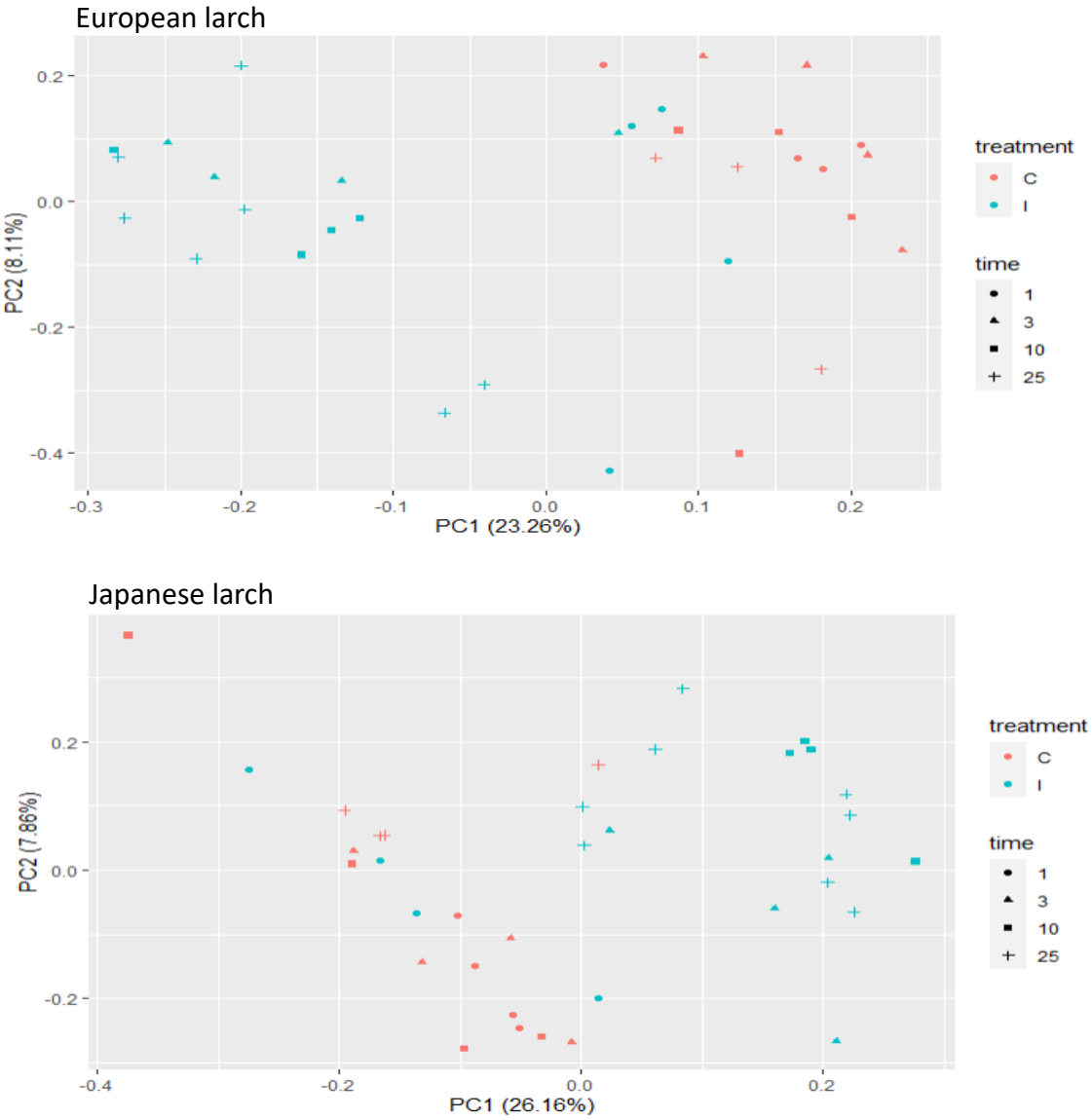


Figure 38: Principal component analysis of the overall set of gene expression separated by species. For treatment C=control and I= inoculated, Time is days post inoculation.

3.3.2.2 Analysis of differentially expressed genes (DEGs)

Overall, more genes were differentially expressed between treatment and control in JL than EL. The DEseq2 analysis found 7,500 DEGs in JL and 4,964 DEGs in EL through the modelling of differential expression between the inoculated and control treatments (Table 2). Fewer DEGs were found for each species through the modelling of differential expression over time and only a small subset of the transcripts were differently expressed when the interaction between time and treatment was modelled.

Table 11: Total number of differentially expressed genes identified for each species through DEG analysis (DEseq2).

Factor	Japanese larch	European larch
treatment	7500	4964
time	5966	5189
interaction between treatment and time	479	152

3.3.3 Genes with a significant interaction of treatment and time

The numbers of DEGs with a significant treatment x time interaction were 479 in JL and 152 in EL, with 22 found in both species (Figure 39a), and functional annotations were found for a large fraction of the DEGs (Figure 39b). Enrichment analysis with DAVID only identified significant GO terms for cellular component in both species (Table 12) and KEGG pathway only in JL (Table 13).

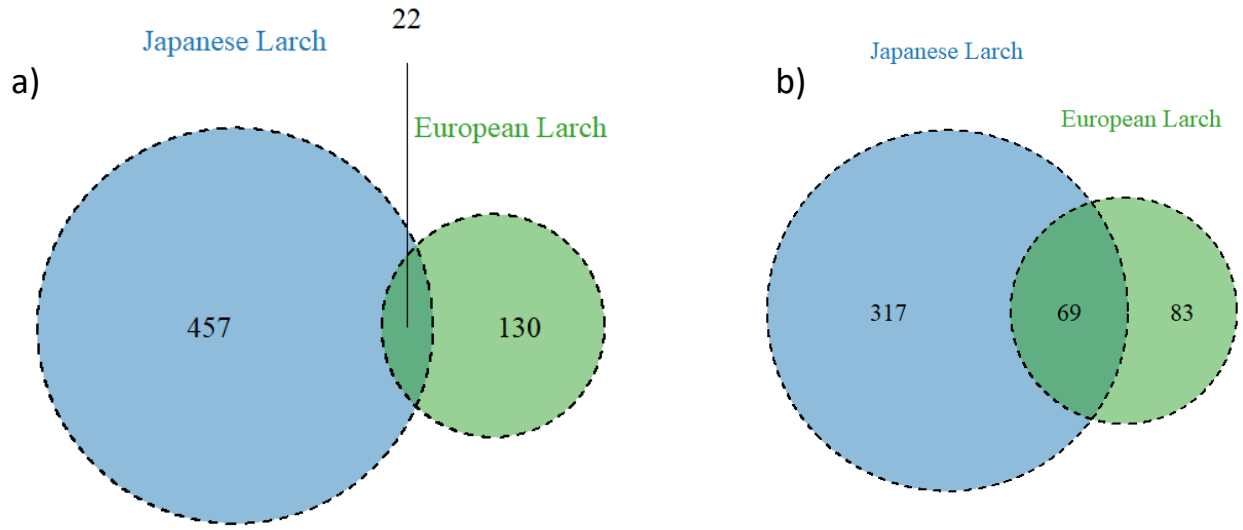


Figure 39: a) Numbers of differentially expressed genes identified through the modelling of the interaction between treatment and time by DESeq2 for Japanese larch and European larch, b) Numbers of the differentially expressed genes that have TAIR annotations.

Table 12: Significantly enriched cellular component GO terms in Japanese larch and European larch. Enriched terms from DEG transcripts identified through the modelling of the interaction between treatment and time Corrected P value is Benjamini Hochberg correction.

	GO term	Gene count	Percentage of total	Corrected P value
Japanese larch	extracellular region	46	14.6	6.47E-04
	apoplast	19	6.1	0.0063
	Chloroplast stroma	30	9.6	0.0063
	Cell wall	20	6.4	0.0063
	membrane	46	14.4	0.0063
	integral component of plasma membrane	12	3.8	0.0432
European larch	thalakoid	8	7.8	0.0287

Table 13: Significantly enriched KEGG pathways in Japanese larch. Enriched terms from DEG transcripts identified through the modelling of the interaction between treatment and time. Corrected P value is Benjamini Hochberg correction.

KEGG Pathway	Gene count	Percentage of total	Corrected P value
Phenylpropanoid biosynthesis	12	3.8	0.0019
Metabolic pathways	49	15.6	0.0439
Biosynthesis of secondary metabolites	32	10.2	0.0439

3.3.3.1 Genes that are differentially expressed by treatment

There was large overlap in DEGs with a significant treatment effect between the two species (Figure 40). In total there were more downregulated than upregulated genes for both species; 3,873 compared to 3,627 upregulated in JL and 2,849 compared to 2,115 upregulated in EL.

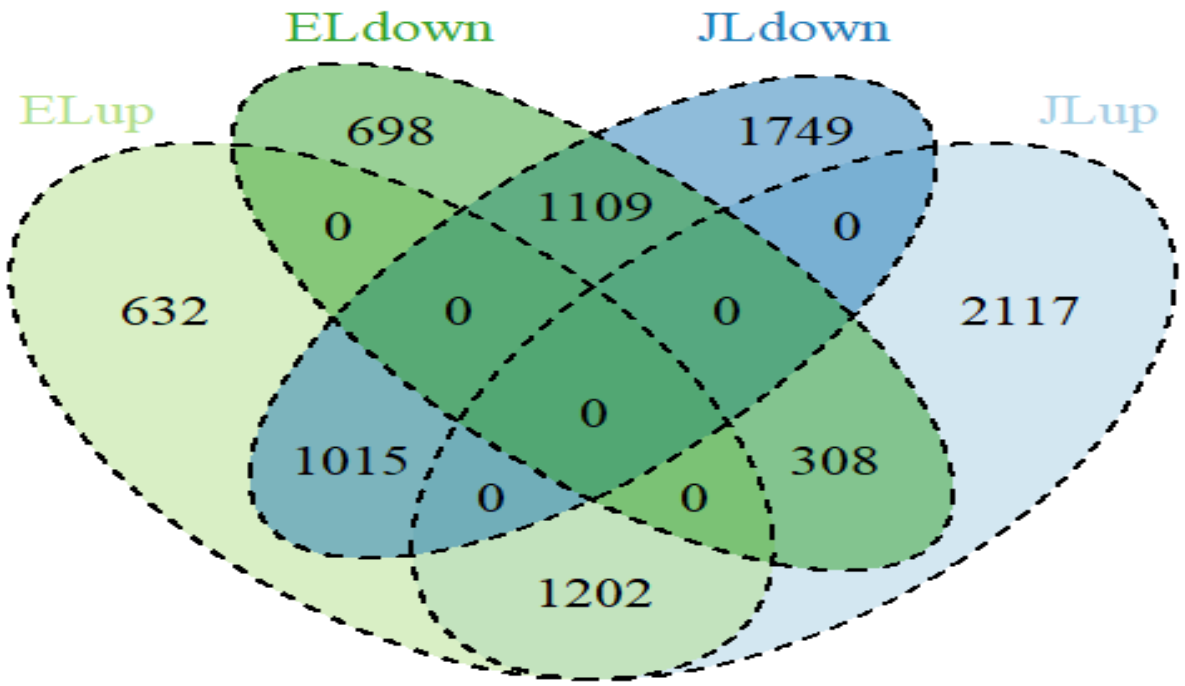


Figure 40: Numbers of differentially expressed genes with up or down regulation by log2 fold change, identified through the modelling of the effect of treatment by DESeq2 for European larch (EL) and Japanese larch (JL).

The functional annotations associated with the DEGs (5,690 JL genes and 3,768 EL genes) were categorized into 34 and 32 main functional groups for JL and EL, respectively, by DAVID analysis, across GO categories of Molecular Function, Cellular Component and Biological Process.

Enrichment analysis identified few statistically significant Molecular Function terms (Table 14) and a moderate number of Biological Processes terms (Figure 41). Many of these Biological Processes terms were shared between the two species including translation, response to salt stress, response to wounding, response to chitin and jasmonic acid biosynthetic process. For the Cellular Component terms there were over 20 significantly enriched terms in both JL and EL, many being related and many being different between the species (Figure 42). The significantly enriched KEGG pathway terms include six pathways shared between JL and EL: biosynthesis of antibiotics, biosynthesis of amino acids, ribosome, carbon metabolism, cysteine and methionine metabolism and flavonoid biosynthesis (Figure 43).

Table 14: Significantly enriched Molecular Function GO terms in Japanese and European larch. Enriched terms identified from DEG genes identified through the modelling of the effect of treatment by DESeq2. Corrected P value is Benjamini Hochberg correction.

	GO term	Gene count	Percentage of total	Corrected P value
Japanese larch	structural constituent of ribosome	143	3.6	4.59E-06
European larch	structural constituent of ribosome	122	4.4	9.36E-11
	mRNA binding	90	3.3	0.0027
	copper ion binding	56	2.0	0.041

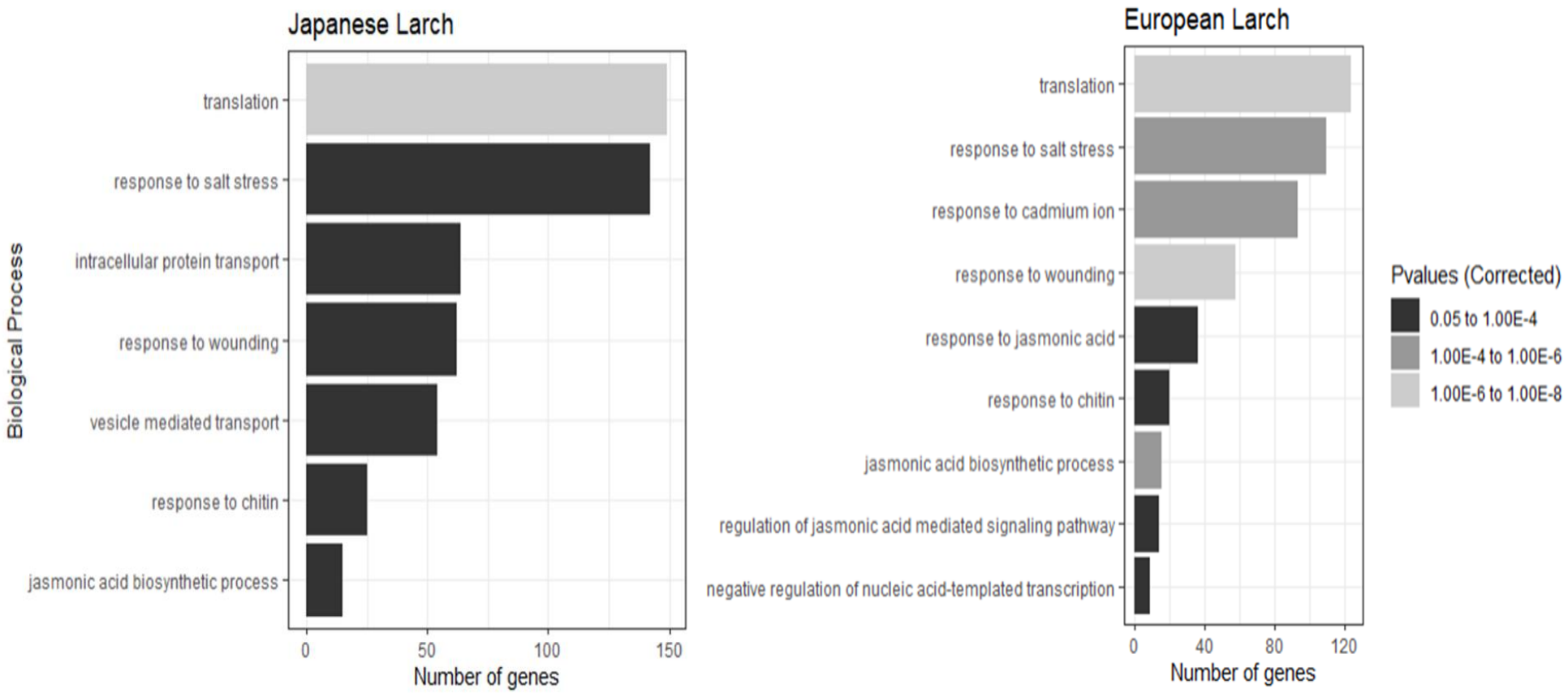


Figure 41: Significantly enriched Biological Process GO terms for each species of larch. Enriched terms identified from DEGs identified through the modelling of the effect of treatment by DESeq2. Corrected P value is Benjamini Hochberg correction.

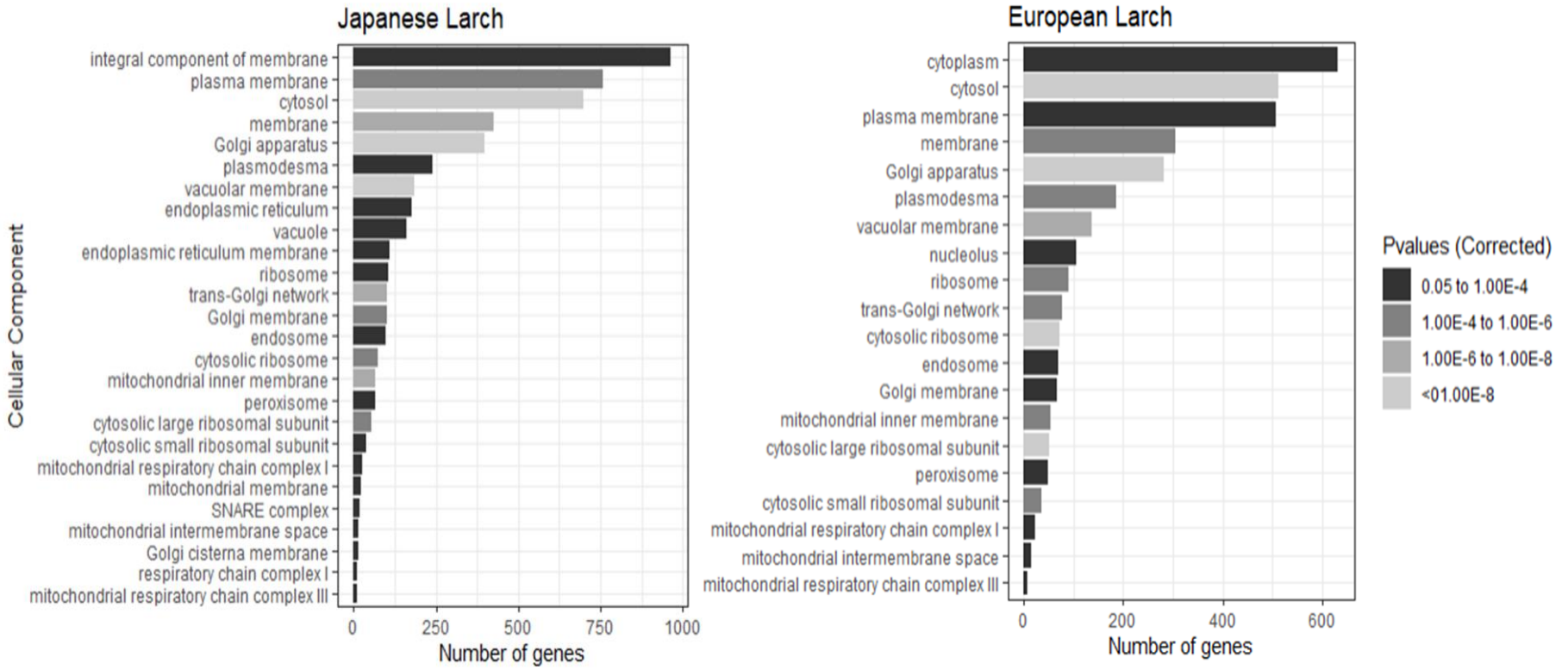


Figure 42 : Significantly enriched Cellular Component GO terms in Japanese larch and European larch. Enriched terms identified from DEGs identified through the modelling of the effect of treatment by DESeq2Corrected P value is Benjamini Hochberg correction.

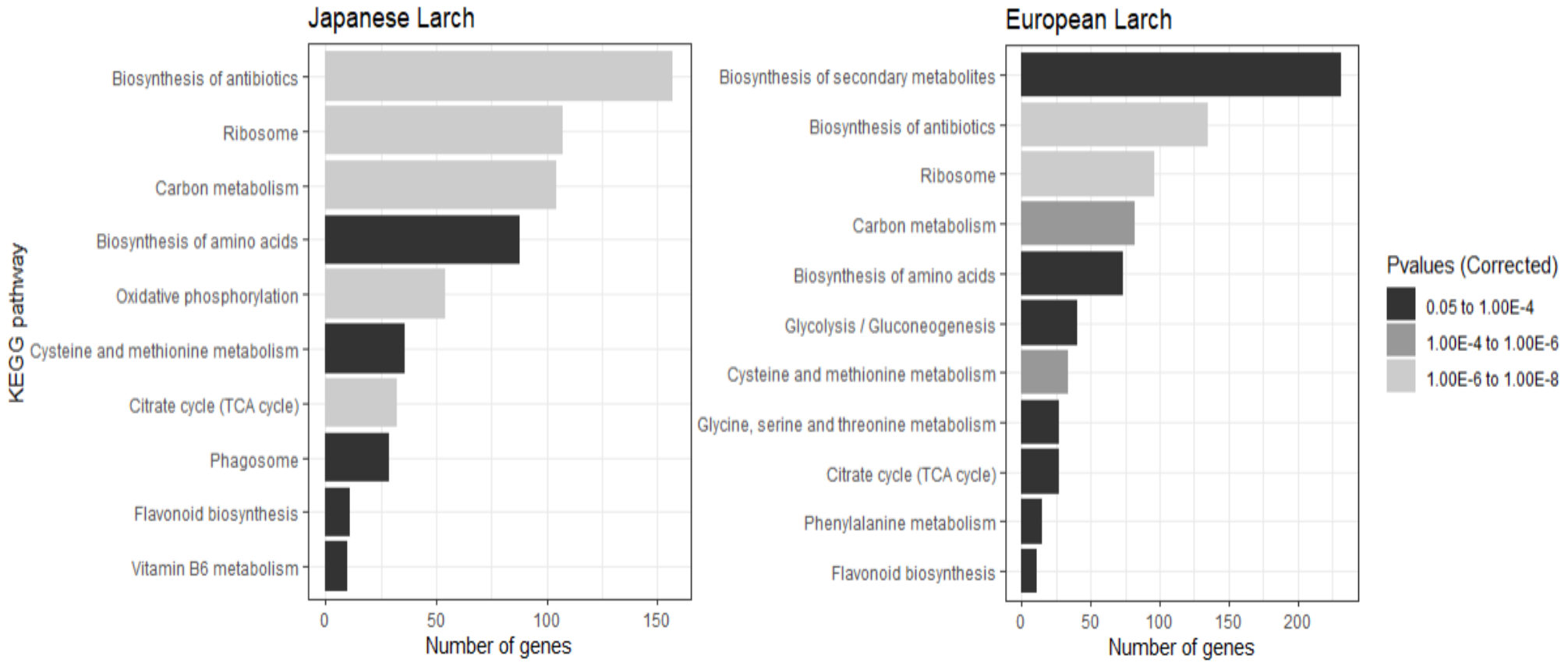


Figure 43: Significantly enriched KEGG pathways in Japanese larch and European larch. Enriched pathways identified from DEGs through the modelling of the effect of treatment by DESeq2 Corrected P value is Benjamini Hochberg correction.

The DEGs were clustered separately for JL and EL by their expression profiles over time, resulting in 24 clusters (Figure 44) and 19 clusters (Figure 46), respectively. The clusters are referred to in the text with an E- or J- prefix to denote their origin. These were used to inform our understanding of the genes from the enriched GO terms and KEGG pathways (Figure 45 and Figure 47).

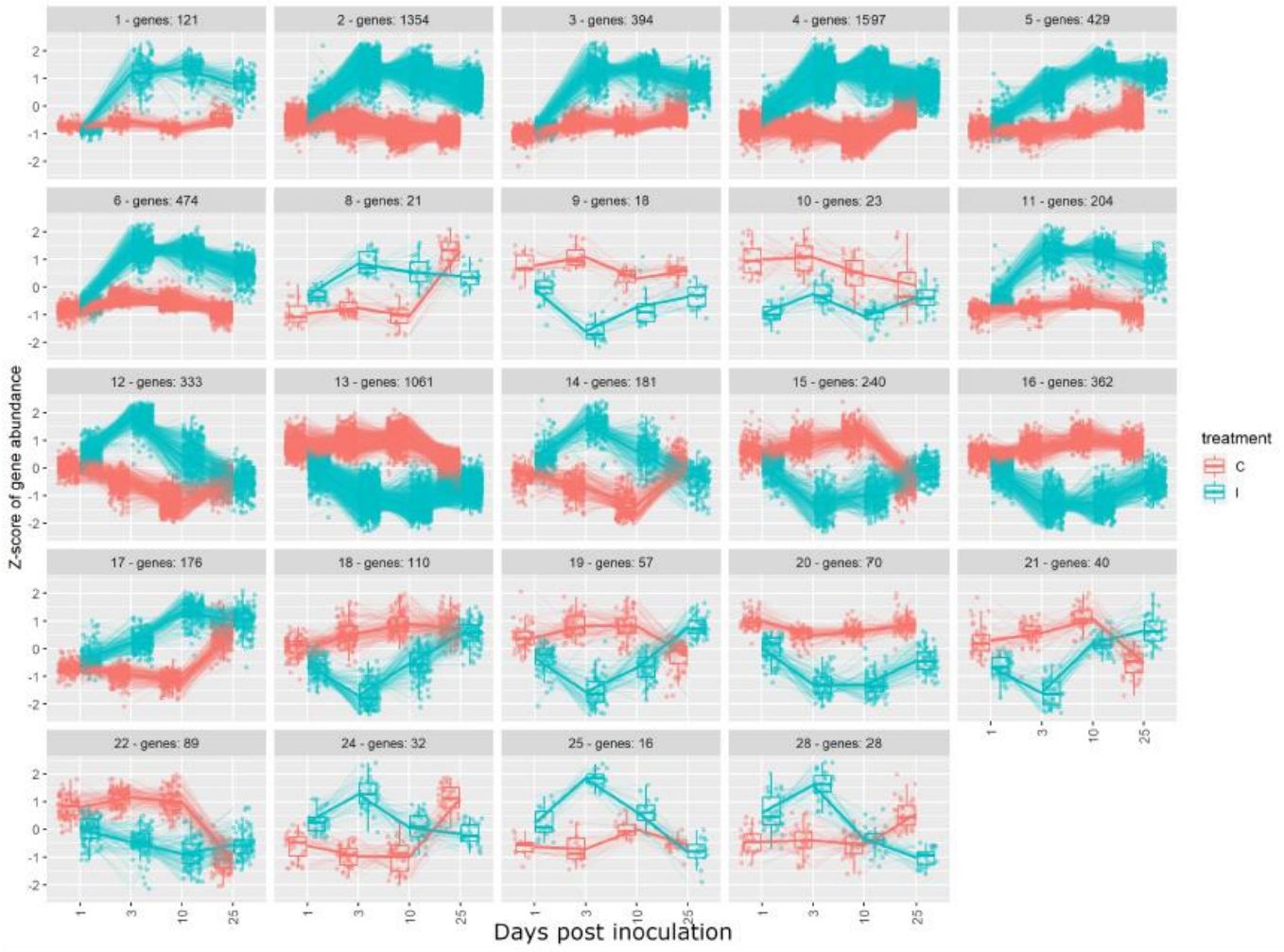


Figure 44: Expression profile clustering of DEGs identified through the modelling of the effect of treatment by DESeq2 in Japanese larch. The first number in each heading is the identifying number of that expression profile cluster, followed by the number of genes within that profile. Red lines represent the control treatment (C) and blue lines represent the inoculated treatment (I).

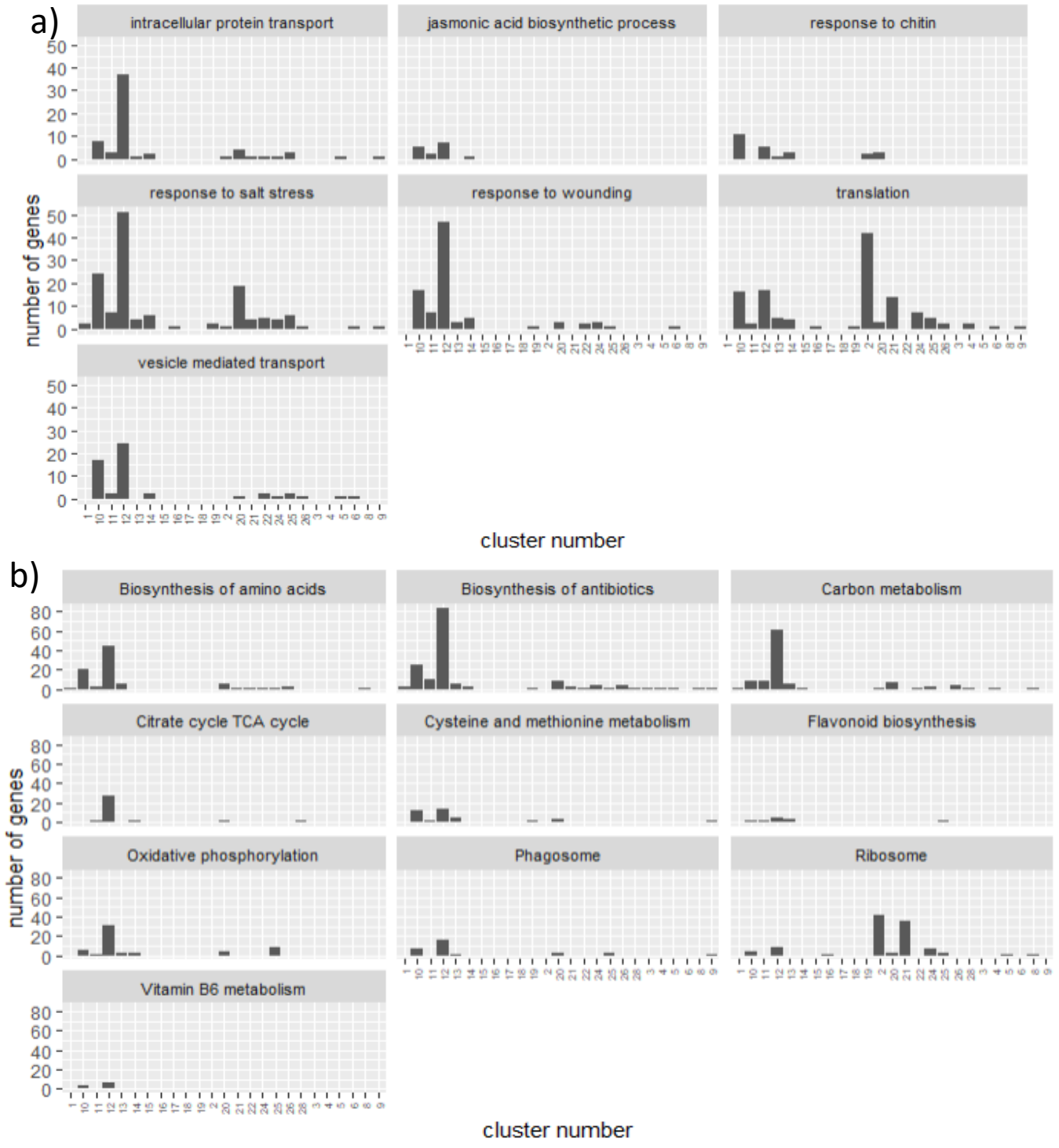


Figure 45: Numbers of genes in overrepresented pathways in Japanese larch. Cluster number refers the Japanese larch specific expression pathway clusters in Figure 44. a) enriched Biological Process GO terms, b) enriched KEGG pathway terms.

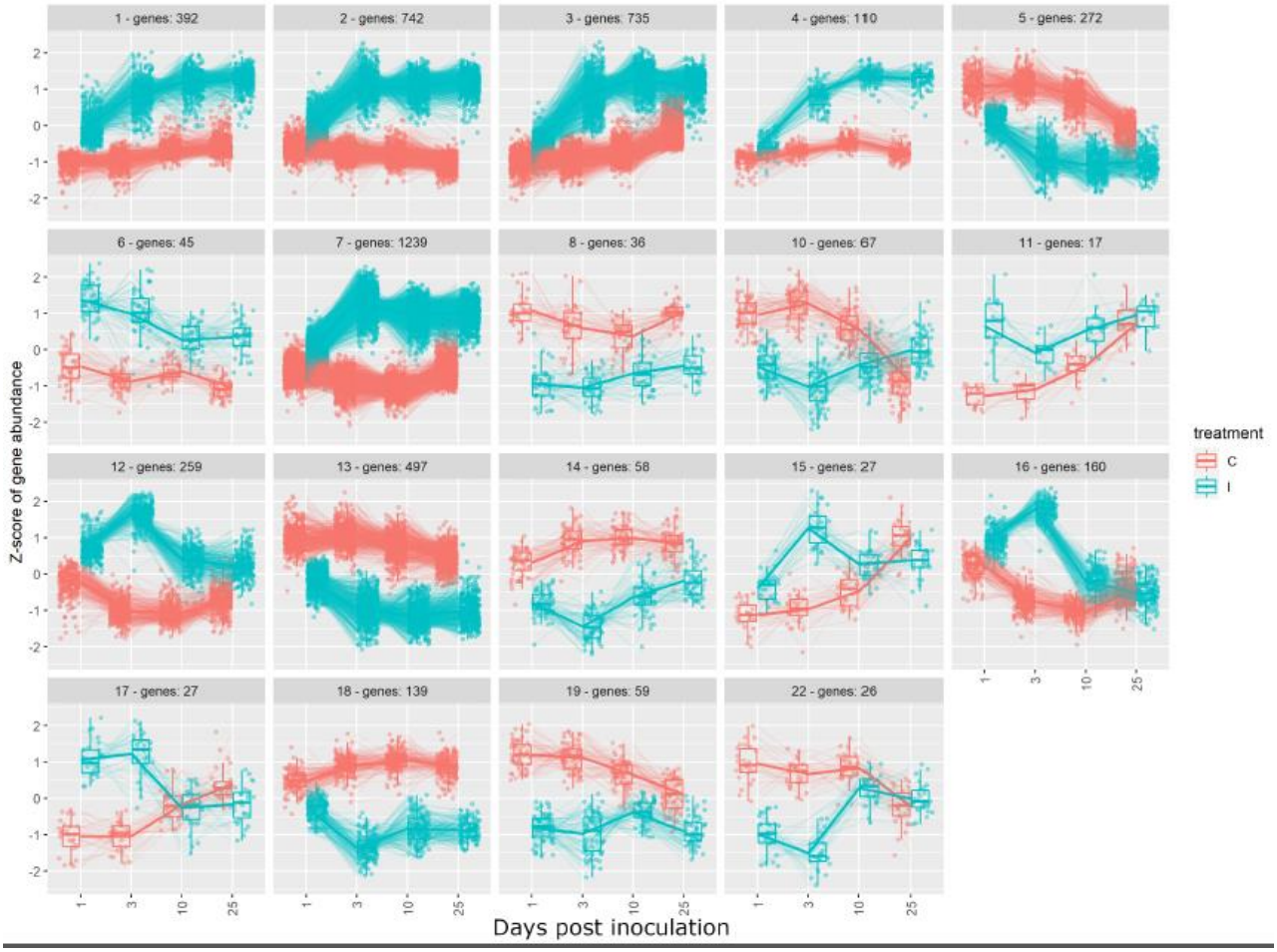


Figure 46: Expression profile clustering of DEGs identified through the modelling of the effect of treatment by DESeq2 in European larch. The first number in each heading is the identifying number of that expression profile cluster, followed by the number of genes within that profile. Red lines represent the control treatment (C) and blue lines represent the inoculated treatment (I).

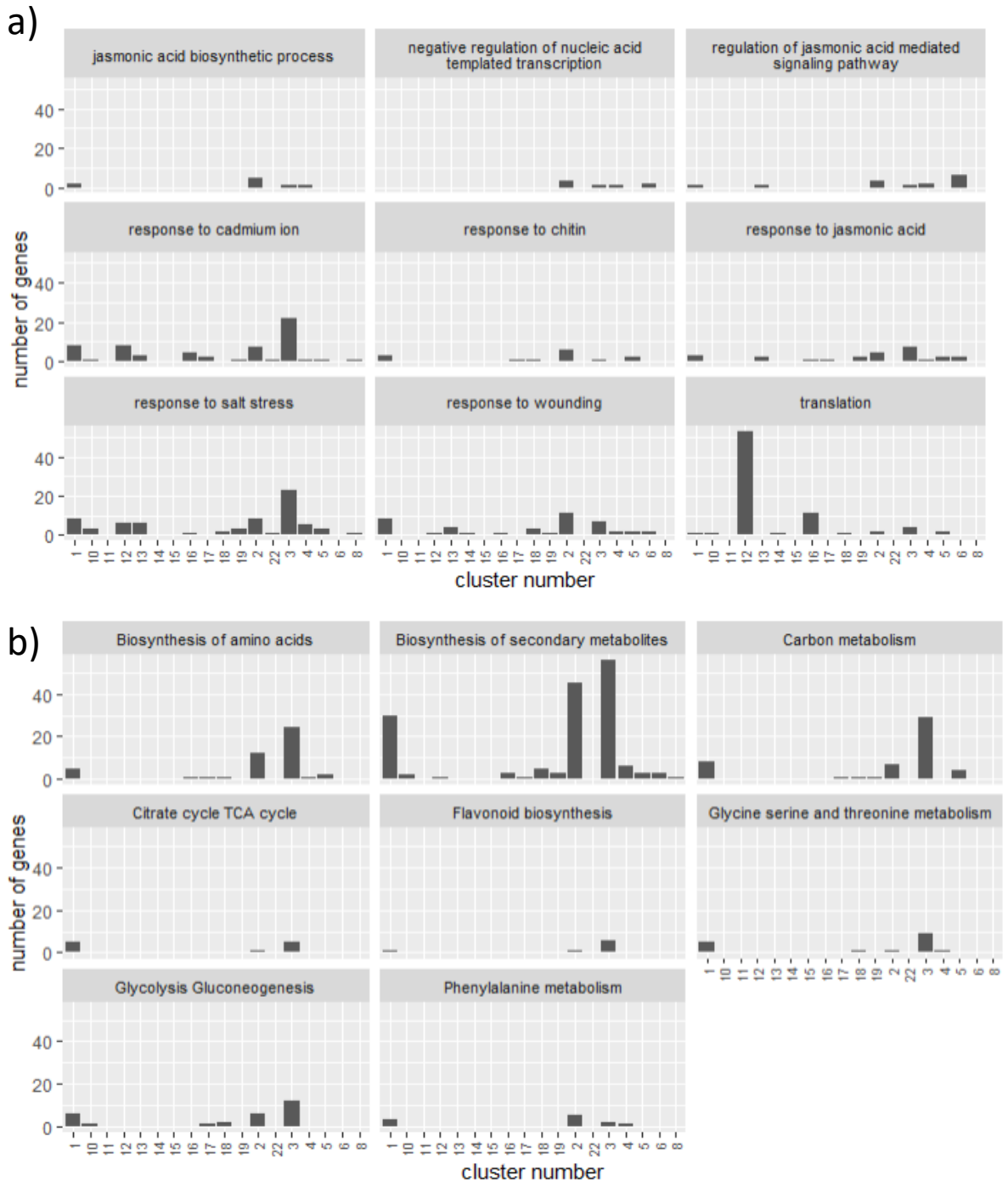


Figure 47: Numbers of genes in overrepresented pathways in European larch. Cluster number refers to the European larch specific expression pathway clusters in Figure 46. a) enriched Biological Process GO terms, b) enriched KEGG pathway terms.

3.3.3.2 Changes in expression related to jasmonic acid

Jasmonic acid (JA) biosynthesis genes were overrepresented in both species, with the same 15 DEGs in both species (Table 15). The JL genes fit into expression profiles J2, J3, J4 and J6 (Figure 44) and the EL genes fit into expression profiles E1, E2, E3, E4 and E7 (Figure 46). All of these clusters follow a similar pattern of rapid increase in expression in the inoculated plants between 1- and 3-dpi. However, after 3 dpi the expression differs slightly between the two species. In EL the expression slowly increases over subsequent days (cluster E1) or is maintained at a high level (clusters E2, E3, E4 and E7) to the end of the experiment at 25 dpi. In JL the rapid increase in expression between 1 and 3 dpi was followed by a slight decrease (clusters J2, J3, J4 and J6) to the end of the experiment at 25 dpi.

Three of the larch genes were similar to *Arabidopsis thaliana* lipoxygenase genes: AT1G55020- *LOX1*, AT1G17420-*LOX3* and AT1G72520- *LOX4*. *LOX* genes are well characterised for their role in rapid jasmonate synthesis after wounding and in response to infection. The expression profiles of putative *LOX* genes show differences between EL and JL.

The expression of *LOX1* homologs are consistently upregulated compared to the control in both species. There is a is a small gene family of sequences that map to *LOX1*; six genes in EL and seven genes in JL (Figure 48), with a range of response levels but all of them have higher expression than the controls.

The most notable increase is in the gene that maps to *LOX3* (Figure 49). The EL sequence increases sharply between 1 and 3 dpi and continues to increase to 25 dpi. In JL there was a greater increase between 1 and 3 dpi followed by a more gradual increase to 10 dpi followed by a decrease, although the final expression level is similar between the two species.

In *LOX4* there is a high level of overexpression in both EL and JL in response to infection whereas the control trees have sharply decreasing expression toward the end of the experiment (Figure 50).

Table 15: Details of the DEGs in Japanese larch (JL) and European larch (EL) that are in the enriched jasmonic acid biosynthetic process GO term. TAIR annotation descriptions come from The Arabidopsis Information Resource (Arabidopsis.org).

TAIR gene name	JL cluster number	EL cluster number	TAIR annotation
AT1G61850	J4	E7	Involved in basal jasmonic acid biosynthesis by releasing the precursor fatty acid from membrane lipids
AT3G25770	J2	E2	Encodes allene oxide cyclase which catalyzes an essential step in jasmonic acid biosynthesis
AT1G55020	J4	E7	*LOX1*- a Lipoxygenase which catalyzes the oxygenation of fatty acids

AT5G42650	J4	E7	Encodes a member of the cytochrome p450 CYP74 gene family that functions as an allene oxide synthase which catalyzes dehydration of the hydroperoxide to an unstable allene oxide in the JA biosynthetic pathway.
AT4G29010	J4	E7	Functions in beta-oxidation of fatty acids
AT1G17420	J2	E1	*LOX3* - a Lipoxygenase which catalyzes the oxygenation of fatty acids
AT4G05160	J2	E2	Encodes a peroxisomal protein involved in the activation of fatty acids through esterification with CoA
AT2G33150	J4	E7	Encodes an organellar (peroxisome, glyoxysome) 3-ketoacyl-CoA thiolase, involved in fatty acid b-oxidation
AT1G05800	J6	E7	*DGL*-Encodes a galactolipase. Located in the chloroplast. Involved in the initial step of jasmonic acid biosynthesis. Expressed in vegetative tissues and is necessary for the biosynthesis of basal-level JAs in vegetative tissues.
AT1G72520	J2	E2	*LOX4* - a Lipoxygenase which catalyzes the oxygenation of fatty acids
AT2G43710	J4	E1	Encodes a stearoyl-ACP desaturase, involved in fatty acid desaturation.
AT1G76690	J2	E2	Encodes one of the closely related 12-oxophytodienoic acid reductases
AT4G16760	J4	E3	Encodes a medium to long-chain acyl-CoA oxidase. Catalyzes the first step of fatty acid beta-oxidation
AT2G06050	J3	E4	Encodes a 12-oxophytodienoate reductase that is required for jasmonate biosynthesis.
AT1G20510	J4	E2	CoA Ligase1

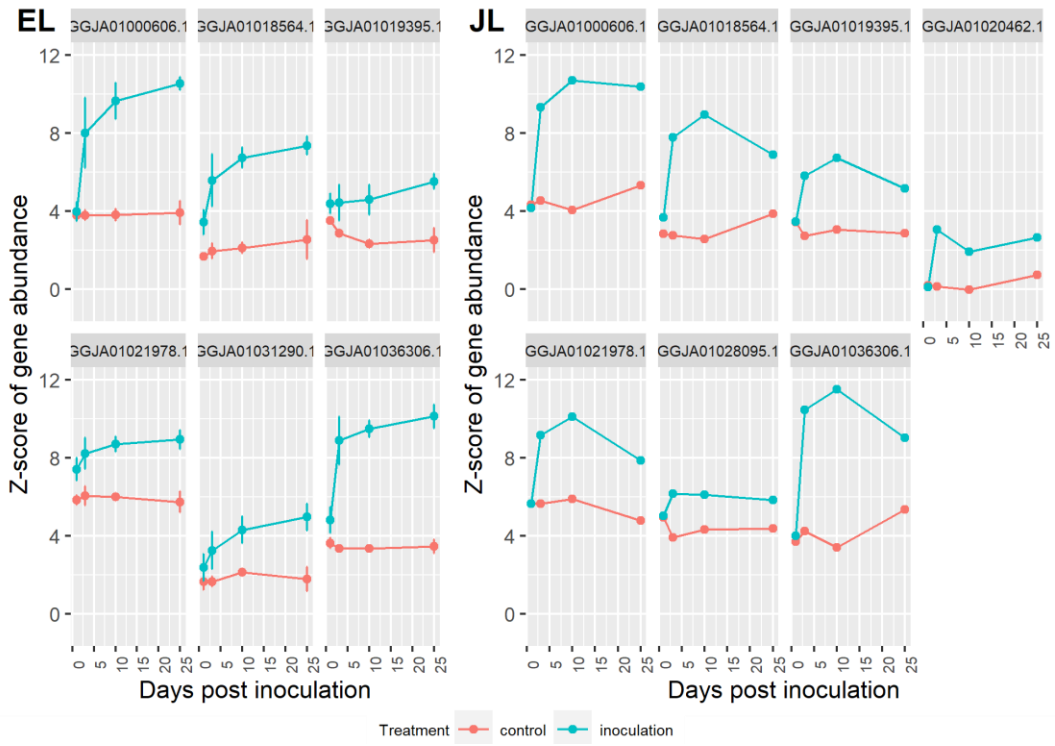


Figure 48: Normalised Z score expression profile of genes which map to the Arabidopsis gene LOX1 in European larch (EL) and Japanese larch (JL) following inoculation with P. ramorum. Error bars are standard error.

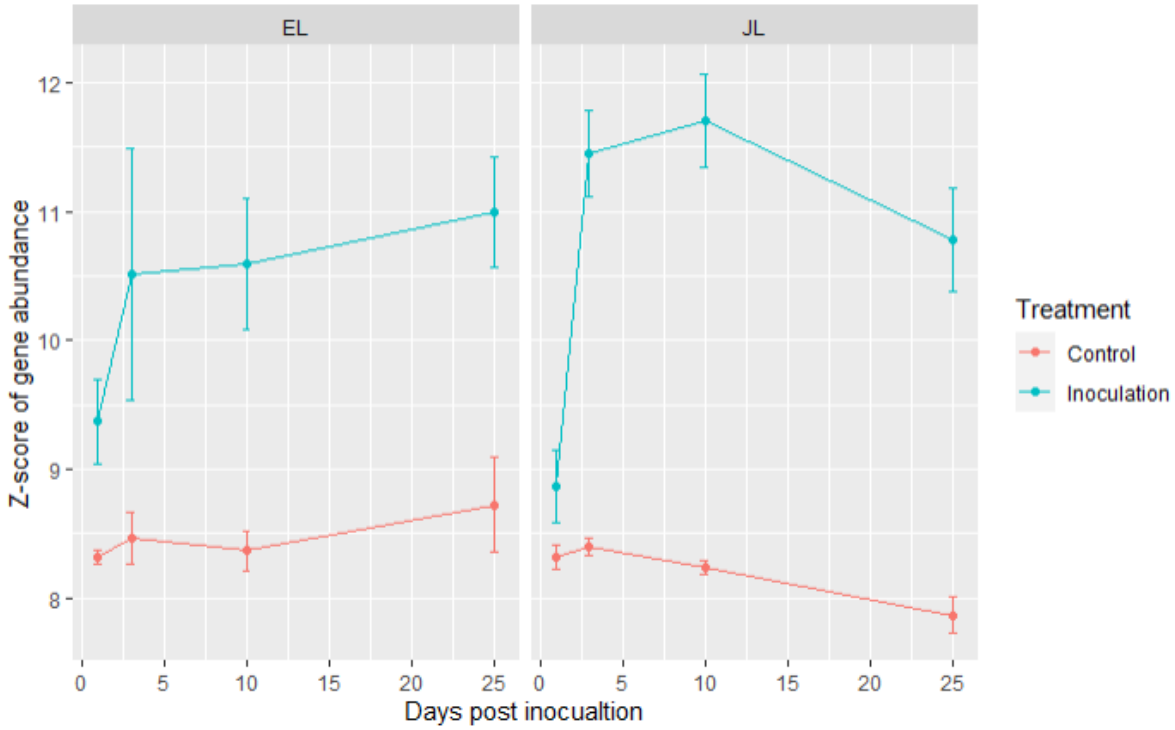


Figure 49: Normalised Z score expression profile of gene GGJA01008964.1 (which maps to the Arabidopsis gene LOX3) in European larch (EL) and Japanese larch (JL) following inoculation with P. ramorum. Error bars are standard error.

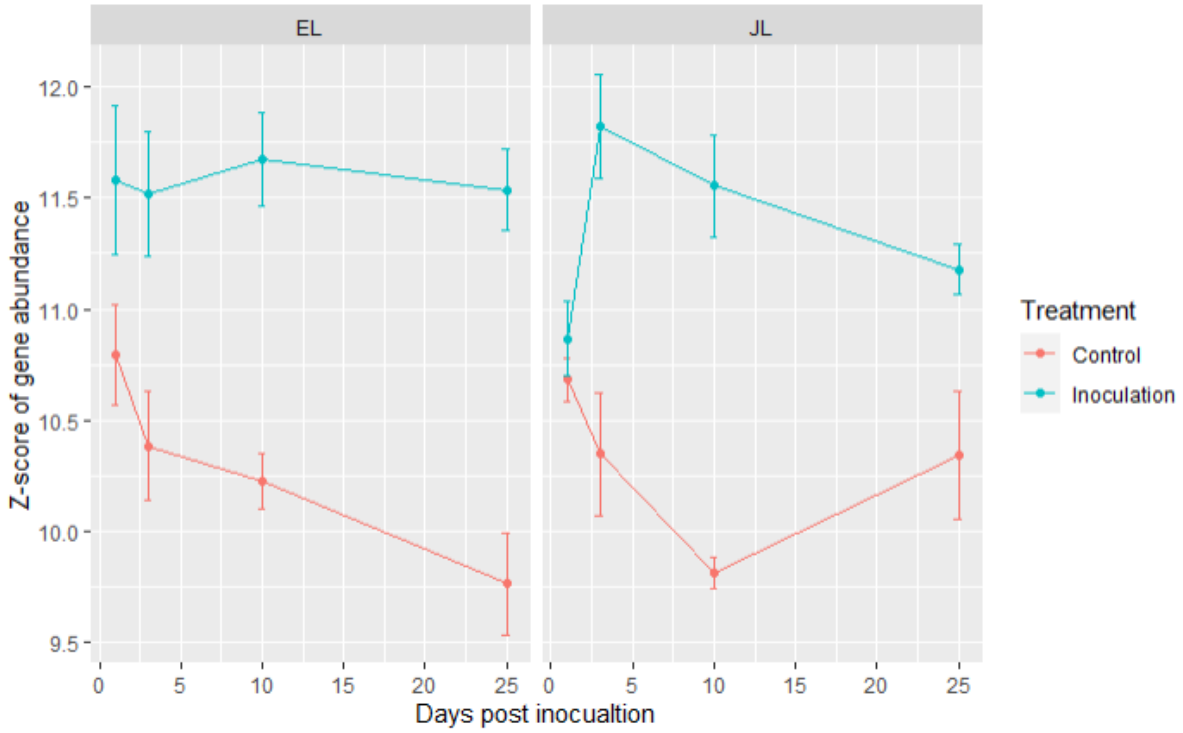


Figure 50: Normalised Z score expression profile of gene GGJA01014840.1 (which maps to the Arabidopsis gene LOX4) in European larch (EL) and Japanese larch (JL) following inoculation with P. ramorum. Error bars are standard error.

European larch also has an overrepresentation of genes in the regulation of jasmonic acid mediated signalling pathway and response to jasmonic acid as indicated by their expression clustering results (Table 16). In the latter, all of the expression clusters have a similar pattern of rapid upregulation after inoculation followed by sustained expression, except for the gene in cluster E13 which has a pattern of downregulation to compared to the control. The gene in cluster E13 (AT1G64280) encodes an *NPR1* homolog, a key regulator of the salicylic acid (SA)-mediated systemic acquired resistance pathway. In regulation of jasmonic acid mediated signalling pathway most of the genes fall into the expression profiles of rapid upregulation after inoculation followed by sustained expression. Here, there are two genes in cluster E13, one of which is similar to AT2G39940, which encodes proteins for the SCF COI1 ubiquitin-ligase complexes and the other is similar to AT4G12570, which codes for ubiquitin protein ligase 5. Clusters E16 and E17 have similar profiles of reduced expression after 3dpi.

Table 16: Gene clustering in jasmonic acid related enriched GO terms in European larch.

	Cluster	Number of genes
regulation of jasmonic acid mediated signalling pathway	E1	1
	E2	3
	E3	1
	E4	2
	E6	6
	E7	4
	E13	1
response to jasmonic acid.	E1	3
	E2	4
	E3	7
	E4	1
	E5	2
	E6	2
	E7	11
	E13	2
	E16	1
	E17	1
	E19	2

3.3.3.3 Enriched KEGG pathways

Several enriched KEGG pathways are likely to be involved in the defence response including biosynthesis of flavonoids in both species, biosynthesis of antibiotics in JL and biosynthesis of secondary metabolites in EL.

The most overrepresented pathways were biosynthesis of secondary metabolites in EL with 232 genes and biosynthesis of antibiotics in JL with 157 genes. These two pathways are similar and have an overlap of 111 shared genes, whilst 121 genes are only found in biosynthesis of secondary metabolites and 46 genes are only found in biosynthesis of antibiotics. The secondary metabolites pathways included the biosynthesis of phenylpropanoids (Figure 51), flavonoids, terpenoid backbone and isoquinoline alkaloids. The antibiotics pathway included the biosynthesis of tropane, piperidine and pyridine alkaloid, terpenoid backbone and isoquinoline alkaloids.

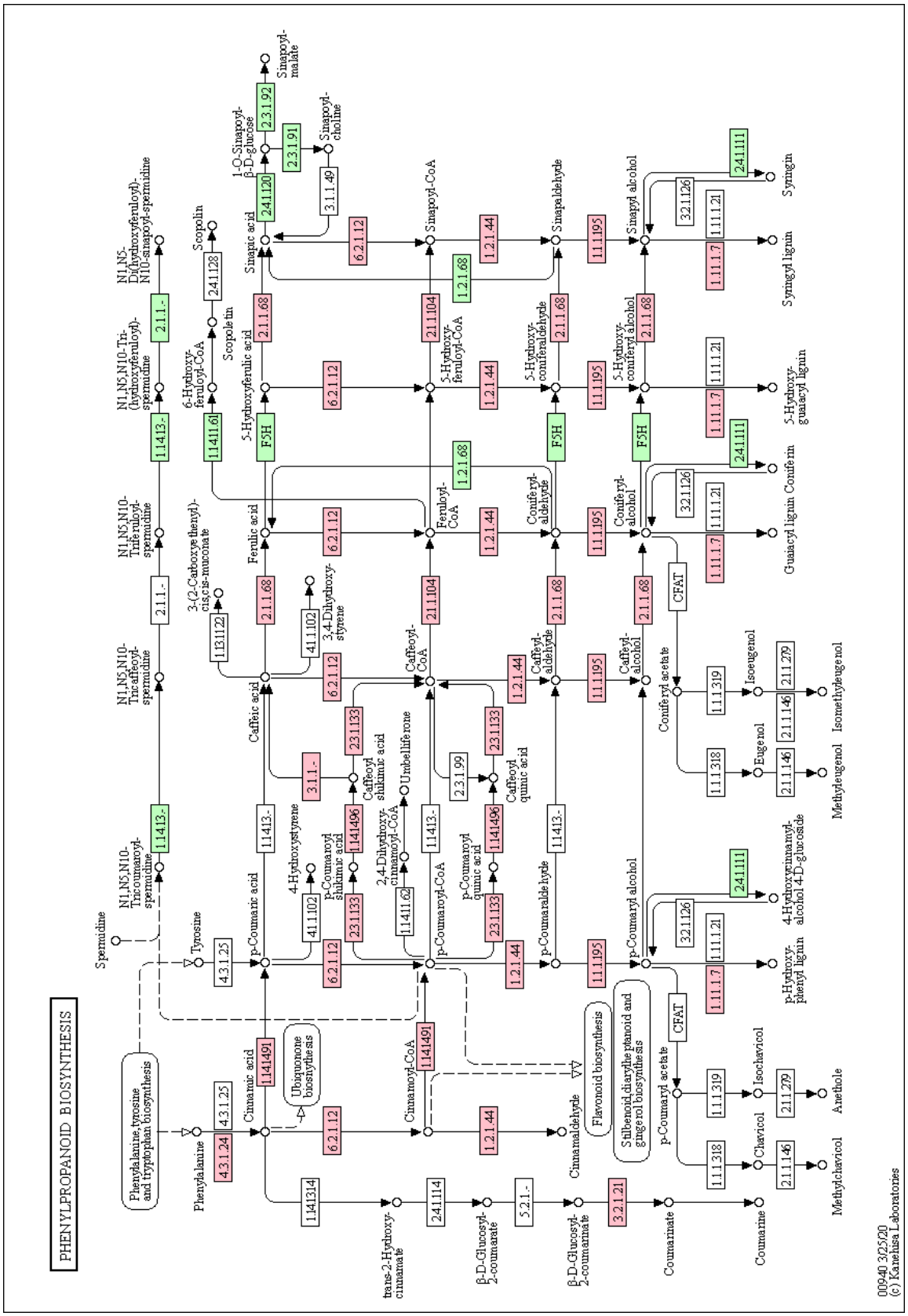
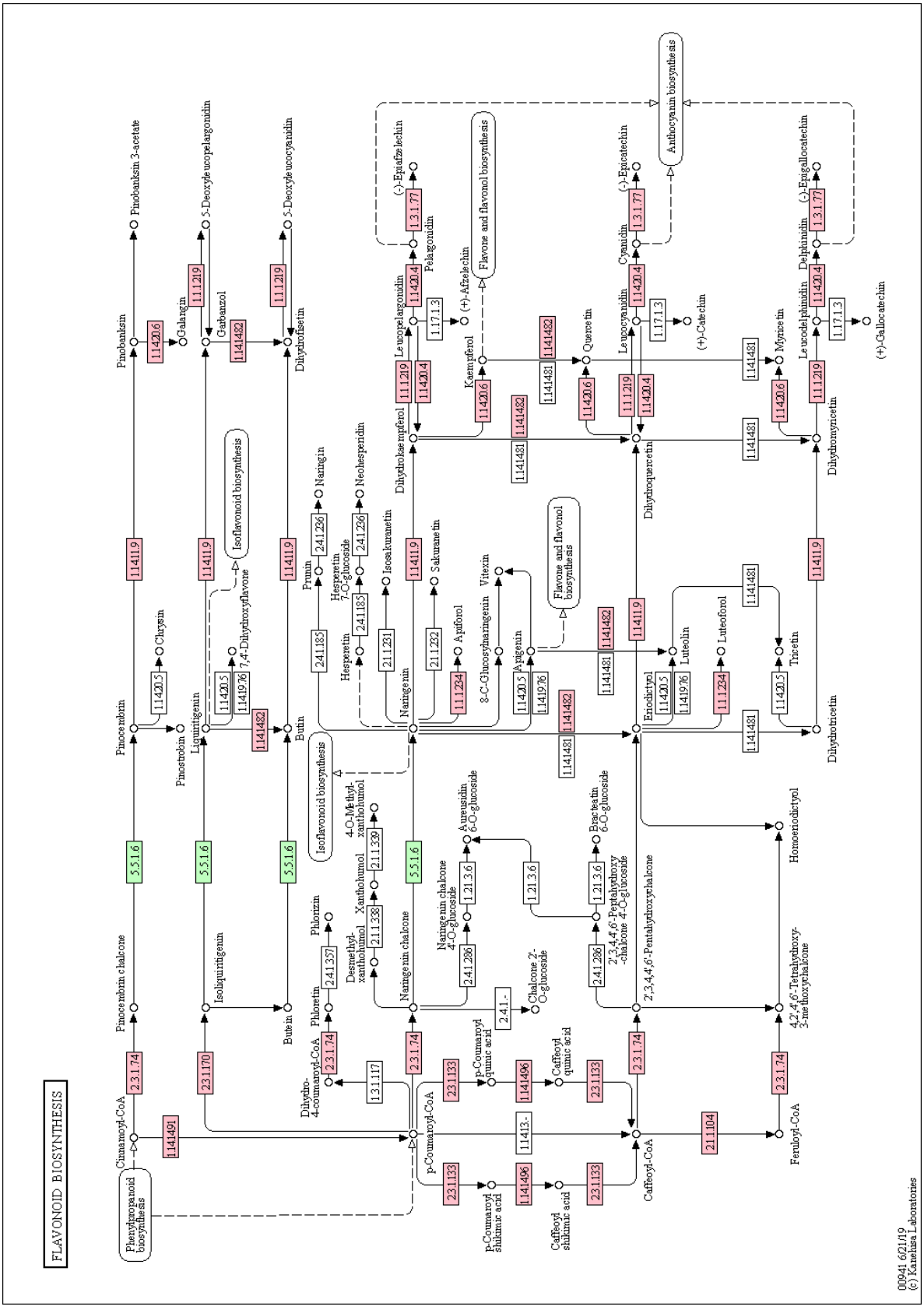


Figure 51: The phenylpropanoid biosynthesis pathway (KEGG pathway ath00940). Pink boxes indicate location within the pathways of the genes that were differentially expressed by Japanese larch and European larch. Green boxes indicate a complete pathway where all required genes are present.

A smaller group of 11 genes resulted in enrichment of the flavonoid biosynthesis pathway (Figure 52) in both JL and EL. Figure 21 shows that these genes cover a large proportion of the flavonoid biosynthesis pathway. In EL the expression profile of these genes matched clusters E1, E2, E3 and E7, with a rapid increase in expression in the inoculated plants between 1- and 3-dpi followed by a slow increase over subsequent days (cluster E1) or maintained a high level of expression (clusters E2, E3 and E7) to the end of the experiment at 25 dpi. In JL the genes were in clusters J2, J3, J4, J5 and J17, which showed an early and rapid increase in expression between 1 and 3 dpi followed by a fall in expression after 3 dpi (clusters J2, J3, J4) or 10 dpi (clusters J5 and J17).

Both species had multiple genes mapping to each enzyme in the pathway, with 4 (min=1, max=15) and 6.4 (min=2, max=26) sequences on average in EL and JL, respectively. Of particular interest is chalcone synthase (AT5G13930), which had the largest associated gene families, with 15 EL genes and 26 JL genes mapping to it (Figure 53). All the transcripts that mapped to chalcone synthase in both species were upregulated in the inoculated trees throughout the time course of the experiment. The expression profiles across all the genes are highly similar.


FLAVONOID BIOSYNTHESIS
Phenylpropanoid biosynthesis
Cinnamoyl-CoA
Pinocembrin chalcone
Isoliquiritigenin
Butein
Dihydro-4-coumaroyl-CoA
Pinocembrin
Pinostrobin
Liquiritigenin
Pinobanksin
Pinobanksin 3-acetate
Galangin
Garbanzol
5-Deoxydelphinidin
Chrysin
7,4'-Dihydroxyflavone
Isoflavonoid biosynthesis
Dihydrofisetin
Pinocembrin chalcone
Naringenin chalcone
Naringenin 4'-O-glucoside
Chalcone 2'-O-glucoside
Pinocembrin
Pinostrobin
Liquiritigenin
Butein
Pinobanksin
Galangin
Garbanzol
Dihydrofisetin

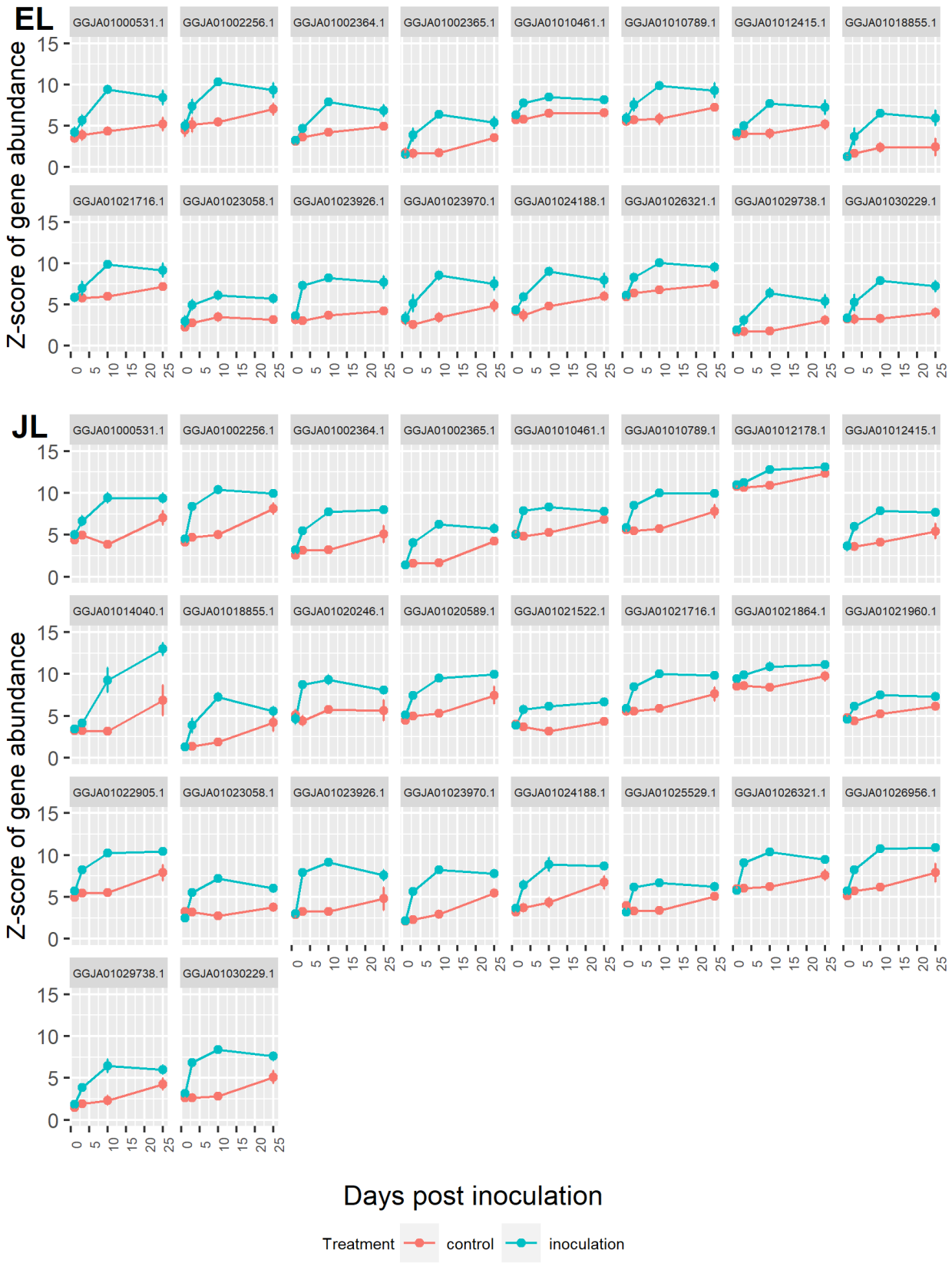


Figure 53: Normalised Z score expression profile of putative chalcone synthase genes in European larch (EL) and Japanese larch (JL) (based on similarity to the Arabidopsis gene AT5G13930) following inoculation with P. ramorum. Error bars are standard error.

Carbon metabolism was enriched in both JL (159 DEGs) and EL (85 DEGs). In JL the largest group of DEGs (38% of the total) are in expression profile J4 which has a rapid increase between 1 and 3dpi before gradually decreasing between 3 and 25dpi. A minority (17) of the DEGs (1% of total) are in expression profiles J12, J13, J15, J16, J18, J29, J21 and J24 where the expression in inoculated plants is lower than in the control, suggesting downregulation of a few parts of the pathway. In EL 63 DEGs (74%) are in expression profiles E3 and E7 with a rapid increase between 1 and 3dpi and sustained expression between 3 and 25dpi.

3.3.3.4 Nucleotide-binding, leucine-rich-repeat genes

Nucleotide-binding, leucine-rich-repeat (*NBS-LRR*) genes form the largest resistance gene family in plants and are the main group of cytoplasmic receptors involved in the recognition of specific pathogens as part of effector-triggered immunity in plants. *NBS-LRR* genes were identified by comparisons of the DEG list for each species to the *NBS-LRR* genes identified in *Larix laricina* by Van Ghelder et al. (2019). In JL, 35 DEGs matched *NBS-LLR* sequences and in EL there were 24 DEGs. The expression of these genes is variable, with some upregulated in the inoculated trees and others upregulated in the control trees (Figure 54). One gene, GGJA01021702.1 was strongly upregulated in the first stages of response in both species and a second, GGJA01019122.1 was also strongly upregulated in EL; several other genes showed upregulation to a lesser extent. These two genes mapped to AT1G27180 and AT1G72930, respectively, and both are in the TIR class of plant *NBS-LRR* genes.

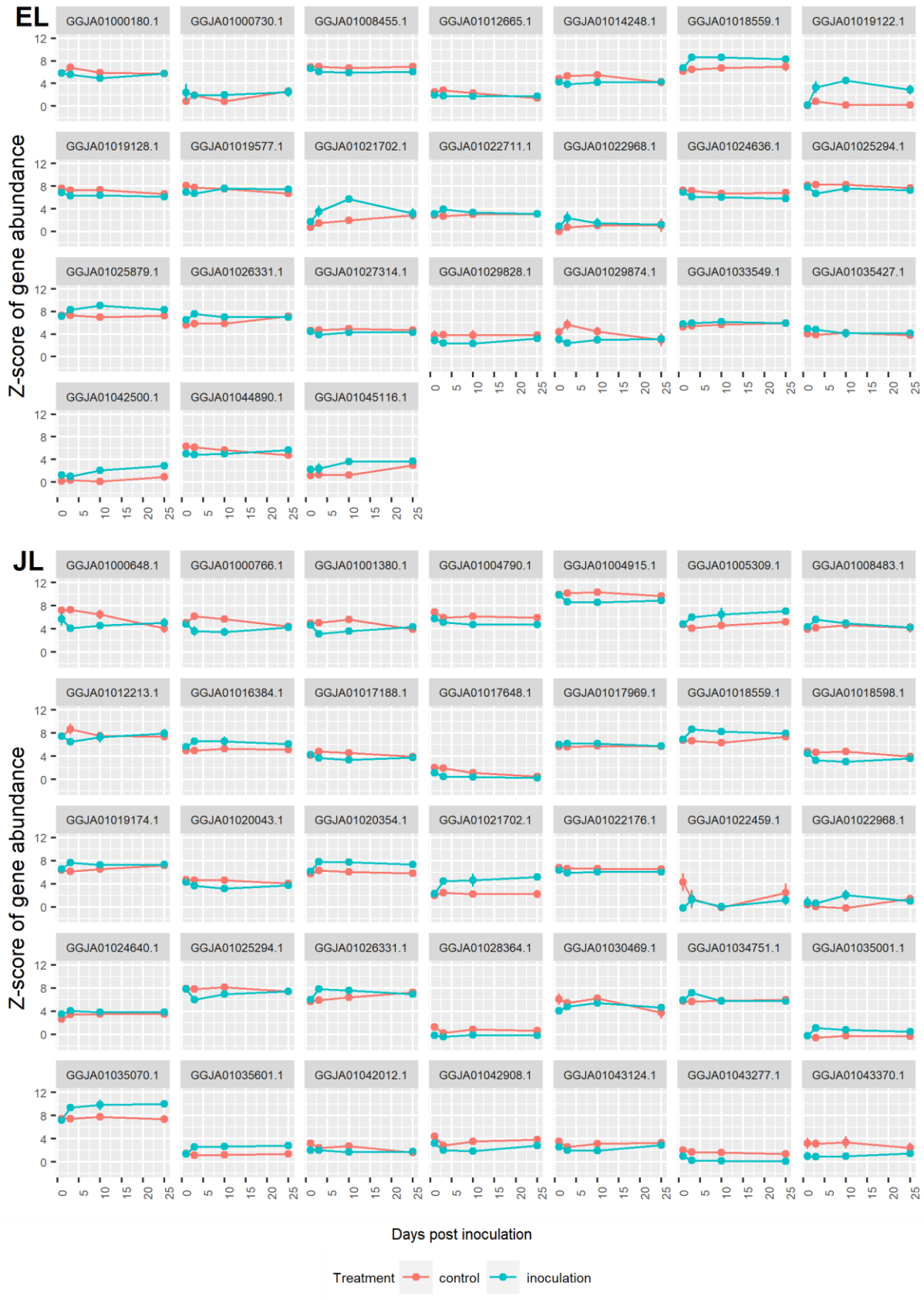


Figure 54:Normalised Z score expression profile of genes in European larch (EL) and Japanese larch (JL) which map to the Nucleotide-binding, leucine-rich-repeat genes, following inoculation with P. ramorum. Error bars are standard error.

3.3.3.5 Genes with the greatest fold change

The average fold change per day of each transcript was extrapolated from the available data over the time course of the experiment. Many of the most upregulated genes with functional annotations were related to hormone response including gibberellin, abscisic acid, brassinosteroids and jasmonic acid (Table 8). Three JL transcripts were annotated as proteins catalysing the conversion of geranylgeranyl pyrophosphate to copalyl pyrophosphate in gibberellin biosynthesis as part of the diterpenoid biosynthetic process. One transcript in both species mapped to a beta-glucosidase involved in xyloglucan metabolism. Two EL transcripts and one JL transcript mapped to genes in the Cytochrome P450 family which has roles in mediating plant growth and development, as well as biotic and abiotic stress responses. The EL sequences are predicted to be involved in the biosynthesis of secondary metabolites pathway. Two EL transcripts match glutathione transferases belonging to the tau class of GSTs, which have a role in response to biotic infections.

Table 17: Details of selected transcripts from those with the highest levels of upregulation in response to infection with P. ramorum in Japanese larch (JL) and European larch (EL).

Species	transcript	baseMean	log2FoldChange	TAIR	other names	Description	GO BP involved in
JL	GGJA01005380.1	1017.2	0.284	AT4G02330	*ATPME41*	Encodes a pectin methylesterase and brassinosteroid regulation.	cell wall modification, pectin catabolic process, response to brassinosteroid, response to cold, response to fungus
JL	GGJA01018863.1	108.4	0.283	AT1G30040	*ATGA2OX2*		gibberellin biosynthetic process, gibberellin catabolic process
JL	GGJA01022010.1	107.1	0.277	AT4G19810	*CHIC*	encodes a Class V chitinase that is a part of glycoside hydrolase family 18. It primarily acts as an exochitinase.	chitin catabolic process, response to abscisic acid, response to jasmonic acid, response to salt stress
JL	GGJA01016722.1	40.6	0.272	AT3G48520	*CYP94B3*	*CYP94B3* is a jasmonoyl-isoleucine-12-hydroxylase that catalyzes the formation of 12-OH-JA-Ile from JA-Ile. By reducing the levels of this the biologically active phytohormone, *CYP94B3* attenuates the jasmonic acid signaling cascade. *CYP94B3* transcript levels rise in response to wounding.	defence response to insects, response to wounding
JL	GGJA01032176.1	9427.5	0.268	AT4G02780	*ABC33*	Catalyzes the conversion of geranylgeranyl pyrophosphate (GGPP) to copalyl pyrophosphate (CPP) of gibberellin biosynthesis	diterpenoid biosynthetic process, gibberellic acid mediated signaling pathway, gibberellin biosynthetic process
JL	GGJA01034699.1	301.8	0.266	AT4G02780			
JL	GGJA01040402.1	1231.0	0.261	AT4G02780			

EL	GGJA01037884.1	104.9	0.341	AT3G13730	CYTOCHROME P450	Encodes a cytochrome P-450 gene that is involved in brassinosteroid biosynthesis the CYP450 family.	brassinosteroid biosynthetic process, brassinosteroid homeostasis, leaf development,
EL	GGJA01022954.1	55.5	0.300	AT5G07990	*CYP75B1*, CYTOCHROME P450 75B1	Required for flavonoid 3' hydroxylase activity. Enzyme abundance relative to Chalcone synthase determines Quercetin/Kaempferol metabolite ratio. The mRNA is cell-to-cell mobile.	flavonoid biosynthetic process, response to auxin
EL	GGJA01015494.1	778.4	0.291	AT1G10360	GLUTATHIONE S-TRANSFERASE 29,	Encodes glutathione-s-transferase (*GST*) belonging to the tau class of *GST*s	glutathione metabolic process, toxin catabolic process
EL	GGJA01049582.1	1357.9	0.283	AT3G57260	β-1,3-GLUCANASE	beta 1,3-glucanase	carbohydrate metabolic process, response to cold, systemic acquired resistance
EL	GGJA01020211.1	1175.6	0.278	AT5G20950	*BGLC1*	Encodes a beta-glucosidase involved in xyloglucan metabolism.	glucan catabolic process, xyloglucan biosynthetic process
EL	GGJA01049373.1	292.8	0.277	AT1G10360	*ATGSTU18*	Encodes glutathione transferase belonging to the tau class of *GST*s	glutathione metabolic process, toxin catabolic process

The genes with the highest upregulation included an EL homolog of AT5G07990, which is required for flavonoid 3' hydroxylase activity and influences the quercetin/kaempferol metabolite ratio. It occupies several key positions in the flavonoid biosynthesis pathway (Figure 55). It had a 7.5 log2 fold change in expression over the time course of the experiment (i.e. >150-fold increase) and a rapid increase between 1- and 3-dpi and remained highly expressed to the end of the experiment at 25dpi. In comparison this gene was in the list of the genes with the greatest downregulation in JL and is in expression cluster J5 (Figure 44). It has a steady increase in expression in the inoculated plants between 1- and 10-dpi followed by a decreased expression to the end of the experiment at 25 dpi.

Several of the downregulated DEGs in both JL and EL were involved in general cell maintenance including transcription factors, amino acid transporter proteins and pump proteins. These genes had rapid decrease in expression between 1 and 3 dpi followed by a slight increase over subsequent timepoints to the end of the experiment. In JL two of the most downregulated DEGs mapped to the gene AT4G11650 which is involved in the bacterial defence response.

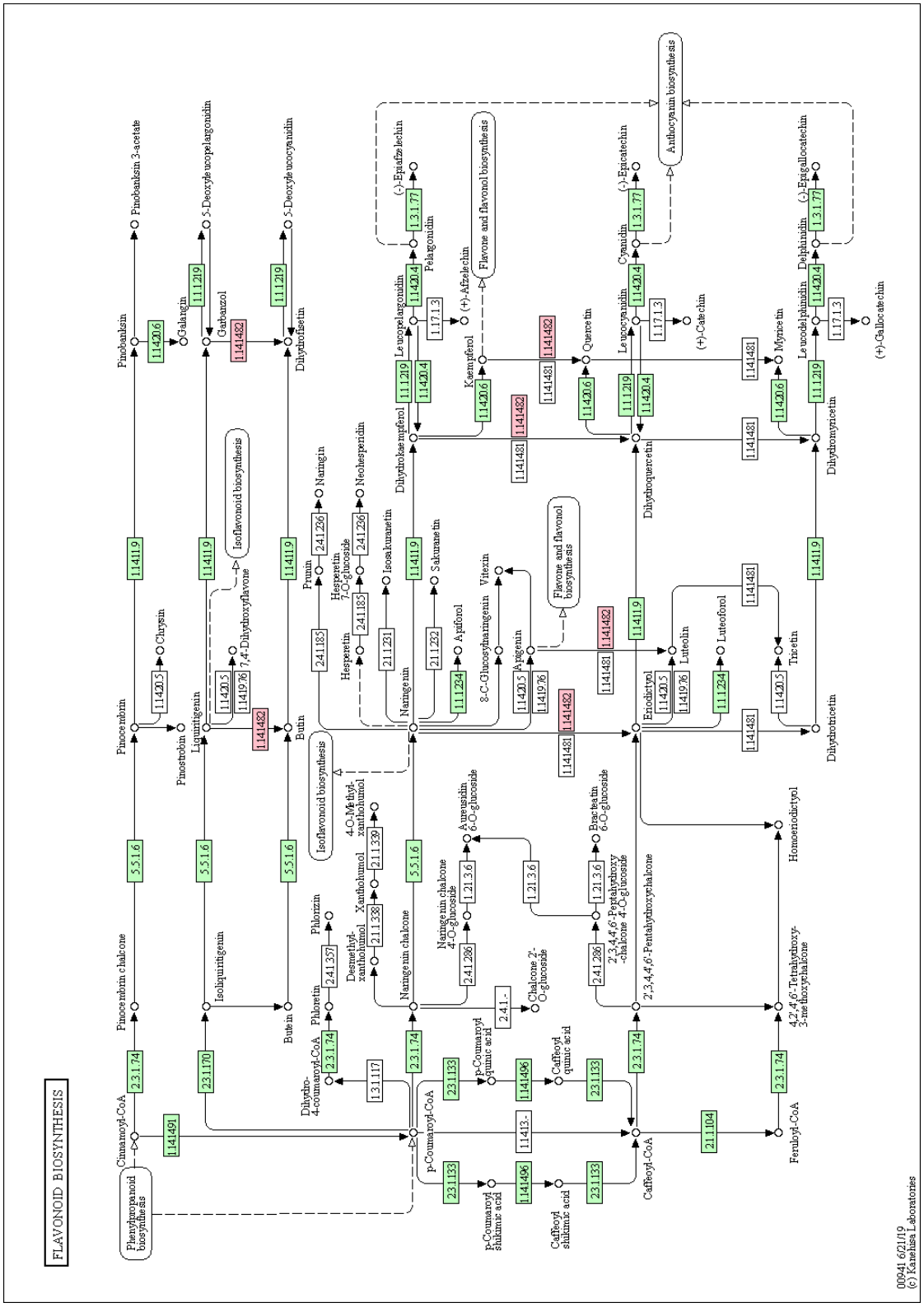


Figure 55: Gene AT5G07990 in the flavonoid biosynthesis pathway. Pink boxes indicate the location of its activity.

3.4 Discussion

The recent development of RNA-seq analysis (Martin & Wang, 2011) has allowed molecular investigations of the host responses to infection in several tree-pathogen interactions including apple (*Malus* × *domestica*) and *Erwinia amylovora* (Kamber et al., 2016), chestnut species (*Castanea mollissima* and *C. dentata*) and *Cryphonectria parasitica* (Barakat et al., 2012), western white pine (*Pinus monticola*) and *Cronartium ribicola* (Liu et al., 2013) and Norway spruce and *Chrysomyxa rhododendri* (Trujillo-Moya et al., 2020). RNA-seq has also given insight into host response to infection by Phytophthoras including *P. nicotianae* in tobacco (J. K. Yang et al., 2017) and *P. infestans* in potato (Gao et al., 2013; Gao & Bradeen, 2016), as well as comparisons between the responses of similar *Solanum* species to *P. infestans* (Frades et al., 2015). There are only two studies which have used RNA-seq to analyse the gene expression patterns in response to infection by *P. ramorum*; one on tanoaks (*Notholithocarpus densiflorus*) (Hayden et al., 2014) and one on larches (De La Mata Saez, 2015). The present study investigated RNA transcript accumulation profiles in the responses of JL and EL to inoculation with *P. ramorum* and identified the defence response pathways that are activated in order to consider the differences in response between JL and EL. Our work develops new insights by using sporangia and zoospores as opposed to mycelium for inoculation and through a longer period of study allowing us to better follow changes in gene expression between the two species of larch from first infection to the development of extensive lesions. This longer time frame of study revealed that responses to infection differed between EL and JL over time. We also found enrichment of similar pathways as well as different suites of secondary metabolites being produced in each species.

3.4.1 Comparing responses to *P. ramorum* infection in European and Japanese larch

We did not observe differences in lesion lengths between EL and JL at any of the time points, suggesting they had similar levels of susceptibility to infection. This is different to reported field observations, which suggested that EL was more resistant to stem infections (Harris and Webber,2012). Previous reports of laboratory inoculations were varied with some reporting larger lesions on JL than EL but that this difference was not statistically significant (Harris, 2014; Harris et al., 2021). Both Harris (2014) and Harris et al. (2021) used a different inoculation method, mycelia inoculation rather than the sporangial suspensions as used in the present study, but also found no statistically significant difference in lesion length between the two species.

Chastagner et al. (2012) found that zoospore inoculation of potted intact seedlings resulted in longer mean lesion lengths at 21 dpi on JL compared with EL (80 mm and 61 mm, respectively). Apart from using the EU1 lineage of *P. ramorum* and dipping the foliage to inoculate, Chastanger's study is the

most similar to our work. Although we had a slightly longer incubation period of 25 days and we reported much longer lesions of 168 mm and 178 mm for JL and EL respectively, the overall results were the same in that no significant difference in lesion length was found between EL and JL in either study.

In the present study, we aimed to explore whether investigating the immune responses of EL and JL could provide clues to explain inconsistencies between differences observed in the field and results from laboratory inoculations.

3.4.2 Overview of transcriptome changes

The transcriptome data clearly indicated a differentiation of samples responding to inoculation compared to the controls according to the PCA, with differences becoming apparent from 3dpi. However, the two species could not be clearly differentiated at any time point with the PCA, suggesting that the differences in their transcriptomes were not as important as the changes induced by infection. The spread of the data points showed that changes in gene expression have not yet developed at 1dpi. A similar inability to differentiate between the control and inoculated treatments at 1dpi was also found in the response of tanoak to *P. ramorum* infection (Hayden et al., 2014) which was linked to the generally slow first stages of disease progression in tanoaks, with lesions only visible 3–5 dpi.

We found the greatest changes in transcript abundance, particularly for upregulated genes, came between 1 and 3 dpi, with less extreme changes in expression over the subsequent time points. More DEGs were identified in JL than EL despite the greater number of raw reads obtained for EL and the slightly higher mapping rate to the reference transcriptome. It is unclear if differences between the two species tested and *Larix laricina* used to develop the reference transcriptome could account for some of the difference in the number of DEGs identified in the two species. *Larix laricina* is in the North American Clade I. However, both EL and JL are in the Eurasian clade II of the Larix phylogeny (Wei & Wang, 2003); although there is some debate as to the exact relationship between them (Gros-Louis et al., 2005; Semerikov et al., 2003), they are generally placed in the same monophyletic clade.

Overall, this study found more downregulated than upregulated genes. However, in the enriched pathways there was mostly upregulation. This suggests that downregulation is a general suppression of genes in many pathways, which could be part of the plants defence response supressing pathways that are not vital to the immune response. Pathogens have evolved mechanisms that can interfere with the plant defence system (Jones & Dangl, 2006; Oßwald et al., 2014) which could inhibit the expression of some of these genes. In comparison, upregulated genes are found in the enriched

pathways suggesting that there is an activation of specific pathways by the plant in response to infection.

Hayden et al. (2014) portrayed the interaction between tanoak and *P. ramorum* by simultaneous RNA-sequencing of both the host and pathogen. They found that tanoaks showed enrichment of proteins associated with necrosis and disease response and *P. ramorum* expressed a variety of cellulose-binding and lectin-like proteins and some avirulence homologs at 5 dpi. Variation in avirulence homologs can suppress the host's effector-triggered immunity as not all avirulence homologs will be recognised by the host, allowing the pathogen to infect (Jones & Dangl, 2006). Therefore, the presence of avirulence homologs in *P. ramorum* could be part of its effective infection strategy. A study of the interaction between larches and *P. ramorum* by De La Mata Saez (2015) found more defence pathway genes upregulated in EL than in JL . The author suggested that *P. ramorum* might be more successful in turning down the plant´s defence process in JL. We found more genes upregulated in JL but, similarly to De La Mata Saez, more defence pathways enriched in EL.

In general, a large proportion of genes in both species were differentially expressed suggesting a comprehensive change in gene expression in response to infection. Further analysis of these enriched genes allowed us to link the DEGs to the pathways in which they were active and the resulting response in the plant.

3.4.3 Responsive genes and pathways

In the present study, genes related to stress and defence responses were overrepresented among DEGS and many of them were rapidly induced following infection (from 1dpi). Both species of larch had overexpression of the translation genes suggesting that there was an increase in the production of proteins.

This study also found similarities in EL and JL with enrichment in response to wounding, amino acid biosynthesis, jasmonic acid (JA) biosynthesis (as outlined in more detail later) and flavonoid biosynthesis pathways. Variations were found in the representation of metabolic pathways for the biosynthesis of secondary metabolites and antibiotics in EL and JL, respectively. Whilst these two pathways represent similar groups of secondary metabolites, they do have differences, suggesting that the two species produce differing suites of secondary metabolites in response to infection by *P. ramorum.* There were also differences in expression profiles, with upregulated genes in JL tending to have a greater but more transient increase in expression than EL.

This study found that JA biosynthetic genes were overrepresented in both species but JL had higher and more transient expression than EL, which had sustained levels of expression. Jasmonic acid is a signalling compound that can induce expression of defence related proteins in response to pathogen attack (van Loon et al., 2006), in particular in response to necrotrophic pathogens (Glazebrook, 2005). Our data suggest that the JA response is sustained for a longer period of time in EL compared to JL suggesting that EL has a stronger JA-related defence response. This is consistent with the enrichment of the regulation of the JA mediated signalling pathway and the response to JA in EL but not in JL. This could also suggest enhanced JA activated defence responses such as the production of flavonoids, alkaloids and terpenoids (Wasternack & Parthier, 1997). De La Mata Saez (2015) found that EL had a higher number of upregulated genes in the JA pathway in response to *P. ramorum* infection than JL; 24 out of 33 genes (73%) compared to 17 out of 37 genes (46%) for JL.

Within the JA biosynthesis pathway, the *LOX* genes are a family of lipoxygenases involved in initiating wound-induced jasmonate synthesis. The various *LOX* genes contribute to JA, and its derivative jasmonoyl-isoleucine synthesis at different times after wounding (Chauvin et al., 2013). We found DEGS mapping to *LOX3* and *LOX4* and a small gene family mapping to *LOX1*, which were over-expressed over the whole course of the experiment. Our work gives a clearer picture of the expression of *LOX* genes than was previously known, as only *LOX2* and *LOX3* had previously been found in larches in response to *P. ramorum* infection, with *LOX3* upregulated at 3dpi in EL but not in JL and *LOX2* upregulated in EL at 3dpi and downregulated in JL at 3 and 7dpi (De La Mata Saez, 2015). We found that these genes were sharply upregulated in EL between 1 and 3dpi and then maintained or gradually increased in expression whilst there was a more rapid, large and transient induction in JL, which trailed off towards the end of the experiment. However, the expression of the *LOX* genes was maintained longer than previously reported by De La Mata Saez.

Pathways for the biosynthesis of defence compounds were strongly represented by secondary metabolites including the biosynthesis of phenylpropanoids in EL, and the biosynthesis of antibiotics in JL. Phenylpropanoid compounds have many roles in plant defence from preformed or inducible chemical barriers against infection to signal molecules involved in local and systemic signalling for defence gene induction (R. A. Dixon et al., 2002). The antibiotics pathways included the biosynthesis of tropane, piperidine and pyridine alkaloid, terpenoid backbone and isoquinoline alkaloids. The terpenoid backbone is the precursor for the production of monoterpenoids, sesquiterpenoids, and diterpenoids which can then be used to make antimicrobial phytoalexins (Singh & Sharma, 2015) and other terpenoids that have antifungal properties (Kusumoto et al., 2014). Other secondary metabolites than those found here have been identified in Californian coast live oak including higher constitutive levels of the tyrosol derivative ellagic acid in putatively resistant trees (Nagle et al.,

2011) and *P. ramorum* induced accumulation of gallic and ellagic acid in infected tissue with gallic acid and tyrosol found to be inhibitory against *P. ramorum* in vitro (Ockels et al., 2007).

We found that both larch species have large gene families mapping to the genes enriched in the flavonoid biosynthesis pathway. The gene chalcone synthase (*CHS*) catalyses the first step of flavonoid biosynthesis by directing carbon flux from general phenylpropanoid metabolism to the flavonoid pathway (Zhang et al., 2017) and alterations to *CHS* affect the accumulation of flavonoids (Routaboul et al., 2012). The role of *CHS* expression in conifers is indicated by its responsiveness to wounding and exposure to jasmonic acid in white spruce (*Picea glauca*) (Richard et al., 2000) and its association with resistance to the pathogenic fungus *Ceratocystis polonica* in Norway spruce (*Picea abies*) (Nagy et al., 2004). Increased expression of *CHS* lead to increased activation of flavonoid biosynthesis and is suggested to improve resistance of conifers to fungal pathogens (Kolosova & Bohlmann, 2012; Nagy et al., 2004). Both EL and JL have multiple genes that map to *CHS* and expression increased in both control and inoculated trees, suggesting that the expression response is both related to wounding and *P. ramorum* infection.

The activation of the defence response and the production of secondary metabolites is energy intensive and needs a redistribution of energy toward the defence response leading to the so called "fuel for the fire" (Bolton, 2009) that allows a strong defence response to be produced. Primary metabolism genes were responsive to infection in both EL and JL, including carbon metabolism and the tricarboxylic acid cycle. Increased primary metabolism would provide more energy to support the defence response (Rojas et al., 2014).

The most highly responsive DEGs revealed other aspects of defence metabolism including genes in the Cytochrome P450 family which have roles in mediating biotic and abiotic stress responses. The Cytochrome P450 family includes catalases that are involved in the production of diterpene resin acids in the oleoresin defence pathway of conifers (Hamberger et al., 2011). Two EL genes map to a glutathione transferase belonging to the tau class of *GST*s. These are induced in response to biotic infections, with some being specifically upregulated by microbial infections and others being linked to protection from oxidative stress (D. P. Dixon et al., 2002; Gullner et al., 2018) and to the intracellular binding and stabilization of flavonoids (D. P. Dixon et al., 2002). It is possible that the high expression of *GST* is related to upregulation of the biosynthesis of flavonoids in EL. Numerous transcriptome-wide investigations have shown that distinct groups of *GST*s are markedly induced in the early phase of bacterial, viral, fungal and oomycete infections including potato infected with *P. infestans* (Gullner et al., 2018).

One of the highly responsive DEGs in JL mapped to a class V chitinase and both EL and JL had enrichment of the response to chitin pathway. Hayden et al. (2014) found that *P. ramorum* infection in tanoaks led to differentially expressed transcripts matched to pathogenesis-related families of chitinases. However, Phytophthoras are oomycetes and have a cell wall comprised mainly of cellulose with very little chitin, unlike most fungal pathogens, which have chitin-based cell walls (Meijer et al., 2006). It is therefore unclear if the expression of chitin responses and chitinases is related to *P. ramorum* infection or if it is related to other physiological processes such as ethylene synthesis or a response to abiotic factors (Hamid et al., 2013).

3.4.4 Large gene families and evolution of defence responses in conifers

In our study, some of the DEGs annotated as having defensive roles are part of small gene families with several homologs matching a single *Arabidopsis* gene. Chalcone synthase (AT5G13930) had 11 to 15 sequences with remarkably similar upregulation profiles, suggesting that the accumulation of extra gene copies is under positive selection. There were also many homologs for *LOX1* (in contrast to *LOX2* and *LOX3*) but with more variable upregulation, suggesting diversification in function or regulation. This interpretation may be supported by the observation that some *LOX1* homologs were unique to EL or JL. The large number of putative *NBS-LRR* genes also varied in their expression. The *NBS-LRR* genes form the largest resistance gene family in plants and are the main group of cytoplasmic receptors involved in the detection and recognition of specific pathogens (DeYoung & Innes, 2006; Jones & Dangl, 2006; Van Ghelder et al., 2019). In response to pathogen perception the NBS-LRR proteins undergo conformational alterations, such as phosphorylation, which activates downstream signalling (DeYoung & Innes, 2006), thus most of their action comes through constitutive signal transduction by the protein rather than induced increased gene expression. *NBS-LRR* genes are often *R* genes that give resistance to Phytophthoras such as *P. infestans* in tomatoes (Zhang et al., 2014) and in domesticated and wild potatoes (Ballvora et al., 2002; Gu et al., 2020). Some of these *R* genes are specific and others give broad-spectrum resistance to many strains of *P. infestans* (van der Vossen et al., 2003). The presence of a few strongly upregulated *NBS-LRR* genes in larch is of interest as these have been induced in response to the infection by *P. ramorum* and so could be involved in the detection and recognition of *P. ramorum*. *NBS-LRR* genes need to be finely regulated to ensure correct resistance responses, while limiting their metabolic cost and any detrimental effects on plant growth (Marone et al., 2013) so the downregulation observed in some of the *NBS-LRR* genes in larch suggests that they are not responsive to *P. ramorum* infection. However, it is known that pathogens have evolved mechanisms that can interfere with the plant defence system (Jones & Dangl, 2006; Oßwald et al., 2014) which could account for the downregulation of some of these *NBS-LRR* genes.

Conifers are well known for having very large genome sizes ranging from 18 to 35 gigabases (Gb) (Mackay et al., 2012) approximately 200x bigger than the much smaller genome of 125-megabases (Mb) in *Arabidopsis thaliana* (Kaul et al., 2000). Zonneveld (2012) found that the genomes of EL and JL contain 26.6 and 26.4 pg DNA per nucleus, respectively, allowing a calculation of genome size as 26.6 and 26.4 Gb, respectively. This places the two species close to the genus average of 27 Gb and slightly above the *Pinaceae* average of 23 Gb (Leitch et al., 2019).

Conifers' large genomes are thought to have resulted from periods of retrotransposon expansion and the accumulation of many neutral or slightly detrimental mutations which led to further genome inflation (Mackay et al., 2012; Morse et al., 2009). Conifers also have a number of very large gene families, particularly related to secondary metabolites and defence responses (Keeling et al., 2008; Mackay et al., 2016; Van Ghelder et al., 2019; Warren et al., 2015), although it is not a general rule for all gene families. These gene families arise from multiple gene duplications, which can give rise to subsequent neofunctionalization and subfunctionalization (Keeling et al., 2008) leading to functional diversification and phenotypic plasticity.

3.4.5 Comparison of the present study with previous RNA-seq work on larch

A previous study has been carried out on the EU2 lineage of *P. ramorum* in EL and JL (De La Mata Saez, 2015). Our study differs from the work of De La Mata Saez in several key aspects including inoculation method, study timeframe, sampling aproach and enrichment analysis.

We used sporangial suspension inoculation to better mimic the proposed natural infection process rather than the mycelial inoculation as in De La Mata Saez. We also had an extended timeframe in our experiment, with sampling starting earlier and extending later (1 to 25 dpi) compared to De La Mata Saez (3 and 7dpi). This allowed us to more closely follow the changes in gene expression, in particular the early upregulation between 1 and 3dpi, and show the distinctive pattern in the expression profiles between JL and EL, with differences in gene expression at later time points such as the downregulation between 10 and 25dpi in many key genes in JL. We also maintained each plant as a biological replicate during RNA extraction and sequencing giving us a larger sample size than De La Mata Saez who pooled samples and this allowed us to carry out robust statistical analysis. We approached data interpretation without prior assumptions and carried out enrichment analysis to identify differentially expressed genes rather than investigating the expression of previously selected defence related pathways as De La Mata Saez did. Overall, these differences allowed us to investigate additional aspects of the interaction between *P. ramorum* and EL and JL from De La Mata Saez, with more focus on the changes over time.

3.4.6 Conclusions

Although there were no significant differences between EL and JL in the development of *P. ramorum* lesions, at a transcriptomic level there were both similarities, with the same JA and flavonoid biosynthesis pathways being enriched, and differences, with different suites of secondary metabolite genes being induced in each species and more JA related pathways enriched in EL. The expression profiles of the DEGs also differed with upregulated genes in JL tending to have a greater, but more transient, increase in expression than EL. Whilst we cannot link any of these transcriptomic differences to possible greater resistance to infection in EL, as was suggested by field observations, it is worth noting that the expression of key defence pathways and genes remained high in EL, or was increasing at the end of the experiment compared to the more transient expression in JL. These observations along with the greater expression of JA-related pathways could suggest a stronger defence response in EL. However, other factors are also likely to affect the differences in infection seen in the field, from the greater area of JL planted to the greater sporulation of *P. ramorum* on JL (Harris & Webber, 2016) resulting in higher inoculum loads in JL stands and thus more infection.

The results of this work pave the way for future work on the host response to *P. ramorum* infection and the RNAseq data will be made available through an open access platform. It would be possible to use this data to analyse the *P. ramorum* side of this pathosystem and investigate intra-species variation in EL and JL. We have identified a large number of highly responsive genes, including two *NBS-LRR* genes in EL that are strongly upregulated, that could be further investigated for better understanding of defences to *P. ramorum* and the immune response. The insights gained from this work could inform further work to study differences in response to infection within species, in particular survivor trees and help identify trees suitable for breeding to improve resistance to *P. ramorum*.

Chapter 4- Investigating the possible resistance to *P. ramorum* in putatively tolerant Japanese larch trees.

Abstract

There is great interest from the forestry industry in the possibility of selection for resistance to *P. ramorum* in Japanese larch (JL) in order to retain it as a commercially viable species. This study is the first to investigate variation in resistance to *P. ramorum* in the JL population through inoculation of excised material from putatively tolerant survivor trees. We found quantitative variation in susceptibly to *P. ramorum* within the JL population planted in the Galloway forest with some trees showing significantly shorter lesion development. We propose that survivor trees include a higher proportion of resistant or low susceptibility trees and would be a useful starting point for further work investigating natural resistance in larch.

4.1 Introduction

The Galloway Forest sits within the Dumfries and Galloway region of Scotland where forestry is largely comprised of commercial conifers and contains significant plantations of Japanese larch (JL), with 10,400 hectares of JL accounting for 7% of all woodland, as well as 400 hectares of European larch (EL) with extensive planting starting in the 1940s and 1950s (Forestry Commission, 1999). In spring 2013 a major outbreak of *P. ramorum* occurred in the Galloway Forest with extensive areas of dying larch observed, particularly in the Glen Trool area. It was estimated that 5,000-6,000 hectares of larch were affected (Forestry Commission Scotland, 2017). There was widespread mortality in larch stands across the forest; many of these stands suffered >95% mortality and some stands were killed entirely (Figure 56a). However, some JL trees in otherwise heavily affected stands appeared healthy two years after the initial epidemic (Figure 56b). Such observations raised questions as to whether these trees had simply 'escaped' infection or whether they indeed had natural resistance to *P. ramorum*. Due to the interest in retaining larch as a viable commercial forestry species, Forestry Commission Scotland (now Scottish Forestry) funded the establishment of a clonal trial of putatively tolerant and known susceptible JL trees from scions collected in 2015/2016 in order to investigate natural resistance. This trial forms the basis of this chapter.



Figure 56: Mortality in mature Japanese larch in the Galloway Forest. a) whole stand mortality, b) single surviving tree with foliage in the crown surrounded by the bare crowns of the dead trees.

Natural variation in susceptibility to disease has been documented for many tree species. The ability of a tree to resist a pathogen depends on the existence of a genetic variation that confers resistance (White *et. al.* 2007). Variation in susceptibility to pathogens has been reported in loblolly pine (*Pinus taeda*) to the pathogens *Fusarium circinatum* (Quesada et al., 2010) and *Cronartium quercuum* (Powers & Kraus, 1983); Scots pine (*Pinus sylvestris*) to *Dothistroma septosporum* (Perry et al., 2016); western white pine (*Pinus monticola*) to *Cronartium ribicola* (Liu et al., 2013); ash (*Fraxinus excelsior*) to *Hymenoscyphus fraxineus* (Sollars et al., 2017), and European larch to *Lachnellula willkommii* (Kulej, 2006).

Even in seemingly devastating disease outbreaks there have been reports of survivor trees, trees that remain healthy and show no, or fewer, signs of infection than those around them despite high disease pressure. The prevalence of survivor trees varies between different pathosystems, from 1 in 100,000 American elms (*Ulmus americana*) which appear to be resistant to Dutch elm disease (*Ophiostoma ulmi and 0. nova-ulmi*) (Schlarbaum et al., 1998) and the occasional American chestnut (*Castanea dentata*) surviving chestnut blight (*Cryphonectria parasitica*) infection (Hebard, 2005) to the estimated 1- 5% of ash trees that are thought to be resistant to ash dieback (ADB) (*Hymenoscyphus fraxineus*) (McKinney et al., 2014). Survivor trees are often thought to be resistant

to infection. However, it is possible that some trees miss infection by chance, or due to their position within the stand, and so inoculations must be carried out to test this putative resistance. Inoculation trials allow the identification of true survivor trees with viable naturally occurring resistance and these trees form the basis of many resistance breeding programmes. Hansen et al., (1989) developed inoculation methods for testing the putative resistance of Port-Orford-cedar (*Chamaecyparis lawsoniana*) to *Phytophthora lateralis* using mycelium to inoculate wounded seedlings and excised material from survivor trees. Their work showed that excised material was suitable for preliminary screening of survivor trees to submit to more intensive testing. Subsequent work has built on almost 40 years of selection and testing of survivor trees and their progeny and found both qualitative major gene and quantitative disease resistance that is sufficiently strong to now be used for restoration and reforestation (Sniezko et al., 2020). Similar preliminary testing with excised material has also been used to investigate natural resistance in Juniper (*Juniperus communis*) to *Phytophthora austrocedri*, leading to the finding that survivor trees had higher levels of resistance than known susceptible controls (Green et al., 2020) and results were validated in whole plant testing (Green et al., 2020).

The usual management strategy to attempt to control the spread of invasive pathogens is preventative felling around infected sites, as well as felling infected trees. However, this is often counterproductive in the search for disease resistance as it removes possible survivor trees before they can be identified (Budde et al., 2016). *Phytophthora ramorum* is classed as a notifiable disease in the UK and as such is subject to statutory plant health notices (SPHNs). These require the mandatory felling of infected trees, along with a 250 m buffer zone of healthy trees around them (Forest Research, 2020b). The creation of the Galloway forest Management Zone allowed the phased removal of larch inside the zone and this delay in felling allowed the identification of survivor trees and the collection of scion material for inoculation trials.

Variation in susceptibility to *P. ramorum* in Californian coast live oak (*Quercus agrifolia*) populations has been found (Nagle et al., 2011; Ockels et al., 2007). In areas of natural infection some oaks were found to survive and some inoculated trees recovered from infection, thus complete or partial resistance might be found across the population, which gives hope for the survival of coast live oaks in the face of *P. ramorum* spread. In contrast, resistance to *P. ramorum* in larch remains to be identified. The aim of this study was to compare the response to inoculation with *P. ramorum* in grafted scions from putatively resistant and known susceptible mature JL trees. The hypothesis is that grafts from putatively tolerant trees are more likely to show resistance than grafts from known susceptible trees. This will allow insight into levels of naturally-occurring resistance to *P. ramorum* in the JL population, which is a pre-requisite for studying its level of genetic control (or heritability) and

using it in a breeding programme. This work breaks new ground and, although limited in scope, represents a first step to investigate variation in resistance to *P. ramorum* in the JL population.

4.2 Methods

4.2.1 Identification of putatively tolerant Japanese larch trees, scion collection and grafting

In December 2015 and February 2016, scions (healthy young shoots) were collected from ten mature JL with no symptoms of *P. ramorum* infection within three infected stands, with high mortality, in the Galloway Forest; Glen Trool, planted in 1956, Glen of the Bar and Black Loch, both planted in 1946 (Figure 57). These 'putatively tolerant' trees appeared healthy and had no evidence of stem cankers or epicormic recovery growth, were dominant with generally good growth form, and not located at stand edges. In January 2017, healthy scions were also collected from the lower crown of fourteen 'known susceptible' JL with top dieback due to cankers in the upper main stem and with healthy branches in the middle and lower crown, in a moderately infected stand at Glen Trool. All scion material was bagged and stored at 4°C until grafting, which was carried out in February and March 2016 for the putatively tolerant trees and in February 2017 for the known susceptible trees. Grafts comprised the ten most vigorous looking scions per parent tree joined on to 1+1 EL rootstocks in 5" square pots using the side veneer technique. Each individual ramet was labelled and the grafts were maintained in a heated polytunnel at Gatehouse of Fleet, to ensure frost free conditions with natural daylight, before being moved outside after one year. To provide a duplicate set of grafts for inoculation, scions from the original grafts were grafted onto new EL rootstocks in winter 2017/18, and maintained in the polytunnel as previously described (Figure 58).

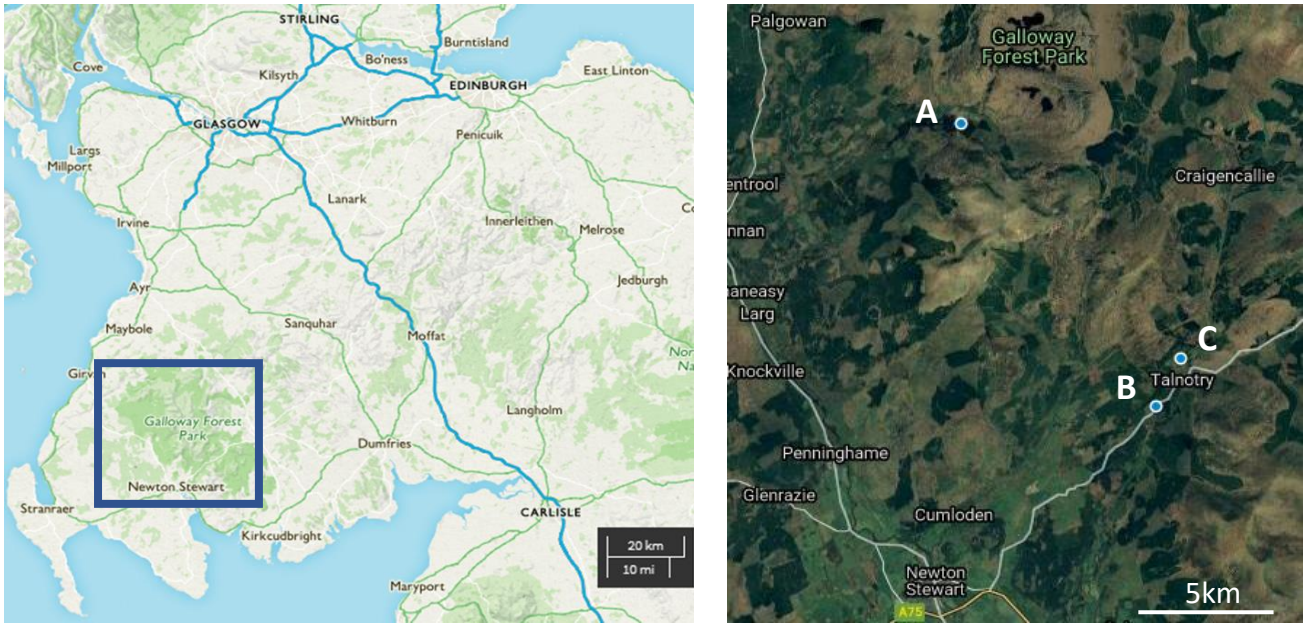


Figure 57:Location of the Galloway Forest Park in south-west Scotland (left) and location of the two stands of mature JL within the Forest Park from which scions were taken (right); A) Glen Trool, B) Glen of the Bar, C) Black Loch. Base map- Google maps.



Figure 58: Grafted Japanese larch trees in the polytunnel at Gatehouse of Fleet prior to sample collection in October 2019.

4.2.2 Inoculation of excised material

In October 2019 ten ramets from each of the putatively tolerant and susceptible clones were selected for inoculation. In the putatively resistant group, eight clones (Tol1 to Tol8) were from parent trees located at Glen of the Bar, whereas one clone (Tol9) was from Black Loch and one clone (Tol10) originated from Glen Trool. All trees in the known susceptible group were from Glen Trool.

For inoculation, one or two thirty-centimetre-long shoots were excised from the leading shoot of the main stem or large branch of each ramet ensuring that selected shoots were well-developed with a basal diameter of ~1 cm. The cut shoots were labelled and each end was wrapped with cotton wool which had been soaked in sterile distilled water (SDW). The cotton wool was secured in place with Parafilm and tin foil. The shoots were then double bagged and placed in a fridge box for transport back to the pathology laboratory at Forest Research's Northern Research Station, Roslin.

The experiment comprised of twenty JL clones (ten putatively tolerant and ten known susceptible clones) and two treatment types (*P. ramorum* and control) with 3 replicate shoots per clone for the control treatment and 6-8 replicate shoots per clone for the inoculation treatment, depending on the number of suitable shoots as the trees varied in size. Sporangial suspension was prepared as described in section 2.2.4 from the *P. ramorum* EU2 lineage isolate 16.13.a. To inoculate each shoot the bark was pre-wounded immediately before inoculation by removing a 5 mm diameter circle of bark from the midpoint of the shoot using a scalpel (Figure 59a). A 15 µL droplet of sporangial suspension was pipetted onto the wound and left for 15 minutes to allow zoospore encystment. The wounded area was then loosely wrapped in tin foil to mark the inoculation point (Figure 59b) and also to prevent inoculum cross contamination. Control inoculations were carried out as above but using SDW. The shoots were double bagged and placed in an incubator in the dark at 15 ^{o}C. The shoots were allocated to bags in a randomized complete block design with one inoculated shoot from each clone per bag excluding the controls which were blocked separately to prevent cross-contamination of the inoculum. The experiment was conducted twice; in October 2019 and in August 2020.

Plants were destructively sampled and assessed for disease at 21 days post inoculation (dpi). To measure lesions the needles were removed, the inner and outer bark slit with a scalpel and removed from the stem, and the length of any lesions which had developed in the phloem measured (Figure 60). DNA was extracted from bark tissue at the live-dead margins of the lesion from one ramet per clone. The presence of *P. ramorum* in the lesion was confirmed through real-time PCR amplification with *P. ramorum* specific primers as previously detailed in section 2.2.2.



Figure 59: Methodology of inoculation of the excised shoots. a) Wound in bark of Japanese larch shoot prepared for inoculation, b) inoculated shoots prepared for incubation.

4.2.3 Data analysis

Statistical analyses were conducted using R version 4.0.4 (R Core Team, 2020) in RStudio (RStudio Team, 2020). To compare the tolerant and susceptible groups a linear mixed model fit by REML was fitted to the lesion length data with group and trial repeat treated as fixed effects and clone as a random effect. To compare between clones a linear model was fitted with lesion length as the outcome variable and with trial repeat and clone as predictor variables. The estimated marginal means were then calculated from the linear models using emmeans (Lenth, 2020) and pairwise comparisons of the clones were analysed using the 'cld' function in the 'multcomp' package (Hothorn et al., 2008).'cld' creates a compact letter display of the pairwise comparisons of marginal means so that clones which share letter grouping are not significantly different from one another. The Tukey method was used to adjust the p-values to control for the number of statistical tests performed. To test whether there were significant differences between clones across the two trials an interaction was fitted between clone and trial and an anova showed that this was not significant and so it was excluded from the final model.

4.3 Results

To investigate if the grafts from putatively tolerant trees are more likely to show resistance than grafts from known susceptible trees we examined lesion lengths on excised material from clones of trees in the two groups, 21 days after inoculation with *P. ramorum*. Lesions on control shoots ranged from 1-4 mm in length but these were of a pale colour uncharacteristic *of P. ramorum*, and were likely discolouration related to wounding (Figure 60a and Figure 61). Lesions were observed in all inoculated shoots (Figure 60b and Figure 61) and ranged from 2-240 mm in length. The effect of experimental trial was significant (Table 18) with shorter lesions developing in the second trial in August 2020 (mean overall lesion length 66.5 mm, SD=37.4) compared to the first trial in October 2019 (mean overall lesion length 128mm, SD=53.7). However, no significant differences were found between clones across trials (Anova, F=1.6, P=0.06). Comparing the two clone groups (putatively tolerant vs known susceptible), the putatively tolerant clones tended to have shorter lesions (Figure 61) but the lesion lengths in both groups were variable and the mixed linear model showed that the differences were not significant (Table 18).

Table 18: Parameter estimates and fit statistics of the linear mixed model of lesion development.

Fixed effect	Estimate	Std error	T-value	P-value
Intercept	201.1	11.0	18.21	<0.05
Grouping (tolerant/susceptible)	-22.7	11.8	-1.92	0.07
Trial	-62.1	4.7	-13.46	<0.05



Figure 60: Examples of lesion development a) control shoot showing no lesion, b) P. ramorum inoculated shoot showing extensive lesion development, the black arrows indicate the edges of the lesion.

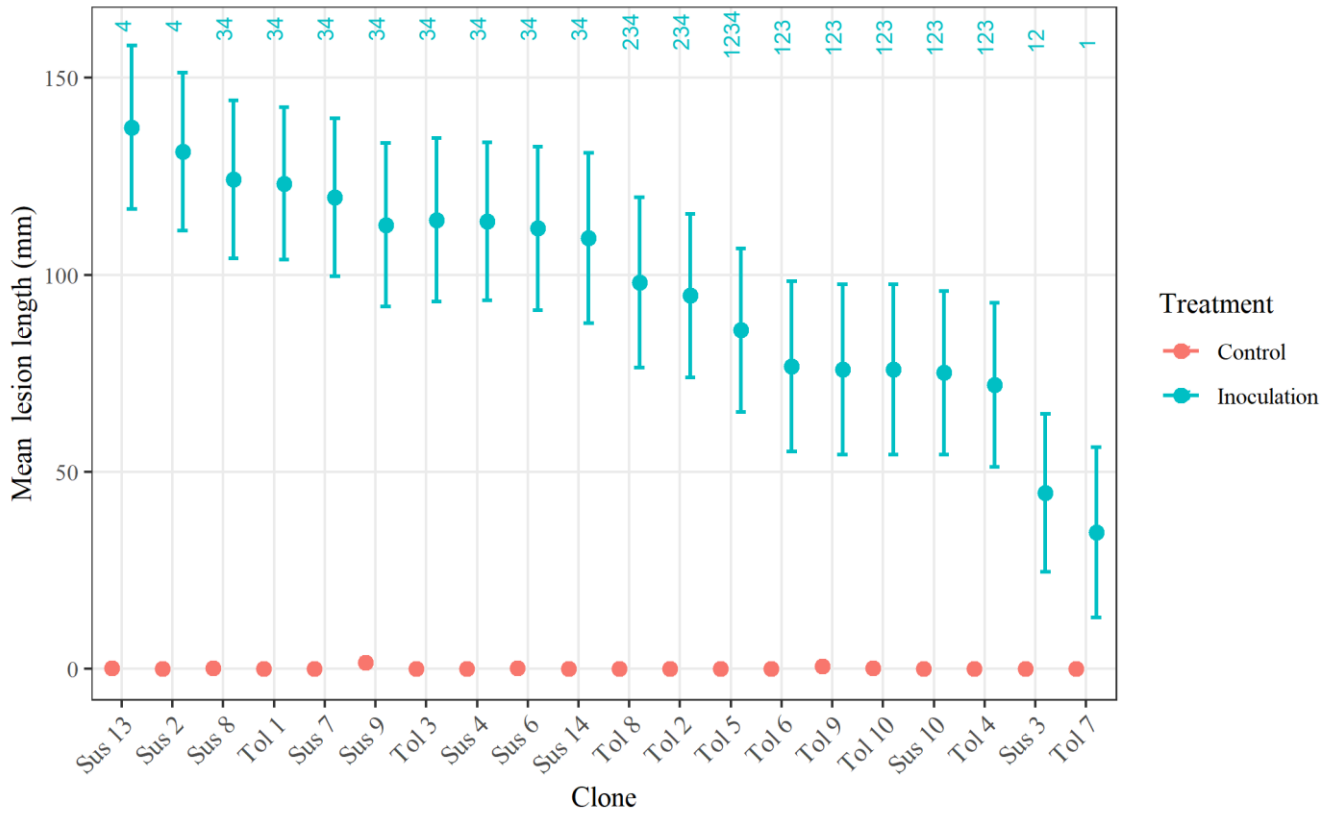


Figure 61: Mean lesion length for each clone in the P. ramorum inoculations ordered by decreasing mean lesion length. Clones named Sus are from the known susceptible group and Tol are from the putatively tolerant group. Adjusted marginal means for lesion length averaged over the levels of experiment. Error bars show the 95% unadjusted confidence intervals. Numbers denote statistical significance (p <= 0.05 Tukey adjustment for multiple comparisons), so that clones which share a number are not significantly different. n=3 for controls and n=6-8 for inoculations.

The lesion lengths in the *P. ramorum* inoculated clones showed continuous variation from the longest, clone Sus13 at 135 mm (SD=57.2), to the shortest, clone Tol7 at 34.7 mm (SD=22.8) (Figure 61). The pairwise comparisons between the clones showed that although there are significant differences between the clones at each end of the range, these overlap in the middle. The seven clones to the left of Figure 61 are significantly shorter than the 11 clones with longer lesions on the right-hand side.

There was also variation between the clones in needle colour and retention. In the controls the shoots remained green and only lost a few needles (Figure 62). In the inoculated shoots there was wide variation between clones with more needle loss, and the majority of the needles becoming yellowed or even completely brown, in the clones with the longest lesions (see top row of Figure 63). In comparison, clones that developed shorter lesions had better needle retention and were greener and looked more similar to the control treatment (see bottom row of Figure 63).



Figure 62: Representative selection of the controls 21 days post inoculation with one shoot per clone, the shoots are arranged in numerical order from left to right, a) clones from the "tolerant" group of clones, b) clones from the "susceptible" group of clones.

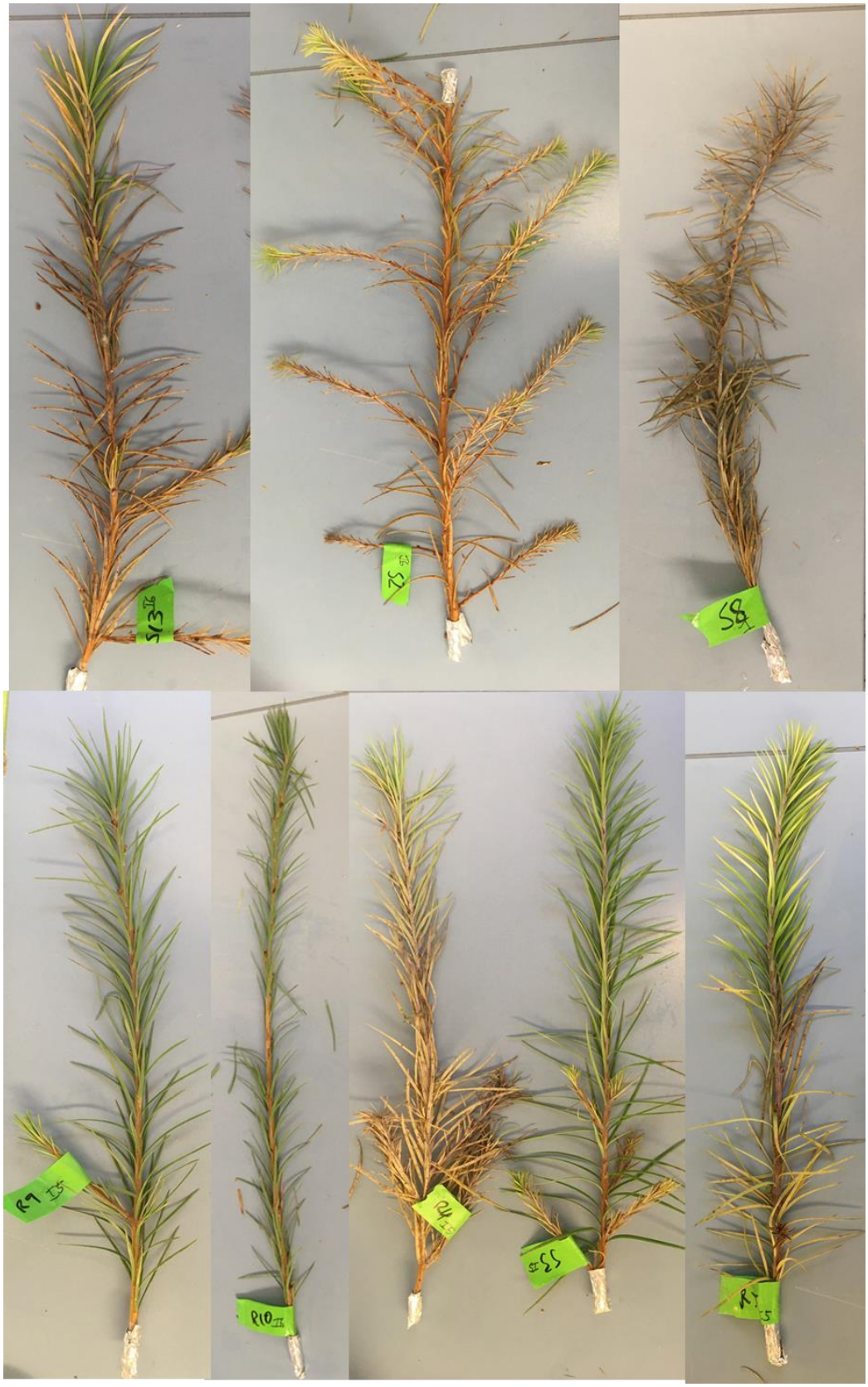


Figure 63: Representative examples of P. ramorum-inoculated shoots 21 days post inoculation, ordered by lesion length. Top row: three clones with the longest lesion lengths, L-R, Sus13, Sus2 and Sus8. Bottom row: five clones with the shortest lesion lengths L-R, Tol9, Tol10, Tol4, Sus3 and Tol7. All shoots are 30 cm long.

4.4 Discussion

This is the first study to investigate variation in susceptibility to *P. ramorum* among JL individuals. The experimental approach was based on inoculations of excised material from multiple grafts of putatively tolerant (survivor trees) and known susceptible trees, which were repeated over two years. The hypothesis for this work was that survivor trees had higher levels of resistance to *P. ramorum*. The data showed that clones from the putatively tolerant group tended to have shorter lesions. The statistical analyses indicated that the difference in lesion lengths between the putatively tolerant survivors and known susceptible trees was nearly significant. The hypothesis may be revised to state that survivor trees include a higher proportion of resistant or low susceptibility trees, accounting for observations that at least some survivors appear to have escaped infection by chance. These observations indicate that using survivor trees would be a useful starting point for further work investigating natural resistance in larch and suggest that a larger number of trees is needed in screening to filter out putative disease escapes.

The inoculation data indicate that lesion length is on a continuum showing that there is variation within the JL population. The development of smaller lesions on excised material suggests lower susceptibility on an intact tree as the lesions are more contained and so are less likely to progress to girdling, and to killing the tree. The clone with the shortest lesions, Tol7, had an average lesion length four times shorter than the longest, Sus13. The difference in lesion length among clones suggests that there might be varying levels of tolerance within the population of JL. The definition of tolerance or partial resistance can vary but is often considered as a reduction in disease severity rather than the absence of disease (González et al., 2012; Pagan & Garcia-Arenal, 2020) often including incomplete resistance conditioned by multiple genes of partial effect (Poland et al., 2009). As lesion length in response to *P. ramorum* infection is on a continuum of varying levels of damage it raises the possibility of minor gene contributions, where multiple quantitative trait loci (QTLs) are associated with the tolerance. QTLs have been found related to other Phytophthoras; eight QTLs have been associated with tolerance to P*hytophthora sojae* in Soybean (*Glycine max*) (Han et al., 2008) and multiple QTLs have been associated with resistance to *Phytophthora parasitica* in citrus (Siviero et al., 2006). With the current high levels of mortality in JL, identification of tolerance or partial resistance could be potentially useful for a breeding programme. Further insight into the molecular responses of plants to pathogen infection could further inform strategies for resistance breeding.

Durability in pathogen resistance is expected to be greater in quantitative (as opposed to qualitative) traits that are controlled by multiple genes (Lindhout, 2002), as selective pressures in the pathogen

are spread over multiple loci. Palloix et al. (2009) proposed that polygenic host resistance is more durable than monogenic resistance as the greater the number of resistance factors the pathogen has to overcome, the greater the number of virulence mutations required in the pathogen genome and the less probable their occurrence. Even so, in pathogens with highly plastic genomes such as *P. infestans,* polygenic resistance can be overcome. The 'pyramiding' of three *R* genes within the Pentland Dell potato cultivar in the 1960s was thought to maintain the efficacy of *R* genes against the most common race of *P. infestans* at the time. However, within four years of commercial production there was widespread and severe damage to the crop by new strains of *P. infestans* (Fry, 2008). Bourguet et al. (2016) proposed that the durability of disease resistance genes can be improved through management strategies such as the use of several different resistance genes in different plants within fields (multiline and variety mixtures) or between fields (regional or landscape deployment) and the periodic rotation of different resistance genes at the same site. Given the long generation times and cropping rotations in forest trees, the durability of pathogen resistance is a key aspect in producing healthy timber trees and management strategies should be developed to help maintain the durability of the disease resistance genes.

Genetic diversity within a population would provide a broader resource for the identification of resistant individuals. Juniper is a native species in the UK with high genetic diversity (Merwe et al., 2000). The testing of survivor trees from two sites heavily affected by *P. austrocedri* has found large variation in susceptibility (Green et al., 2020) within the two populations with some clones developing minimal lesions and thus being considered resistant. The genetic diversity available in the juniper populations likely exceeds that found in the JL plantations. The provenance of the stands at Glen of the Bar and Glen Trool is unknown but the original JL provenance trials in the UK, planted in 1934, sourced seeds from the Nagano Prefecture in Japan (Lines, 1987). A subsequent provenance trial in Scotland at Fetteresso, in 1956, included material from throughout the natural range (Lines & Mitchell, 1989). Lines (1987) identified a 'first choice' and two 'acceptable' provenances for subsequent planting in the UK. The founder effect and the subsequent narrowing of the gene pool through the selection and breeding of plus trees is known to reduce genetic variation within populations (Ingvarsson & Dahlberg, 2019). It is therefore possible that greater variation in JL exists in the 1956 provenance trials at Fetteresso, which remain standing (R. Whittet, 2020, personal communication), or in the native range, and could be subsequently tested for resistance to *P. ramorum.*

Phytophthora ramorum has recently been discovered in the laurosilva forests of Shikoku and Kyushu in south-west Japan (Jung et al., 2021) and in Vietnam (Jung et al., 2020). The high level of phenotypic and phylogenetic diversity in the populations and the absence of overt host symptoms

suggests that *P. ramorum* is probably endemic to the laurosilva forests of Indochina (eastern Myanmar, northern Laos and Vietnam, and southwest China) and Japan (Jung et al., 2020, 2021). The evergreen subtropical laurosilva forests where *P. ramorum* has been found in Japan are very different to the natural subalpine habitat of JL, which exists mainly at elevations between 1,200-2,400 m, on scattered volcanic mountains between 35-37°N and 137-141°E in the central island (Honshu) of Japan (Lines, 1987). It is therefore unlikely that JL has co-existed with *P. ramorum* in its native range, suggesting that tolerance through co-evolution may not occur in JL. The Japanese lineages of *P. ramorum* have not been tested for their ability to infect larch and it is unknown if they may cause severe infection in JL. The lineages of *P. ramorum* are also very diverse. The EU2 lineage (which causes extensive mortality in larch) is estimated to have diverged from the others more than 1 million years ago (Jung et al., 2021), and host preference and virulence may have also diverged in the process. However, the likely wider genetic variation in the native range of JL should still be considered when looking for possible tolerance to *P. ramorum*.

Whilst the significance of the genetic basis of disease resistance in plants has been well established, there are other fields of research that have raised interesting possibilities. Epigenetics, including chromatin modifications, nucleosome positioning, and DNA methylation have been recognised as important components of adaptation and defence mechanisms (Amaral et al., 2020; Lämke & Bäurle, 2017). The microbiome of fungal endophytes had been found to influence the severity of *Cronartium ribicola* infection on *Pinus albicaulis* (Bullington et al., 2018) and Phytophthora infection on *Theobroma cacao* (Arnold et al., 2003). Further investigation of the interaction between *P. ramorum* and larches could consider these factors and the possible influence they might have on disease severity and tolerance to infection. However, this would require deeper and more extensive studies.

The Living Ash Project (*Living Ash Project*, 2020) was developed in response to reports of tolerance in the natural population of common ash (*Fraxinus excelsior*) to ash dieback (ADB) (*Hymenoscyphus fraxineus*). The project screened and selected ash survivor trees for resistance to ADB, which identified 412 trees with a level of tolerance, as well as mass screening of 155,000 commercially produced seedlings. Symptom scoring of 28,160 trees found variation in *H. fraxineus* damage across provenances, with ~3% of trees remaining healthy. The work led to the conclusion that a breeding programme could produce ash trees with lower ADB susceptibility (Plumb et al., 2020; Stocks et al., 2017). The Living Ash Project, alongside other projects on ADB, shows the level of input required to identify tolerant individuals and evaluate the feasibility of a breeding programme. Breeding programmes for resistance to *Cronartium ribicola* in North American white pines (*Pinus* L. subgenus *Strobus*) have been running for over 40 years and have managed to moderately increase resistance

(Sniezko, 2006). In comparison, the scope of our study is limited but it is an important first step into investigating variation in resistance in the JL population.

Further work to investigate resistance or tolerance to *P. ramorum* in JL is already being undertaken. The expansion of *P. ramorum* in the Galloway Forest in spring 2018 allowed the identification of an additional 50 'putatively tolerant' survivor trees in newly affected stands. Scion material was collected from these trees in January 2020. Clones from these trees will be tested with a broader range of isolates and from both EU1 and EU2 lineages (S. Green, personal communication, 2020). Further testing of the clones with lower susceptibility found in this study using a range of *P. ramorum* isolates from both EU1 and EU2 lineages would also be of great interest. Testing against multiple isolates and lineages is important for identifying the robust resistance required to be useful in trees, especially due to their long generation times and prolonged cropping rotations. The EU2 lineage was found to be significantly more pathogenic on larch than the EU1 lineage, but EU1 lesion development was still extensive (Harris et al., 2021). The lesion development on rhododendron is similar between lineages but varied greatly between isolates although not significant statistically (Harris et al., 2021). Variation in resistance to different *P. austrocedri* isolates has also been found in inoculated juniper clones (Green et al., 2020) and is very well characterised in the relationship between *P. infestans* and potato (Fry, 2008). Therefore, any JL identified as being resistant or tolerant to *P. ramorum* infection should be tested against multiple isolates and lineages.

4.4.1 Conclusions

Our study suggests quantitative variation in susceptibly to *P. ramorum* within the JL population planted in the Galloway forest, with some clones having significantly shorter lesions than other clones. If further testing could prove this variation was stable against a range of isolates and lineages then this could lead to future work to establish a progeny trial from JL seed, from the less susceptible clones and from different provenances, in order to establish whether variation in resistance is heritable and able to be exploited in a breeding programme.

Chapter 5- General Discussion

5.1 Discussion of major findings

The aim of this study was to expand our understanding of *P. ramorum* on larch through an extended field study and associated inoculation experiments. We focused on the factors affecting the epidemiology of *P. ramorum* in JL plantations and developed a methodology to allow inoculation using sporangial suspension to investigate its infection pathway. We subsequently used this methodology to identify the defence response pathways in JL and EL that are activated following inoculation, through analysis of the RNA transcript accumulation profiles, and to investigate possible resistance to *P. ramorum* in the JL population.

5.1.1 The epidemiology of *P. ramorum* in Japanese larch

Prior to this work, no multi-year studies of the natural infection processes of *P. ramorum* and factors influencing disease progression in larch had been carried out. The period of increased spread in spring 2018 allowed us to fulfil the objectives of this study- to investigate the pathway of natural infection, how *P. ramorum* spreads between trees and the climatic factors affecting spread. Through a detailed field-based examination of infection in individual trees and within stands, we gained a unique insight into the processes involved. These observations identified fine shoots as the site of first infection, which then spreads down the branches and into the main stem, causing whole tree mortality through girdling. Primary infection most likely occurs in autumn during periods of high sporulation around needle senescence with the new infections becoming apparent in the failure to flush during the subsequent spring. Most new infections occurred broadly across the upper portion of the trees, resulting in widespread dieback of leaders and upper shoots in many trees within a stand at the same time, rather than disease spread from tree to tree. These observations are consistent with prior work showing that infection takes place high in the larch canopy and results from aerially transmitted inoculum which can potentially spread long distances between stands (Dhuli et al., 2014; Webber, Turner, et al., 2010). This pattern, along with the climatic conditions observed, suggests that in suitable conditions spread can be expansive, but also not absolute with some stands remaining infection-free throughout the study period. This observation is supported by the lack of correlation between initial infection level in a site and mortality levels at the end of the study period, and also the variability in speed and severity of infection. Together, these findings clearly suggest that sources of inoculum in the wider environment are important for influencing the extent of infections within the stand.

We identified various climatic factors as important to the spread of *P. ramorum,* including high summer precipitation, strong winds from an atypical easterly direction during spring storms, low winter temperatures and snowfall in spring. Our work showed that all these factors occurred prior to the 2013 and 2018 disease outbreaks. However, we were not able to separate out the individual factors to consider their individual contribution to infection spread. Higher than average summer rainfall might have induced sporulation of *P. ramorum* in infected needle litter and provided the surface moisture required for further successful infection of the needles. Strong winds might have helped move inoculum but, combined with low temperature and late season snowfall, may have resulted in the trees being stressed through a mismatch of phenology to the climatic conditions. The atypical easterly winds might also cause greater damage to the canopy, leading to wounds that could become a conduit for *P. ramorum* infection. Larches are known to be susceptible to frosts during flushing (Forestry Commission, 1957) and so cold and snowfall, as the trees transition from dormancy towards flushing, could stress the trees, even if not resulting in bud mortality. High levels of stress can compromise the trees' ability to produce an effective defence response and make them more susceptible to infection by necrotrophic and opportunistic pathogens (Ennos, 2015). The melting of late season snowfall also increases ground water availability, potentially leading to higher than normal levels of tissue moisture which might favour the rapid spread of the pathogen within the tree.

Whilst the influence of temperature and precipitation upon the infection of *P. ramorum* in American oaks has been well studied (Davidson et al., 2005; Dodd et al., 2008; Hüberli et al., 2012; Kozanitas et al., 2017) this work was the first to investigate their effect on the infection of larch. There were anecdotal suggestions that the wetter summers of 2012 and 2017 might be related to epidemics in the subsequent year and this work provides an evidence base to support that theory. Prior reports suggested, based on laboratory experiments on optimum growth temperatures, that mild or warmer than average winters might result in more infections (Forest Research & FERA, 2010). Our results suggest the opposite, that colder temperatures in late winter and early spring are followed by periods of high infections. The identification of easterly storm winds and late season snowfall are new factors that have not been considered before.

5.1.2 Differing responses to *P. ramorum* infection in European and Japanese larch

Prior work had shown variation in susceptibility between the two species with EL thought to be less severely affected than JL in the field but the results of experimental trials are inconsistent (Chastagner et al., 2013; De La Mata Saez, 2015; Harris, 2014; Harris et al., 2021). This work expanded upon this by studying the RNA transcript accumulation profiles in response to inoculation,

which indicated the molecular interactions involved in the defence response to *P. ramorum* infection. Although there was no significant difference in lesion development between the two species of larch following controlled inoculations, there were differences in both the suites of genes activated and the magnitude and duration of gene expression. European larch has a greater number of over-expressed defence related pathways compared with JL, in particular related to jasmonic acid. European larch also had rapidly induced expression of defence genes within 24 hours of infection followed by sustained or increasing expression until 25 days after inoculation. Japanese larch had fewer key defence pathways clearly responding to infection and the observed gene expression increases were both larger and more transient compared to EL. Although the lesions in EL were not significantly different to JL, the factors mentioned above may have some influence in field conditions that were not apparent in our experimental setup and thus might account for the less severe effects seen on EL in the field. This work covers a longer timeframe after inoculation than previous work by De La Mata Saez (2015) and so has been able to show temporal differences in gene expression between the two species of larch, which were unknown before. Previous work on *P. ramorum* in tanoaks by Hayden et al. (2014) found enrichment of disease response proteins in excised material up to 5 days after inoculation, but they did not investigate the enrichment of gene pathways and there was no widespread enrichment of secondary metabolites, unlike our observations in larch. This could be due to the limitations of the short experimental timeframe and inoculation of excised material from saplings used by Hayden et al. (2014), as increased expression of secondary metabolites, including ellagic acid and tyrosol, were observed in mature coast live oak trees during infection (Nagle et al., 2011; Ockels et al., 2007). However, it is also likely that larches, as conifers, may express different defence responses than oaks. The suites of phenolic metabolites expressed in response to pathogen attack are known to differ between species, and between conifers and deciduous angiosperm trees (Witzell & Martín, 2008). Many of the over-expressed genes we observed in larch were part of very large gene families that may be indicative of differences between conifers and oaks. These include secondary metabolite genes (*CHS*) and jasmonic acid pathway related genes. The large size of these gene families suggests that accumulation of extra gene copies is under positive selection and that diversification in function or regulation may be occurring.

5.1.3 Possible resistance to *P. ramorum* in putatively tolerant Japanese larch trees.

We are the first to report the presence of quantitative variation in susceptibility to *P. ramorum* within the JL population, with some clones having significantly shorter lesions than other clones. It was hypothesised that the post-epidemic survivor larch trees might have a greater level of tolerance to *P. ramorum* infection (as has been found in other pathosystems) but, prior to this work, no evidence was available to support this hypothesis. This work found that, although clones from the

putatively resistant group (survivor trees) tended to have shorter lesions than clones from trees known to be susceptible, they were not statistically significantly different and so the hypothesis was not supported. There were also some clones from the known susceptible group that had shorter than average lesions overall. Therefore, there appears to be a continuum and wide range of tolerance within the population, consistent with quantitative factors controlling disease responses in larch. This variation provided evidence for some level of tolerance in larch in the Galloway Forest and could be useful for further exploration as detailed below. In addition, the observation of variability among clones from the known susceptible group clearly suggests that there is variation in the wider population in general.

5.2 Future research directions

5.2.1 Epidemiology of *P. ramorum* on larch

Our field observations generated the hypothesis that infection occurs in autumn. We showed experimentally that intact bark is an effective barrier in winter and that infection does not occur via healthy buds during flushing. Therefore, it is likely that infection is already established within shoots prior to flushing in the spring. However, due to time constraints, we were not able to test infection in autumn. Further work to investigate this could confirm our field observations and explore the role of senescing needles, buds and the abscission zone as conduits for infection of shoots. Experiments could involve direct inoculation of needles at different stages of senescence on young trees, from late summer into autumn in controlled conditions, supplemented by field surveys looking specifically at needle infections in autumn. Factors favouring the extension of infection within the tree during spring would also be of interest. Late season snowfall, and subsequent melting, increases soil moisture availability and potentially leads to higher levels of tissue moisture as sap flow and water transport increase during flushing (di Francescantonio et al., 2018). This might favour the rapid spread of the pathogen within the tree. In coast live oaks it was suggested that the medullary rays in the xylem which conduct water radially though the stem may serve as pathways of infection for *P. ramorum* (Florance, 2005). Further work could investigate the effects of tissue moisture levels and ground water availability on *P. ramorum* infection.

We identified higher than average summer and autumn rainfall in the previous year to each epidemic and low temperatures, easterly winds and late lying snowfall in early spring (March) of each epidemic year. We observed that these factors all occurred prior to infection outbreaks. Whilst some conditions, such as snowfall and low temperatures, are intrinsically linked, future work could

consider each factor in isolation and investigate whether all of these climatic factors are necessary or if some have greater influence on infections and spread of *P. ramorum*. Multivariate analyses could be used to consider the relationships between the variables and their respective contributions to infection spread.

Identification of the climatic variables influencing *P. ramorum* spread on larch in the UK could be further expanded upon to inform the development of models to predict future outbreaks. Ecological niche modelling by Kluza et al. (2007) used climatic records and data of the occurrences of *P. ramorum* in California to identify regions that had the highest potential for the establishment of *P. ramorum*. This included the south-east of the USA, where *P. ramorum* is not yet currently found. Kluza et al. (2007) also modelled suitability across Europe and made particular note of the high potential risk in the west and south of the UK, with potential hotspots in southern Wales and Cornwall. These hotspots are where the first outbreaks of *P. ramorum* in larch were subsequently discovered in 2009 (Webber, Mullett, et al., 2010), showing the predictive power of modelling. Modelling can also inform management strategies. Václavík et al. (2010) modelled the of spread *P. ramorum* in Oregon using climate conditions, topographical factors, abundance and susceptibility of host vegetation, and dispersal pressure to identify areas of risk and recommend priority areas for early detection and invasion control. Modelling should be considered a priority research objective for further work, not only to enhance our understanding of the pathosystem but also to assist in management decisions. Modelling of *P. ramorum* on oaks in the USA differs from the UK situation as it involves natural populations rather than plantation forestry but it is still of use, particularly if modelling could be done on a finer scale to give localised outbreak predictions in specific regions of the UK based on real-time climatic patterns. This is because the spread of *P. ramorum* on larch has been highly variable from year to year and accounting for these changes would be important for accurate disease modelling. This would be of use to the UK forestry industry and could inform management strategies, as discussed later.

5.2.2 The response of larches to *P. ramorum* infection

We identified differences in expression of jasmonic acid (JA) related genes in response to infection, with EL having overexpression of more JA related pathway genes. The role of JA is well known as a signalling compound, that can induce expression of defence related proteins in response to pathogen attack (van Loon et al., 2006) and in particular in response to necrotrophic pathogens (Glazebrook, 2005). Further work could give better understanding of its role in relation to *P. ramorum* infection and the downstream pathways it activates. This could be carried out by investigating the effect of priming with JA before inoculation. Jasmonic acid treatment of tomato

(*Solanum lycopersicum*) seeds primes the JA defence response, leading to lower susceptibility to the fungal pathogen *Botrytis cinerea*, and was associated with enhanced defence gene expression during pathogen attack for at least eight weeks post treatment (Worrall et al., 2012). Norway spruce (*Picea abies*) seedlings grown from JA treated seeds show reduced attack by pine weevils (*Hylobius abietis*) through improved defence response (Berglund et al., 2016).

Analysis of RNA transcript accumulation profiles found different suites of secondary metabolite pathway genes expressed in JL and EL in response to infection. Chemical profiling could be used to investigate differences and changes in phenolic compounds by using HPLC to discover variations and GC-MS to verify the identity of compounds produced in response to infection. Secondary metabolites with inhibitory effects against *P. ramorum* have been found to vary constitutively and to be induced in response to infection in American oaks (Nagle et al., 2011; Ockels et al., 2007). Similar studies could be carried out in larches to investigate the secondary metabolites identified in the RNA transcript accumulation profiles. Phenotypic and genetic studies of variations in secondary metabolites would be of interest for understanding the molecular basis of natural variation in disease susceptibility and could support resistance breeding work (see 5.2.3 and 5.3).

5.2.3 Intraspecific and interspecific variation in *P. ramorum* tolerance in larch

We found quantitative variation in susceptibility to *P. ramorum* in excised material from JL in the Galloway Forest. Our work has provided the evidence to justify the subsequent establishment of a larger clonal trial of JL trees which will be evaluated for resistance to *P. ramorum*. In January 2020, scion material was collected from an additional 50 'putatively tolerant' survivor JL trees in the Galloway Forest (S. Green, personal communication, 2020). Further work will need to establish if this variation is stable against a range of *P. ramorum* isolates and lineages, through inoculation of whole plants. This would provide a more robust analysis of the variation in susceptibility and potential for utilising resistance in commercial forestry. A progeny trial of seed originating from different provenances, as well as from the less susceptible clones, could establish whether variation in resistance is heritable and able to be exploited in a breeding programme. More genetic variation within JL would be available in the original provenance trials planted in the UK between 1930 and 1950 or by gathering seed from the native range of JL.

Our work has considered intraspecific variation in JL and interspecific differences between JL and EL. The genus *Larix* comprises 10–15 species that are known to hybridize (Semerikov et al., 2003), but only three, EL and JL and their hybrid (*Larix × marschlinsii* Syn. L. *× eurolepis*) (HL), are grown commercially in the UK. This raises the possibility of investigating other larches for their suitability to replace EL and JL. Any alternative larch species would have to have low susceptibility to *P. ramorum*

plus the growth characteristics required for commercial production. Chastagner et al. (2012) carried out inoculation trials on EL, JL and two North American larches, eastern (*L. laricina*) and western larch (*L. occidentalis*). They found that western larch developed the longest lesions, averaging 12.6 cm, whilst the other species tested developed lesions between 2.4 and 3.7 cm long. Western larch also had the highest mortality at 53.3% at 55dpi compared to 46.7%, 33.3% and 0% for EL, JL and eastern larch, respectively. Chastagner et al. (2012) only used a small sample size but demonstrated differences between the species, with the lack of mortality in eastern larch of particular note.

Although there are specimens of all larch species grown in botanic gardens and arboreta around the UK, only EL, JL and HL have been formally trialled in the UK (C. Reynolds, personal communication, 2021). Most larch species were disregarded due to their severe susceptibility to spring frosts (Forestry Commission, 1957). These assessments were not based on formal organised trials but rather on reports from small plantings on private estates (Atholl, 1832). This included Siberian larch (*L. sibirica*) which is adapted to continental conditions and flushes early in the UK, becoming damaged by late winter frosts every year (Lines, 1987). Future climate change is likely to result in earlier flushing and more variable weather patterns, including late frosts, thus making frost susceptible larches unlikely candidates for planting in the UK. Western larch was planted experimentally in the early 1900s and although it grew quite well, it was discontinued as its growth was inferior to that of EL and JL (Forestry Commission, 1957). Eastern larch was also considered but found to be of poor stature and prone to larch canker (Forestry Commission, 1957) and, although well grown specimens have raised interest recently, there are currently no plans to carry out formal trials (C. Reynolds, personal communication, 2021). However, the lower mortality seen by Chastagner et al. (2012) in eastern larch could be further investigated to provide support for trials and hybridizations to be carried out.

5.3 Impacts and potential applications of the research.

We found climatic conditions were important in driving infection spread, principally high summer precipitation, strong winds from an atypical easterly direction during spring storms, low winter temperatures and snowfall in early spring. This has implications for three aspects of forest management- allocation of risk zones, disease surveillance and felling strategies.

As part of the management strategy for *Phytophthora ramorum,* the Forestry Commission has separated Great Britain into three risk zones based upon climatic suitability and the previous occurrences of *P. ramorum* in each area (Table 19 and Figure 64) (Forest Research, 2020b). Planting

of larch still occurs in zone 3, on the drier east coast, as it is less likely to get infected (C. Reynolds, personal communication, 2021). Based on the evidence from this research we suggest that the zone boundaries are kept under review, but an assessment of the impact of climatic factors upon *P. ramorum* spread on a larger geographic scale could be used to inform modelling and give the zones active rather than static boundaries. The risk zones should be periodically reviewed and the evidence of climatic factors from our work should be included in future reviews. Disease surveillance could be tailored if modelling was implemented on a fine enough scale to inform individual forest managers of the risks to their stands, thus enabling them to carry out heightened surveillance at periods of increased risk, such as after a particularly wet summer, in areas with the highest likelihood of outbreaks, rather than relying on country-wide surveillance. A simple recommendation, without the need for complex modelling, would be to disseminate the observations made in this work to industry stakeholders. Forest managers, especially in zone 1, should build an understanding of rainfall patterns in their areas and monitor levels of summer rainfall in order to identify excessive rainfall. This would enable them prepare for a potential expansion of infections the following spring. These predictions could also be used to plan ahead and inform contractors and sawmills of a possible requirement for increased capacity for felling and timber processing.

During widespread outbreaks, such as in 2013, the area of larch affected has exceeded the capacity of the forestry industry to fell and process the timber, which was one of the contributing factors to the establishment of the Galloway Forest management zone (Forestry Commission Scotland, 2017). Modelling could allow the identification of stands to be prioritised for felling in areas where the climatic conditions are likely to result in high levels of sporulation and increased spread from the infected trees. We observed healthy JL stands rapidly succumbing to infection despite being geographically isolated from other larches, suggesting that long distance spread of *P. ramorum* occurs in suitable climatic conditions. These findings also indicate that the management of *P. ramorum* by felling infected trees is of greater importance in years when the climatic conditions are favourable for sporulation and subsequent spread.

Table 19: Factors determining the areas covered by each Phytophthora ramorum risk zone.

Zone	Climatic risk	*P. ramorum* infection	Overall risk
1	High	confirmed	High
2	High	none	Medium
3	Low	none	Low



Figure 64: Phytophthora ramorum risk zones in Great Britain. Map generated using data from Forestry Commission Open Data (2016) *in ArcGIS.*

The variation in susceptibility to *P. ramorum* found in this study represents the first step to start the process of developing a breeding programme for resistance to *P. ramorum* in larch. There has not been any breeding for *P. ramorum* resistance in larch or in any other *P. ramorum* hosts, although there is some evidence of natural resistance in American oaks (Nagle et al., 2011), and this highlights the importance of our work. Breeding programmes for disease resistance are underway in many tree species, from multi-decade long programmes on resistance to *Cronartium ribicola* in North American white pines (*Pinus* L. subgenus *Strobus*) (Sniezko, 2006), to new projects on ash dieback (*Hymenoscyphus fraxineus*) in common ash (*Fraxinus excelsior*) (*Living Ash Project*, 2020). These programmes have established methodologies and protocols, from inoculation and disease scoring to

strategies for eventual wide-scale deployment, that provide a wealth of knowledge that could inform and guide a breeding project for resistance to *P. ramorum* in larch. The progress of resistance breeding in North American white pines suggests that it would be possible to improve resistance in larch.

Identifying quantitative trait loci (QTL) related to traits of interest, in this case disease resistance, and estimating their effect on the phenotype has been used to develop marker-assisted selection (MAS) tools in pines and poplars since the 1990s (Grattapaglia, 2014) which are useful to understand the genetic basis of a trait. However, more recently, Genomic Selection (GS) has been developed, which scans the whole genome and uses a dense genome-wide panel of markers, whose effects on the phenotype are estimated simultaneously as forecasters of phenotype in prediction models. This is then used to suggest breeding values related to various characteristics and thus increase the rate of genetic gain over time (Isik et al., 2015). GS consequently produces a single breeding or genotypic value for each individual, moving away from the classical trait dissection approach by de-emphasizing the contribution of individual genes or QTLs to the target quantitative trait (Grattapaglia, 2014). Thus, the quantitative variation in susceptibility to *P. ramorum* found in our work could be a basis for assisted selection, either through further work to identify QTLs related to disease resistance, or by using the identified trees as part of a select population to undergo GS and hopefully give higher breeding values for disease resistance than the general population.

References

Adorada, D. L., Biles, C. L., Liddell, C. M., Fernandez-Pavi'a, S., Waugh, K. O., & Waugh, M. E. (2000). Disease development and enhanced susceptibility of wounded pepper roots to Phytophthora capsici. *Plant Pathology*, *49*(6), 719–726. https://doi.org/10.1046/j.1365-3059.2000.00510.x

Amaral, J., Ribeyre, Z., Vigneaud, J., Sow, M. D., Fichot, R., Messier, C., Pinto, G., Nolet, P., & Maury, S. (2020). Advances and promises of epigenetics for forest trees. *Forests*, *11*(9), 1–21. https://doi.org/10.3390/f11090976

Andrews, S. (2018). *FastQC: a quality control tool for high throughput sequence data*. http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Armstrong, C. (2018). *Chemotaxis and inhibition of the kauri killer, Phytophthora agathidicida*. University of Otago.

Arnold, A. E., Mejía, L. C., Kyllo, D., Rojas, E. I., Maynard, Z., Robbins, N., & Herre, E. A. (2003). Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(26), 15649–15654. https://doi.org/10.1073/pnas.2533483100

Atholl, J. M. (1832). *Account of the larch plantations on the estates of Atholl & Dunkeld, executed by the late John, Duke of Atholl; drawn up from papers and documents, communicated by his Grace's trustees to the Highland Society of Scotland. - University of Oxford*. C.G. Sidey.

Ballvora, A., Ercolano, M. R., Weiss, J., Meksem, K., Bormann, C. A., Oberhagemann, P., Salamini, F., & Gebhardt, C. (2002). The R1 gene for potato resistance to late blight (Phytophthora infestans) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. *The Plant Journal*, *30*(3), 361–371. https://doi.org/10.1046/j.1365-313X.2001.01292.x

Barakat, A., Staton, M., Cheng, C.-H., Park, J., Yassin, N., Ficklin, S., Yeh, C.-C., Hebard, F., Baier, K., Powell, W., Schuster, S. C., Wheeler, N., Abbott, A., Carlson, J. E., Sederoff, R., Oh, S., Manos, P., Anagnostakis, S., Hill, J., … Yellanki, P. (2012). Chestnut resistance to the blight disease: insights from transcriptome analysis. *BMC Plant Biology*, *12*(1), 38. https://doi.org/10.1186/1471-2229-12-38

Bassani, I., Larousse, M., Tran, Q. D., Attard, A., & Galiana, E. (2020). Phytophthora zoospores: From perception of environmental signals to inoculum formation on the host-root surface. *Computational and Structural Biotechnology Journal*, *18*, 3766–3773. https://doi.org/10.1016/j.csbj.2020.10.045

Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, *57*(1), 289–300.

Bennett, R. N., & Wallsgrove, R. M. (1994). Secondary metabolites in plant defence mechanisms. *New Phytologist*, *127*(4), 617–633. https://doi.org/10.1111/j.1469-8137.1994.tb02968.x

Berglund, T., Lindström, A., Aghelpasand, H., Stattin, E., & Ohlsson, A. B. (2016). Protection of spruce seedlings against pine weevil attacks by treatment of seeds or seedlings with nicotinamide, nicotinic acid and jasmonic acid. *Forestry*, *89*(2), 127–135. https://doi.org/10.1093/forestry/cpv040

Bolton, M. D. (2009). Primary Metabolism and Plant Defense-Fuel for the Fire. */ 487 MPMI*, *22*(5), 487–497. https://doi.org/10.1094/MPMI

Bourguet, D., Delmotte, F., Franck, P., Guillemaud, T., Reboud, X., Vacher, C., & Walker, A. S. (2016). Combining Selective Pressures to Enhance the Durability of Disease Resistance Genes. *Frontiers in Plant Science*, *7*(1916), 1–8. https://doi.org/10.3389/fpls.2016.01916

Brasier, C. M., Denman, S., Rose, J., Kirk, S. A., Hughes, K. J. D., Griffin, R. L., Lane, C. R., Inman, A. J., & Webber, J. F. (2004). First report of ramorum bleeding canker on Quercus falcata, caused by Phytophthora ramorum. *Plant Pathology*, *53*(6), 804–804. https://doi.org/10.1111/j.1365-3059.2004.01079.x

Brasier, C. M., & Webber, J. F. (2010). Plant pathology: Sudden larch death. *Nature*, *466*(7308), 824–825. https://doi.org/10.1038/466824a

Bray, N. L., Pimentel, H., Melsted, P., & Pachter, L. (2016). Near-optimal probabilistic RNA-seq quantification. *Nature Biotechnology*, *34*(5), 525–527. https://doi.org/10.1038/nbt.3519

Browning, M., Englander, L., Tooley, P. W., & Berner, D. (2008). Survival of Phytophthora ramorum Hyphae after Exposure to Temperature Extremes and Various Humidities. *Mycologia*, *100*(2), 236–245. https://www.jstor.org/stable/20444944

Budde, K. B., Nielsen, L. R., Ravn, H. P., & Kjær, E. D. (2016). The Natural Evolutionary Potential of Tree Populations to Cope with Newly Introduced Pests and Pathogens—Lessons Learned From Forest Health Catastrophes in Recent Decades. In *Current Forestry Reports* (Vol. 2, Issue 1, pp. 18–29). Springer International Publishing. https://doi.org/10.1007/s40725-016-0029-9

Bullington, L. S., Lekberg, Y., Sniezko, R., & Larkin, B. (2018). The influence of genetics, defensive chemistry and the fungal microbiome on disease outcome in whitebark pine trees. *Molecular Plant Pathology*, *19*(8), 1847–1858. https://doi.org/10.1111/MPP.12663

Carleson, N. C., Daniels, H. A., Reeser, P. W., Kanaskie, A., Navarro, S. M., LeBoldus, J. M., & Grünwald, N. J. (2021). Novel Introductions and Epidemic Dynamics of the Sudden Oak Death Pathogen Phytophthora ramorum in Oregon Forests . *Phytopathology*, *111*(4), 731–740. https://doi.org/10.1094/phyto-05-20-0164-r

Chastagner, G., Riley, K., & Elliott, M. (2013). Susceptibility of Larch , Hemlock , Sitka Spruce , and Douglas-fir to Phytophthora ramorum 1. In S. J. Frankel, J. T. Kliejunas, K. M. Palmieri, & J. M. Alexander (Eds.), *Proceedings of the Sudden Oak Death Fifth Science Symposium. Gen. Tech. Rep. PSW-GTR-243.* (pp. 77–79). U.S. Department of Agriculture: Forest Service. https://doi.org/https://doi.org/10.2737/PSW-GTR-243

Chauvin, A., Caldelari, D., Wolfender, J. L., & Farmer, E. E. (2013). Four 13-lipoxygenases contribute to rapid jasmonate synthesis in wounded Arabidopsis thaliana leaves: A role for lipoxygenase 6 in responses to long-distance wound signals. *New Phytologist*, *197*(2), 566–575. https://doi.org/10.1111/nph.12029

Chen, X., Sun, X., Dong, L., & Zhang, S. (2018). Mating patterns and pollen dispersal in a Japanese larch (Larix kaempferi) clonal seed orchard: a case study. *Science China Life Sciences 2018 61:9*, *61*(9), 1011–1023. https://doi.org/10.1007/S11427-018-9305-7

Cobb, R. C., Haas, S. E., Kruskamp, N., Dillon, W. W., Swiecki, T. J., Rizzo, D. M., Frankel, S. J., & Meentemeyer, R. K. (2020). The Magnitude of Regional-Scale Tree Mortality Caused by the Invasive Pathogen Phytophthora ramorum. *Earth's Future*, *8*(7). https://doi.org/10.1029/2020EF001500

Cohen, L. I. (1967). The Pathology of Hypodermella laricis on Larch, Larix Occidentalis. In *Source: American Journal of Botany* (Vol. 54, Issue 1).

Committee on Climate Change. (2020). Land use: Policies for a Net Zero UK. In *Committee on Climate Change* (Issue January). https://www.theccc.org.uk/publication/land-use-policies-for-a-net-zero-uk/%0A

Da Ronch, F., Caudullo, G., Tinner, W., & de Rigo, D. (2016). Larix decidua and other larches in Europe: distribution, habitat, usage and threats. In J. San-Miguel-Ayanz, D. de Rigo, G. Caudullo, T. Houston Durrant, & A. Mauri (Eds.), *European Atlas of Forest Tree Species* (pp. 108–110). Publication Office of the European Union.

Dale, A. L., Feau, N., Everhart, S. E., Dhillon, B., Wong, B., Sheppard, J., Bilodeau, G. J., Brar, A., Tabima, J. F., Shen, D., Brasier, C. M., Tyler, B. M., Grünwald, N. J., & Hamelin, R. C. (2019). Mitotic Recombination and Rapid Genome Evolution in the Invasive Forest Pathogen Phytophthora ramorum. *MBio*, *10*(2). https://doi.org/10.1128/MBIO.02452-18

Davidson, J. M., & Shaw, C. G. (2003). Pathways of movement for Phytophthora ramorum, the causal agent of Sudden Oak Death. *Sudden Oak Death Online Symposium*. https://doi.org/10.1094/SOD-2003-TS

Davidson, J. M., Wickland, A. C., Patterson, H. A., Falk, K. R., & Rizzo, D. M. (2005). Transmission of Phytophthora ramorum in Mixed-Evergreen Forest in California. *Phytopathology*, *95*(5), 587–596. https://doi.org/10.1094/PHYTO-95-0587

De La Mata Saez, L. (2015). Phylogenetic and molecular studies of the populations of Phytophthora ramorum, lineages EU1 and EU2, in Ireland and gene expression during infection of Larix spp. In *PhD Thesis, Queen's University of Belfast*. https://doi.org/10.1017/CBO9781107415324.004

De Schepper, V., van Dusschoten, D., Copini, P., Jahnke, S., & Steppe, K. (2012). MRI links stem water content to stem diameter variations in transpiring trees. *Journal of Experimental Botany*, *63*(7), 2645–2653. https://doi.org/10.1093/jxb/err445

Denman, S., Moralejo, E., Kirk, S. A., Orton, E., & Whybrow, A. (2007). Sporulation of Phytophthora ramorum and P. kernoviae on Asymptomatic Foliage and Fruit. *Proceedings of the Sudden Oak Death Third Science Symposium. Gen. Tech. Rep. PSW-GTR-214*, 201–207. https://doi.org/https://doi.org/10.2737/PSW-GTR-214

Department for Environment Food & Rural Affairs. (2015). *List of natural hosts for Phytophthora ramorum with symptom and location*. https://planthealthportal.defra.gov.uk/assets/uploads/P-ramorum-host-list-finalupdate-NOV-20-15.pdf

DeYoung, B. J., & Innes, R. W. (2006). Plant NBS-LRR proteins in pathogen sensing and host defense. *Nature Immunology*, *7*(12), 1243–1249. https://doi.org/10.1038/ni1410

Dhuli, P., Rohloff, J., & Strimbeck, G. R. (2014). Metabolite changes in conifer buds and needles during forced bud break in Norway spruce (Picea abies) and European silver fir (Abies alba). *Frontiers in Plant Science*, *5*, 706. https://doi.org/10.3389/fpls.2014.00706

di Francescantonio, D., Villagra, M., Goldstein, G., & Campanello, P. I. (2018). Leaf phenology and water-use patterns of canopy trees in Northern Argentinean subtropical forests. *Tree Physiology*, *38*(12), 1841–1854. https://doi.org/10.1093/treephys/tpy072

Dixon, D. P., Lapthorn, A., & Edwards, R. (2002). Plant glutathione transferases. *Genome Biology*, *3*(3), 1–10. https://doi.org/10.1186/gb-2002-3-3-reviews3004

Dixon, R. A., Achnine, L., Kota, P., Liu, C.-J., Reddy, M. S. S., & Wang, L. (2002). The phenylpropanoid pathway and plant defence-a genomics perspective. *Molecular Plant Pathology*, *3*(5), 371–390.

https://doi.org/10.1046/j.1364-3703.2002.00131.x

Dobson, A., Barker, K., & Taylor, S. L. (2013). *Biosecurity : the Socio-Politics of Invasive Species and Infectious Diseases.* Taylor and Francis. https://doi.org/https://doi.org/10.4324/9780203113110

Dodd, R. S., Hüberli, D., Douhovnikoff, V., Harnik, T. Y., Afzal-Rafii, Z., & Garbelotto, M. (2004). Is variation in susceptibility to Phytophthora ramorum correlated with population genetic structure in coast live oak (Quercus agrifolia)? *New Phytologist*, *165*(1), 203–214. https://doi.org/10.1111/j.1469-8137.2004.01200.x

Dodd, R. S., Hüberli, D., Mayer, W., Harnik, T. Y., Afzal-Rafii, Z., & Garbelotto, M. (2008). Evidence for the role of synchronicity between host phenology and pathogen activity in the distribution of sudden oak death canker disease. *New Phytologist*, *179*(2), 505–514. https://doi.org/10.1111/j.1469-8137.2008.02450.x

Dumfries and Galloway Council. (2014). *Dumfries and Galloway Forestry and Woodland Strategy*.

Elliott, M., Rollins, L., Bourret, T., & Chastagner, G. (2021). First report of leaf blight caused by phytophthora ramorum on cherry laurel (Prunus laurocerasus) in Washington State, U.S.A. *Plant Disease*, *105*(3), 712. https://doi.org/10.1094/PDIS-07-20-1489-PDN

Elliott, M., Sumampong, G., Varga, A., Shamoun, S. F., James, D., Masri, S., Brière, S. C., & Grünwald, N. J. (2009). PCR-RFLP markers identify three lineages of the North American and European populations of Phytophthora ramorum. *Forest Pathology*, *39*(4), 266–278. https://doi.org/10.1111/j.1439-0329.2008.00586.x

Englander, L., Browning, M., & Tooley, P. W. (2006). Growth and sporulation of Phytophthora ramorum in vitro in response to temperature and light. *Mycologia*, *98*(3), 365–373. https://doi.org/10.3852/mycologia.98.3.365

Ennos, R. A. (2015). Resilience of forests to pathogens: an evolutionary ecology perspective. *Forestry*, *88*(1), 41–52. https://doi.org/10.1093/forestry/cpu048

Erwin, D. C., & Ribeiro, O. K. (1996). Phytophthora diseases worldwide. In *Phytophthora diseases worldwide.* American Phytopathological Society (APS Press).

Everhart, S. E., Tabima, J. F., & Grünwald, N. J. (2014). Phytophthora ramorum. In R. Dean, A. Lichens-Park, & C. Kole (Eds.), *Genomics of plant associated fungi and oomycetes: Dicot Pathogens.* (pp. 159–174). Springer. https://doi.org/10.1007/978-3-662-44056-8_8

Feau, N., Ojeda, D. I., Beauseigle, S., Bilodeau, G. J., Brar, A., Cervantes-Arango, S., Dale, A. L., Dhillon, B., Hammett, C., Herath, P., Shamoun, S. F., Tsui, C. K. M., Tanguay, P., & Hamelin, R. C. (2019). Improved detection and identification of the sudden oak death pathogen Phytophthora ramorum and the Port Orford cedar root pathogen Phytophthora lateralis. *Plant Pathology*, *68*(5), 878–888. https://doi.org/10.1111/ppa.13015

FERA. (2012). *Phytophthora ramorum and Phytophthora kernoviae diseases on bilberry (Vaccinium myrtillus)*. https://planthealthportal.defra.gov.uk/assets/factsheets/phytophthoraBilberry.pdf

Fichtner, E. J., Lynch, S. C., & Rizzo, D. M. (2007). Detection, Distribution, Sporulation, and Survival of Phytophthora ramorum in a California Redwood-Tanoak Forest Soil. *Phytopathology*, *97*(10), 1366–1375. https://doi.org/10.1094/PHYTO-97-10-1366

Fichtner, E. J., Lynch, S. C., & Rizzo, D. M. (2009). Survival, Dispersal, and Potential Soil-Mediated Suppression of Phytophthora ramorum in a California Redwood-Tanoak Forest. *Phytopathology*, *99*(5), 609–618. https://doi.org/10.1094/PHYTO-99-5-0608

Florance, E. R. (2005). Magnetic Resonance Imaging (MRI) of Oak Trees Infected With Phytophthora ramorum to Determine Potential Avenues of Infection in Bark. In S. J. Frankel, P. J. Shea, & M. I. Haverty (Eds.), *Proceedings of the sudden oak death second science symposium: the state of our knowledge. Gen. Tech. Rep. PSW-GTR-196* (pp. 91–99). U.S. Department of Agriculture, Forest Service. https://doi.org/https://doi.org/10.2737/PSW-GTR-196

Forest Research. (2001). *National inventory of woodland and trees - Scotland - Forest Research*. https://www.forestresearch.gov.uk/tools-and-resources/national-forest-inventory/national-inventory-of-woodland-and-trees/national-inventory-of-woodland-and-trees-scotland/

Forest Research. (2020a). *Forestry Statistics 2020-Chapter 1: Woodland Area and Planting*. https://www.forestresearch.gov.uk/documents/7769/Ch1_Woodland_FS2020_cgadFu3.pdf

Forest Research. (2020b). *Ramorum disease (Phytophthora ramorum) - Forest Research*. https://www.forestresearch.gov.uk/tools-and-resources/pest-and-disease-resources/ramorum-disease-phytophthora-ramorum/

Forest Research. (2020c). *Tree Species Database European larch (EL)*. https://www.forestresearch.gov.uk/tools-and-resources/tree-species-database/european-larch-el/

Forest Research. (2021a). *9. Licences to move and process wood from trees with ramorum disease - Forest Research*. Phytophthora Manual. https://www.forestresearch.gov.uk/tools-and-resources/fthr/pest-and-disease-resources/ramorum-disease-phytophthora-ramorum/phytophthora-manual-9-licences-move-and-process-wood-trees-ramorum-disease/

Forest Research. (2021b). *Tree improvement - the first 50 years of Tree Breeding in Britain - Forest Research*. https://www.forestresearch.gov.uk/research/tree-improvement/tree-improvement-50-years-of-tree-breeding-in-britain/

Forest Research, & FERA. (2010). *Report on Phytophthora ramorum incited dieback of larch (Larix kaempferi)*.

Forestry and Land Scotland. (2017). *Galloway Forest District: Shalloch Forest Block Land Management Plan*. https://forestryandland.gov.scot/images/corporate/design-plans/galloway/shalloch/Shalloch-LMP-Final.pdf

Forestry Commission. (1957). *Bulletin 30: Exotic Forest Trees in Great Britain*. https://www.forestresearch.gov.uk/research/archive-exotic-forest-trees-in-great-britain/

Forestry Commission. (1999). *National inventory of woodland and trees (1995-99)- Dumfries and Galloway Region*. http://www.forestry.gov.uk/pdf/nigreatbritain.pdf/$FILE/nigreatbritain.pdf

Forestry Commission. (2020). *Science and innovation strategy for forestry in Great Britain*. https://gov.wales/sites/default/files/publications/2020-10/science-and-innovation-strategy-for-forestry-in-great-britain.pdf

Forestry Commission Open Data. (2016). *Phytophthora Ramorum Risk Zones GB - Overview*. https://www.arcgis.com/home/item.html?id=7bc7d089a0d64bca8b3d3d81725bdd54

Forestry Commission Scotland. (2017). *Ramorum (on larch) Action Plan for Scotland (2017)*. 1–13. http://scotland.forestry.gov.uk/supporting/forest-industries/tree-health

Frades, I., Abreha, K. B., Proux-Wéra, E., Lankinen, Å., Andreasson, E., & Alexandersson, E. (2015). A novel workflow correlating RNA-seq data to Phythophthora infestans resistance levels in wild Solanum species and potato clones. *Frontiers in Plant Science*, *6*, 718. https://doi.org/10.3389/fpls.2015.00718

Franceschi, V. R., Krokene, P., Christiansen, E., & Krekling, T. (2005). Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytologist*, *167*(2), 353–376. https://doi.org/10.1111/j.1469-8137.2005.01436.x

Frankel, S. J., & Palmieri, K. (2014). Sudden Oak Death, Phytophthora ramorum: a Persistent Threat to Oaks and Other Tree Species. *International Oaks*, *25*, 43–56.

Frederickson-Matika, D., Riddell, C., Armstrong, A., Hendry, S., & Green, S. (2019). *Validation of wind vane traps for investigating the dispersal potential of P. ramorum. Final report to Scottish Forestry, November 2020*.

Fry, W. (2008). Plant diseases that changed the world- Phytophthora infestans: The plant (and R gene) destroyer. *Molecular Plant Pathology*, *9*(3), 385–402. https://doi.org/10.1111/j.1364-3703.2007.00465.x

Fry, W., Birch, P. R. J., Judelson, H. S., Grünwald, N. J., Danies, G., Everts, K. L., Gevens, A. J., Gugino, B. K., Johnson, D. A., Johnson, S. B., McGrath, M. T., Myers, K. L., Ristaino, J. B., Roberts, P. D., Secor, G., & Smart, C. D. (2015). Five reasons to consider phytophthora infestans a reemerging pathogen. *Phytopathology*, *105*(7), 966–981. https://doi.org/10.1094/PHYTO-01-15-0005-FI

Gao, L., & Bradeen, J. M. (2016). Contrasting Potato Foliage and Tuber Defense Mechanisms against the Late Blight Pathogen Phytophthora infestans. *PLOS ONE*, *11*(7), e0159969. https://doi.org/10.1371/journal.pone.0159969

Gao, L., Tu, Z. J., Millett, B. P., & Bradeen, J. M. (2013). Insights into organ-specific pathogen defense responses in plants: RNA-seq analysis of potato tuber-Phytophthora infestans interactions. *BMC Genomics*, *14*(1), 340. https://doi.org/10.1186/1471-2164-14-340

Garbelotto, M., & Hayden, K. J. (2012). Sudden oak death: Interactions of the exotic oomycete phytophthora ramorum with naïve North American hosts. *Eukaryotic Cell*, *11*(11), 1313–1323. https://doi.org/10.1128/EC.00195-12

Garbelotto, M., Schmidt, D., Swain, S., Hayden, K., & Lione, G. (2017). The ecology of infection between a transmissive and a dead-end host provides clues for the treatment of a plant disease. *Ecosphere*, *8*(5). https://doi.org/10.1002/ecs2.1815

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology*, *43*, 205–227. https://doi.org/10.1146/annurev.phyto.43.040204.135923

González, A. M., Marcel, T. C., & Niks, R. E. (2012). Genetics and Resistance Evidence for a Minor Gene-for-Minor Gene Interaction Explaining Nonhypersensitive Polygenic Partial Disease Resistance. *Phytopathology*, *102*(11), 1086–1093. https://doi.org/10.1094/PHYTO-03-12-0056-R

Goss, E. M., Carbone, I., & Grünwald, N. J. (2009). Ancient isolation and independent evolution of the three clonal lineages of the exotic sudden oak death pathogen Phytophthora ramorum. *Molecular Ecology*, *18*(6), 1161–1174. https://doi.org/10.1111/j.1365-294X.2009.04089.x

Goss, E. M., Larsen, M., Vercauteren, A., Werres, S., Heungens, K., & Grünwald, N. J. (2010). Population Biology Phytophthora ramorum in Canada: Evidence for Migration Within North America and from Europe. *Phytopathology*, *101*(1), 166–171. https://doi.org/10.1094/PHYTO-05-10-0133

Gouveia, B. C., Calil, I. P., Machado, J. P. B., Santos, A. A., & Fontes, E. P. B. (2017). Immune Receptors and Co-receptors in Antiviral Innate Immunity in Plants. *Frontiers in Microbiology*, *7*,

2139. https://doi.org/10.3389/fmicb.2016.02139

Grattapaglia, D. (2014). Breeding forest trees by genomic selection: Current progress and the way forward. In Tuberosa R., Graner A., & Frison E. (Eds.), *Genomics of Plant Genetic Resources: Volume 1. Managing, Sequencing and Mining Genetic Resources* (pp. 651–682). Springer Netherlands. https://doi.org/10.1007/978-94-007-7572-5_26

Green, S., Hendry, S., Riddell, C., & Armstrong, A. (2019). *Investigation into the use of wind vane traps for detection of P. ramorum. Final report to Forestry Commission Scotland, January 2019*.

Green, S., James, E. R., Clark, D., Clarke, T. K., & Riddell, C. E. (2020). Evidence for natural resistance in Juniperus communis to Phytophthora austrocedri. *Journal of Plant Pathology*, *103*(1), 55–59. https://doi.org/10.1007/s42161-020-00693-1

Green, S., Lee, S., Sykes, R., & Clark, D. (2016). Establishment of a clonal/progeny trial of Japanese larch trees growing in the Galloway district showing putative tolerance to P. ramorum. In *Final Report to Forestry Commission Scotland, April 2016*.

Gros-Louis, M. C., Bousquet, J., Pâques, L. E., & Isabel, N. (2005). Species-diagnostic markers in Larix spp. based on RAPDs and nuclear, cpDNA, and mtDNA gene sequences, and their phylogenetic implications. *Tree Genetics and Genomes*, *1*(2), 50–63. https://doi.org/10.1007/s11295-005-0007-z

Grünwald, N. J., Goss, E. M., Larsen, M. M., Press, C. M., McDonald, V. T., Blomquist, C. L., & Thomas, S. L. (2008). First Report of the European Lineage of Phytophthora ramorum on Viburnum and Osmanthus spp. in a California Nursery . *Plant Disease*, *92*(2), 314–314. https://doi.org/10.1094/pdis-92-2-0314b

Grünwald, N. J., Goss, E. M., & Press, C. M. (2008). Phytophthora ramorum: A pathogen with a remarkably wide host range causing sudden oak death on oaks and ramorum blight on woody ornamentals. *Molecular Plant Pathology*, *9*(6), 729–740. http://genome.jgi-psf.org/Phyra1_1/

Grünwald, N. J., Larsen, M. M., Kamvar, Z. N., Reeser, P. W., Kanaskie, A., Laine, J., & Wiese, R. (2016). First report of the EU1 clonal lineage of Phytophthora ramorum on tanoak in an oregon forest. *Plant Disease*, *100*(5), 1024. https://doi.org/10.1094/PDIS-10-15-1169-PDN

Gu, B., Cao, X., Zhou, X., Chen, Z., Wang, Q., Liu, W., Chen, Q., & Zhao, H. (2020). The Histological, Effectoromic, and Transcriptomic Analyses of Solanum pinnatisectum Reveal an Upregulation of Multiple NBS-LRR Genes Suppressing Phytophthora infestans Infection. *International Journal of Molecular Sciences*, *21*(9), 3211. https://doi.org/10.3390/ijms21093211

Gullner, G., Komives, T., Király, L., & Schröder, P. (2018). Glutathione S-transferase enzymes in plant-pathogen interactions. *Frontiers in Plant Science*, *9*(1836). https://doi.org/10.3389/fpls.2018.01836

Hamberger, B., Toshiyuki, O., Hamberger, B., Séguin, A., & Bohlmann, J. (2011). Evolution of Diterpene Metabolism: Sitka Spruce CYP720B4 Catalyzes Multiple Oxidations in Resin Acid Biosynthesis of Conifer Defense against Insects. *Plant Physiology*, *157*(4), 1677–1695. https://doi.org/10.1104/pp.111.185843

Hamid, R., Khan, M. A., Ahmad, M., Ahmad, M. M., Abdin, M. Z., Musarrat, J., & Javed, S. (2013). Chitinases: An update. *Journal of Pharmacy and Bioallied Sciences*, *5*(1), 21–29. https://doi.org/10.4103/0975-7406.106559

Han, Y., Teng, W., Yu, K., Poysa, V., Anderson, T., Qiu, L., Lightfoot, D. A., & Li, W. (2008). Mapping QTL tolerance to Phytophthora root rot in soybean using microsatellite and RAPD/SCAR derived

markers. *Euphytica*, *162*(2), 231–239. https://doi.org/10.1007/s10681-007-9558-4

Hanbo, C. (2018). *VennDiagram: Generate High-Resolution Venn and Euler Plots* (1.6.20). https://cran.r-project.org/package=VennDiagram

Hansen, E. M., Hamm, P. B., & Roth, L. F. (1989). Testing Port-Orford-Cedar for resistance to Phytophthora . *Plant Disease* , *73*, 791–794. https://www.apsnet.org/publications/PlantDisease/BackIssues/Documents/1989Articles/PlantDisease73n10_791.PDF

Hansen, E. M., Kanaskie, A., Prospero, S., McWilliams, M., Goheen, E. M., Osterbauer, N., Reeser, P., & Sutton, W. (2008). Epidemiology of Phytophthora ramorum in Oregon tanoak forests. *Canadian Journal of Forest Research*, *38*(5), 1133–1143. https://doi.org/10.1139/X07-217

Hansen, E. M., Parke, J. L., & Sutton, W. (2005). Susceptibility of Oregon Forest Trees and Shrubs to Phytophthora ramorum: A Comparison of Artificial Inoculation and Natural Infection. *Plant Disease*, *89*(1), 63–70. https://doi.org/10.1094/PD-89-0063

Hardham, A. R., & Blackman, L. M. (2018). Phytophthora cinnamomi. *Molecular Plant Pathology*, *19*(2), 260–285. https://doi.org/10.1111/mpp.12568

Harding, T. (1998). *Forestry Commission Bulletin: British softwoods: Properties and uses*. https://www.forestry.gov.uk/PDF/FCBU077.pdf/$FILE/FCBU077.pdf

Harris, A. R. (2014). *The epidemiology of Phytophthora ramorum on Larix in the UK* [Imperial College London]. https://spiral.imperial.ac.uk/handle/10044/1/24862

Harris, A. R., Brasier, C. M., Scanu, B., & Webber, J. F. (2021). Fitness characteristics of the European lineages of Phytophthora ramorum. *Plant Pathology*, *70*(2), 275–286. https://doi.org/10.1111/ppa.13292

Harris, A. R., Mullett, M. S., & Webber, J. F. (2018). Changes in the population structure and sporulation behaviour of Phytophthora ramorum associated with the epidemic on Larix (larch) in Britain. *Biological Invasions*, *20*(9), 2313–2328. https://doi.org/10.1007/s10530-018-1702-7

Harris, A. R., Scanu, B., & Webber, J. F. (2014). Comparative fitness of European lineages of Phytophthora ramorum. *Proceedings of the 7th Meeting of the International Union of Forest Research Organizations IUFRO Working Party*, 135.

Harris, A. R., & Webber, J. F. (2016). Sporulation potential, symptom expression and detection of Phytophthora ramorum on larch needles and other foliar hosts. *Plant Pathology*, *65*(9), 1441–1451. https://doi.org/10.1111/ppa.12538

Harris, A. R., & Webber, J. F. (2012). The New Phytophthora ramorum Dynamic in Europe: Spread to Larch. In S. J. Frankel, J. T. Kliejunas, K. M. Palmieri, & J. M. Alexander (Eds.), *Proceedings of the Sudden Oak Death Fifth Science Symposium. Gen. Tech. Rep. PSW-GTR-243.* U.S. Department of Agriculture: Forest Service. https://doi.org/https://doi.org/10.2737/PSW-GTR-243

Harrison, P. A., Vandewalle, M., Sykes, M. T., Berry, P. M., Bugter, R., de Bello, F., Feld, C. K., Grandin, U., Harrington, R., Haslett, J. R., Jongman, R. H. G., Luck, G. W., da Silva, P. M., Moora, M., Settele, J., Sousa, J. P., & Zobel, M. (2010). Identifying and prioritising services in European terrestrial and freshwater ecosystems. *Biodiversity and Conservation*, *19*(10), 2791–2821. https://doi.org/10.1007/s10531-010-9789-x

Hayden, K. J., Garbelotto, M., Knaus, B. J., Cronn, R. C., Rai, H., & Wright, J. W. (2014). Dual RNA-seq of the plant pathogen Phytophthora ramorum and its tanoak host. *Tree Genetics and Genomes*, *10*(3), 489–502. https://doi.org/10.1007/s11295-014-0698-0

Hebard, F. (2005). The backcross breeding program of the American Chestnut Foundation. *Journal of the American Chestnut Foundation*, *19*(2), 55–78.

Herrero, M. L., Toppe, B., & Brurberg, M. B. (2011). First report of phytophthora ramorum causing shoot dieback on bilberry (Vaccinium myrtillus) in Norway. *Plant Disease*, *95*(3), 355. https://doi.org/10.1094/PDIS-10-10-0709

Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous Inference in General Parametric Models. *Biometrical Journal*, *50*(3), 346–363. https://doi.org/10.1002/bimj.200810425

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009a). Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Research*, *37*(1), 1–13. https://doi.org/10.1093/nar/gkn923

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*, *4*(1), 44–57. https://doi.org/10.1038/nprot.2008.211

Hüberli, D., Hayden, K. J., Calver, M., & Garbelotto, M. (2012). Intraspecific variation in host susceptibility and climatic factors mediate epidemics of sudden oak death in western US forests. *Plant Pathology*, *61*(3), 579–592. https://doi.org/10.1111/j.1365-3059.2011.02535.x

Hudgins, J. W., Christiansen, E., & Franceschi, V. R. (2004). Induction of anatomically based defense responses in stems of diverse conifers by methyl jasmonate: a phylogenetic perspective. *Tree Physiology*, *24*, 251–264. https://doi.org/10.1093/treephys/24.3.251

Ingvarsson, P. K., & Dahlberg, H. (2019). The effects of clonal forestry on genetic diversity in wild and domesticated stands of forest trees. *Scandinavian Journal of Forest Research*, *34*(5), 370–379. https://doi.org/10.1080/02827581.2018.1469665

Isik, F., Kumar, S., Martínez-García, P. J., Iwata, H., & Yamamoto, T. (2015). Acceleration of Forest and Fruit Tree Domestication by Genomic Selection. In C. Plomion & A.-F. Adam-Blondonanne (Eds.), *Advances in Botanical Research* (Vol. 74, Issue March 2016, pp. 93–124). Elsevier. https://doi.org/10.1016/bs.abr.2015.05.002

Ivors, K., Garbelotto, M., Vries, I. D. E. E., Ruyter-Spira, C., Hekkert, B. T., Rosenzweig, N., & Bonants, P. (2006). Microsatellite markers identify three lineages of Phytophthora ramorum in US nurseries, yet single lineages in US forest and European nursery populations. *Molecular Ecology*, *15*(6), 1493–1505. https://doi.org/10.1111/j.1365-294X.2006.02864.x

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature*, *444*(7117), 323–329. https://doi.org/10.1038/nature05286

Judelson, H. S., & Blanco, F. A. (2005). The spores of phytophthora: weapons of the plant destroyer. *Nature Reviews Microbiology*, *3*, 47–58. https://www.nature.com/articles/nrmicro1064.pdf?proof=t

Jung, T., Horta Jung, M., Webber, J. F., Kageyama, K., Hieno, A., Masuya, H., Uematsu, S., Pérez-Sierra, A., Harris, A. R., Forster, J., Rees, H., Scanu, B., Patra, S., Kudláček, T., Janoušek, J., Corcobado, T., Milenković, I., Nagy, Z., Csorba, I., … Brasier, C. M. (2021). The Destructive Tree Pathogen Phytophthora ramorum Originates from the Laurosilva Forests of East Asia. *Journal of Fungi*, *7*(3), 226. https://doi.org/10.3390/jof7030226

Jung, T., Scanu, B., Brasier, C. M., Webber, J. F., Milenković, I., Corcobado, T., Tomšovský, M., Pánek, M., Bakonyi, J., Maia, C., Bačová, A., Raco, M., Rees, H., Pérez-Sierra, A., & Horta Jung, M. (2020). A Survey in Natural Forest Ecosystems of Vietnam Reveals High Diversity of both New

and Described Phytophthora Taxa including P. ramorum. *Forests*, *11*(1), 93. https://doi.org/10.3390/f11010093

Kamber, T., Buchmann, J. P., Pothier, J. F., Smits, T. H. M., Wicker, T., & Duffy, B. (2016). Fire blight disease reactome: RNA-seq transcriptional profile of apple host plant defense responses to Erwinia amylovora pathogen infection. *Scientific Reports*, *6*(1), 1–12. https://doi.org/10.1038/srep21600

Kanehisa, M., & Goto, S. (2000). KEGG: Kyoto Encyclopedia of Genes and Genomes. In *Nucleic Acids Research* (Vol. 28, Issue 1, pp. 27–30). Oxford University Press. https://doi.org/10.1093/nar/28.1.27

Kaul, S., Koo, H. L., Jenkins, J., Rizzo, M., Rooney, T., Tallon, L. J., Feldblyum, T., Nierman, W., Benito, M. I., Lin, X., Town, C. D., Venter, J. C., Fraser, C. M., Tabata, S., Nakamura, Y., Kaneko, T., Sato, S., Asamizu, E., Kato, T., … Somerville, C. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature*, *408*(6814), 796–815. https://doi.org/10.1038/35048692

Keeling, C. I., Weisshaar, S., Lin, R. P. C., & Bohlmann, J. (2008). Functional plasticity of paralogous diterpene synthases involved in conifer defense. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(3), 1085–1089. https://doi.org/10.1073/pnas.0709466105

King, K. M., Harris, A. R., & Webber, J. F. (2015). In planta detection used to define the distribution of the European lineages of Phytophthora ramorum on larch (Larix) in the UK. *Plant Pathology*, *64*(5), 1168–1175. https://doi.org/10.1111/ppa.12345

Kluza, D. A., Vieglais, D. A., Andreasen, J. K., & Peterson, A. T. (2007). Sudden oak death: Geographic risk estimates and predictions of origins. *Plant Pathology*, *56*(4), 580–587. https://doi.org/10.1111/j.1365-3059.2007.01602.x

Kobayashi, T. (1970). An Evidence that the Larch Canker Fungus is Native in Japan. *Journal of Phytopathology*, *69*(4), 366–368. https://doi.org/10.1111/j.1439-0434.1970.tb03120.x

Kolosova, N., & Bohlmann, J. (2012). *Conifer Defense Against Insects and Fungal Pathogens* (pp. 85–109). Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-30645-7_4

Kozanitas, M., Osmundson, T. W., Linzer, R., & Garbelotto, M. (2017). Interspecific interactions between the Sudden Oak Death pathogen Phytophthora ramorum and two sympatric Phytophthora species in varying ecological conditions. *Fungal Ecology*, *28*, 86–96. https://doi.org/10.1016/J.FUNECO.2017.04.006

Kulej, M. (2006). Resistance of larches of Polish provenances to larch canker (lachnellula willkommii (hartig.) Dennis) under mountain conditions of the Sacz Beskid range. *Electronic Journal of Polish Agricultural Universities*, *9*(2). http://www.ejpau.media.pl/volume9/issue2/art-29.html

Kushalappa, A. C., Yogendra, K. N., & Karre, S. (2016). Plant Innate Immune Response: Qualitative and Quantitative Resistance. *Critical Reviews in Plant Sciences*, *35*(1), 35–55. https://doi.org/10.1080/07352689.2016.1148980

Kusumoto, N., Zhao, T., Swedjemark, G., Ashitani, T., Takahashi, K., & Borg-Karlson, A.-K. (2014). Antifungal properties of terpenoids in *Picea abies* against *Heterobasidion parviporum*. *Forest Pathology*, *44*(5), 353–361. https://doi.org/10.1111/efp.12106

Lämke, J., & Bäurle, I. (2017). Epigenetic and chromatin-based mechanisms in environmental stress adaptation and stress memory in plants. *Genome Biology 2017 18:1*, *18*(1), 1–11.

https://doi.org/10.1186/S13059-017-1263-6

Lamour, Kurt (Ed.). (2013). *Phytophthora: A Global Perspective*. CAB International.

Lane, C. R., Beales, P. A., Hughes, K. J. D., Griffin, R. L., Munro, D., Brasier, C. M., & Webber, J. F. (2003). First outbreak of Phytophthora ramorum in England, on Viburnum tinus. *Plant Pathology*, *52*(3), 414. https://doi.org/10.1046/j.1365-3059.2003.00835.x

LeBoldus, J. M., Sondreli, K., Sutton, W., Reeser, P. W., Kanaskie, A., Navarro, S., & Grünwald, N. J. (2017). First Report of Phytophthora ramorum lineage EU1 infecting Douglas-fir and grand fir in Oregon. *Plant Disease*, *2*. https://doi.org/10.1094/PDIS-05-17-0681-PDN

Lee, S. (2003). *Breeding Hybrid Larch in Britain*. https://www.forestresearch.gov.uk/documents/6748/FCIN052.pdf

Leitch, I., Johnston, E., Pellicer, J., Hidalgo, O., & Bennett, M. (2019, April). *Plant DNA C-values Database -Royal Botanic Gardens, Kew*. https://cvalues.science.kew.org/

Lenth, R. (2020). emmeans: Estimated Marginal Means, aka Least-Squares Means. In *The American Statistician* (R package version 1.4.7; Vol. 34, Issue 4). https://doi.org/10.1080/00031305.1980.10483031

Liljegren, S. (2010). Phloroglucinol stain for lignin. *Cold Spring Harbor Protocols*, *5*(1), pdb.prot4954. https://doi.org/10.1101/pdb.prot4954

Lindhout, P. (2002). The perspectives of polygenic resistance in breeding for durable disease resistance. *Euphytica*, *124*(2), 217–226. https://doi.org/10.1023/A:1015686601404

Lines, R. (1987). Choice of Seed Origins for the Main Forest Species in Britain. *Forestry Commission Bulletin*, *66*.

Lines, R., & Mitchell, A. F. (1989). Provenance report. In *Report on forest research 1989*.

Liu, J.-J., Sturrock, R. N., & Benton, R. (2013). Transcriptome analysis of Pinus monticola primary needles by RNA-seq provides novel insight into host resistance to Cronartium ribicola. *BMC Genomics*, *14*(1), 884. https://doi.org/10.1186/1471-2164-14-884

Living Ash Project. (2020). https://livingashproject.org.uk/

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12). https://doi.org/10.1186/s13059-014-0550-8

Ma, Z., Zhu, L., Song, T., Wang, Y., Zhang, Q., Xia, Y., Qiu, M., Lin, Y., Li, H., Kong, L., Fang, Y., Ye, W., Wang, Y., Dong, S., Zheng, X., Tyler, B. M., & Wang, Y. (2017). A paralogous decoy protects Phytophthora sojae apoplastic effector PsXEG1 from a host inhibitor. *Science*, *355*(6326), 710–714. http://science.sciencemag.org/content/355/6326/710.full

Mackay, J. J., Dean, J. F. D., Plomion, C., Peterson, D. G., Cánovas, F. M., Pavy, N., Ingvarsson, P. K., Savolainen, O., Guevara, M. Á., Fluch, S., Vinceti, B., Abarca, D., Díaz-Sala, C., & Cervera, M.-T. (2012). Towards decoding the conifer giga-genome. *Plant Molecular Biology*, *80*(6), 555–569. https://doi.org/10.1007/s11103-012-9961-7

Mackay, J. J., Prunier, J., Verta, J.-P. P., & Mackay, J. J. (2016). Tansley review Conifer genomics and adaptation: at the crossroads of genetic diversity and genome function. *New Phytologist*, *209*, 44–62. https://doi.org/10.1111/nph.13565

Marone, D., Russo, M. A., Laidò, G., De Leonardis, A. M., & Mastrangelo, A. M. (2013). Plant

nucleotide binding site-leucine-rich repeat (NBS-LRR) genes: Active guardians in host defense responses. *International Journal of Molecular Sciences*, *14*(4), 7302–7326. https://doi.org/10.3390/ijms14047302

Martin, F., & Kamoun, S. (2011). *Effectors in Plant-Microbe Interactions.* (F. Martin & S. Kamoun (Eds.)). Wiley-Blackwell.

Martin, J. A., & Wang, Z. (2011). Next-generation transcriptome assembly. *Nature Reviews Genetics*, *12*(10), 671–682. https://doi.org/10.1038/nrg3068

McKinney, L. V., Nielsen, L. R., Collinge, D. B., Thomsen, I. M., Hansen, J. K., & Kjaer, E. D. (2014). The ash dieback crisis: genetic variation in resistance can prove a long-term solution. *Plant Pathology*, *63*(3), 485–499. https://doi.org/10.1111/ppa.12196

Meijer, H. J. G., Van De Vondervoort, P. J. I., Qing, Y. Y., De Koster, C. G., Klis, F. M., Govers, F., & De Groot, P. W. J. (2006). Identification of cell wall-associated proteins from Phytophthora ramorum. *Molecular Plant-Microbe Interactions*, *19*(12), 1348–1358. https://doi.org/10.1094/MPMI-19-1348

Merwe, M. Van Der, Winfield, M. O., Arnold, G. M., & Parker, J. S. (2000). Spatial and temporal aspects of the genetic structure of Juniperus communis populations. *Molecular Ecology*, *9*(4), 379–386. https://doi.org/10.1046/j.1365-294x.2000.00868.x

Morgenstern, E. (1996). *Geographic variation in forest trees: genetic basis and application of knowledge in silviculture.* University of British Columbia Press. https://www.cabi.org/ISC/abstract/19980604261

Morse, A. M., Peterson, D. G., Islam-Faridi, M. N., Smith, K. E., Magbanua, Z., Garcia, S. A., Kubisiak, T. L., Amerson, H. V., Carlson, J. E., Nelson, C. D., & Davis, J. M. (2009). Evolution of Genome Size and Complexity in Pinus. *PLoS ONE*, *4*(2), e4332. https://doi.org/10.1371/journal.pone.0004332

Nagle, A. M., McPherson, B. A., Wood, D. L., Garbelotto, M., & Bonello, P. (2011). Relationship between field resistance to Phytophthora ramorum and constitutive phenolic chemistry of coast live oak. *Forest Pathology*, *41*(6), 464–469. https://doi.org/10.1111/j.1439-0329.2010.00703.x

Nagy, N. E., Fossdal, C. G., Krokene, P., Krekling, T., Lonneborg, A., & Solheim, H. (2004). Induced responses to pathogen infection in Norway spruce phloem: changes in polyphenolic parenchyma cells, chalcone synthase transcript levels and peroxidase activity. *Tree Physiology*, *24*(5), 505–515. https://doi.org/10.1093/treephys/24.5.505

National Forest Inventory-Forestry Commission. (2014). *NFI Statistical Analysis Report 50-year forecast of softwood timber availability Overview of the 50-year forecast of softwood timber availability*. https://www.forestresearch.gov.uk/documents/2744/50_YEAR_FORECAST_OF_SOFTWOOD_AVAILABILITY.pdf

NHS Forest. (2021). *Welcome | NHS Forest*. https://nhsforest.org/

Nilsson, K., Sangster, M., & Konijnendijk, C. C. (2011). Forests, trees and human health and well-being: Introduction. In *Forests, Trees and Human Health* (pp. 1–19). Springer Netherlands. https://doi.org/10.1007/978-90-481-9806-1_1

O'brien, L., Valatin, G., & Bursnell, M. (2020). *Scoping Study on Valuing Mental Health Benefits of Forests* . https://doi.org/10.13140/RG.2.2.13173.60645

O'Hanlon, R., Choiseul, J., Grogan, H., & Brennan, J. M. (2017). In-vitro characterisation of the four lineages of Phytophthora ramorum. *European Journal of Plant Pathology*, *147*(3), 517–525. https://doi.org/10.1007/s10658-016-1019-2

Ockels, F. S., Eyles, A., McPherson, B. A., Wood, D. L., & Bonello, P. (2007). Phenolic chemistry of coast live oak response to Phytophthora ramorum infection. *Journal of Chemical Ecology*, *33*(9), 1721–1732. https://doi.org/10.1007/s10886-007-9332-z

Office for National Statistics. (2020). Woodland natural capital accounts, UK. In *Statistical Bulletin* (Issue March). https://www.ons.gov.uk/economy/environmentalaccounts/bulletins/woodlandnaturalcapitalaccountsuk/2020#cultural-ecosystem-services-quantity-and-value

Official Journal of the European Union. (2016). *Commission Implementing Decision (EU) 2016/1967 of 8 November 2016 amending Article 3 of Decision 2002/757/EC on provisional emergency phytosanitary measures to prevent the introduction into and the spread within the Community of Phytophthora ramorum Werr*. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32016D1967

Oßwald, W., Fleischmann, F., Rigling, D., Coelho, A. C., Cravador, A., Diez, J., Dalio, R. J., Horta Jung, M., Pfanz, H., Robin, C., Sipos, G., Solla, A., Cech, T., Chambery, A., Diamandis, S., Hansen, E., Jung, T., Orlikowski, L. B., Parke, J., … Werres, S. (2014). Strategies of attack and defence in woody plant-Phytophthora interactions. *Forest Pathology*, *44*(3), 169–190. https://doi.org/10.1111/efp.12096

Oven, P., & Torelli, N. (1999). Response of the cambial zone in conifers to wounding. *Phyton; Annales Rei Botanicae*, *39*(3), 134–137.

Pagan, I., & Garcia-Arenal, F. (2020). Tolerance of Plants to Pathogens: A Unifying View. *Annual Review of Phytopathology*, *58*, 77–96. https://doi.org/10.1146/annurev-phyto-010820-012749

Palloix, A., Ayme, V., & Moury, B. (2009). Durability of plant major resistance genes to pathogens depends on the genetic background, experimental evidence and consequences for breeding strategies. *New Phytologist*, *183*(1), 190–199. https://doi.org/10.1111/j.1469-8137.2009.02827.x

Parke, J. L., Oh, E., Voelker, S., Hansen, E. M., Buckles, G., & Lachenbruch, B. (2007). Phytophthora ramorum Colonizes Tanoak Xylem and Is Associated with Reduced Stem Water Transport. *Ecology and Epidemiology*, *97*(12), 1558–1567. https://doi.org/10.1094/PHYTO-97-12-1558

Parliamentary Office of Science and Technology. (2007). *POST NOTE- UK Trees and Forests- facts and figures* (No. 275). http://www.parliament.uk/documents/post/postpn275.pdf

Perez-Sierra, A., & Jung, T. (2013). Phytophthora in woody ornamental nurseries. In K. Lamour (Ed.), *Phytophthora: A Global Perspective* (pp. 166–178). CAB International.

Perry, A., Wachowiak, W., Brown, A. V., Ennos, R. A., Cottrell, J. E., & Cavers, S. (2016). Substantial heritable variation for susceptibility to *Dothistroma septosporum* within populations of native British Scots pine (*Pinus sylvestris*). *Plant Pathology*, *65*(6), 987–996. https://doi.org/10.1111/ppa.12528

Plumb, W. J., R Coker, T. L., Stocks, J. J., Woodcock, P., Quine, C. P., Nemesio-Gorriz, M., Douglas, G. C., Kelly, L. J., A Buggs, R. J., & Richard A Buggs, C. J. (2020). *The viability of a breeding programme for ash in the British Isles in the face of ash dieback Societal Impact Statement*. *2*, 29–40. https://doi.org/10.1002/ppp3.10060

Poland, J. A., Balint-Kurti, P. J., Wisser, R. J., Pratt, R. C., & Nelson, R. J. (2009). Shades of gray: the world of quantitative disease resistance. *Trends in Plant Science*, *14*(1), 21–29. https://doi.org/10.1016/j.tplants.2008.10.006

Potter, C., & Urquhart, J. (2017). Tree disease and pest epidemics in the Anthropocene: A review of the drivers, impacts and policy responses in the UK. *Forest Policy and Economics*, *79*, 61–68. https://doi.org/10.1016/j.forpol.2016.06.024

Powers, H. R., & Kraus, J. F. (1983). Developing fusiform rust-resistant loblolly and slash pines. *Plant Disease*, *67*(2), 187–189. http://cat.inist.fr/?aModele=afficheN&cpsidt=9324386

Press, C. M., Fieland, V. J., Creswell, T., Bonkowski, J., Miles, L., & Grünwald, N. J. (2020). First Report of the NA2 Clonal Lineage of Phytophthora ramorum in Indiana. *Plant Disease*, *104*(6), 1875. https://doi.org/10.1094/pdis-12-19-2543-pdn

Quesada, T., Gopal, V., Cumbie, W. P., Eckert, A. J., Wegrzyn, J. L., Neale, D. B., Goldfarb, B., Huber, D. A., Casella, G., & Davis, J. M. (2010). Association mapping of quantitative disease resistance in a natural population of loblolly pine (Pinus taeda L.). *Genetics*, *186*(2), 677–686. https://doi.org/10.1534/genetics.110.117549

R Core Team. (2020). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.r-project.org/

Raffaele, S., Farrer, R. A., Cano, L. M., Studholme, D. J., MacLean, D., Thines, M., Jiang, R. H. Y., Zody, M. C., Kunjeti, S. G., Donofrio, N. M., Meyers, B. C., Nusbaum, C., & Kamoun, S. (2010). Genome Evolution Following Host Jumps in the Irish Potato Famine Pathogen Lineage. *Science*, *330*(6010). http://science.sciencemag.org/content/330/6010/1540.full

Raum, S. (2020). Land-use legacies of twentieth-century forestry in the UK: a perspective. *Landscape Ecology*, *35*(12), 2713–2722. https://doi.org/10.1007/s10980-020-01126-1

Richard, S., Lapointe, G., Rutledge, R. G., & Séguin, A. (2000). Induction of Chalcone Synthase Expression in White Spruce by Wounding and Jasmonate. *Plant and Cell Physiology*, *41*(8), 982–987. https://doi.org/10.1093/pcp/pcd017

Rizzo, D. M., Garbelotto, M., Davidson, J. M., Slaughter, G. W., & Koike, S. T. (2002). *Phytophthora ramorum* as the Cause of Extensive Mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. *Plant Disease*, *86*(3), 205–214. https://doi.org/10.1094/PDIS.2002.86.3.205

Rizzo, D. M., Garbelotto, M., & Hansen, E. M. (2005). Phytophthora ramorum: Integrative Research and Management of an Emerging Pathogen in California and Oregon Forests. *Annual Review of Phytopathology*, *43*(1), 309–335. https://doi.org/10.1146/annurev.phyto.42.040803.140418

Rojas, C. M., Senthil-Kumar, M., Tzin, V., & Mysore, K. S. (2014). Regulation of primary plant metabolism during plant-pathogen interactions and its contribution to plant defense. *Frontiers in Plant Science*, *5*(17). https://doi.org/10.3389/fpls.2014.00017

Routaboul, J.-M., Dubos, C., Beck, G., Marquis, C., Bidzinski, P., Loudet, O., & Lepiniec, L. (2012). Metabolite profiling and quantitative genetics of natural variation for flavonoids in Arabidopsis. *Journal of Experimental Botany*, *63*(10), 3749–3764. https://doi.org/10.1093/jxb/ers067

RStudio Team. (2020). *RStudio: Integrated Development for R.* https://rstudio.com/

Schenck, N., Saurat, C., Guinet, C., Fourrier-Jeandel, C., Roche, L., Bouvet, A., Husson, C., Saintonge, F. X., Contal, C., & Ioos, R. (2018). First report of phytophthora ramorum causing Japanese larch dieback in France. *Plant Disease*, *102*(10), 2045. https://doi.org/10.1094/PDIS-02-18-0288-PDN

Schlarbaum, S. E., Hebard, F., Spaine, P. C., & Kamalay, J. C. (1998). Three American tragedies: chestnut blight, butternut canker, and Dutch elm disease. In K. O. Britton (Ed.), *Exotic pests of eastern forests conference proceedings* (pp. 45–54). U.S. Forest Service and Tennessee Exotic Pest Plant Council.

Scottish Forestry - Phytophthora ramorum in Scotland. (2020). https://forestry.gov.scot/sustainable-forestry/tree-health/tree-pests-and-diseases/phytophthora-ramorum

Scottish Government-Environment and Forestry Directorate. (2015). *Phytophthora ramorum and Phytophthora kernoviae in Scotland: status report*. https://www.gov.scot/publications/phytophthora-ramorum-phytophthora-kernoviae-scotland-status-report/pages/4/

Semerikov, V. L., Zhang, H., Sun, M., & Lascoux, M. (2003). Conflicting phylogenies of Larix (Pinaceae) based on cytoplasmic and nuclear DNA. *Molecular Phylogenetics and Evolution*, *27*(2), 173–184. https://doi.org/10.1016/S1055-7903(02)00447-5

Singh, B., & Sharma, R. A. (2015). Plant terpenes: defense responses, phylogenetic analysis, regulation and clinical applications. *3 Biotech*, *5*(2), 129–151. https://doi.org/10.1007/s13205-014-0220-2

Siviero, A., Cristofani, M., Furtado, E. L., Garcia, A. A. F., Coelho, A. S. G., & Machado, M. A. (2006). Identification of QTLs associated with citrus resistance to Phytophthora gummosis. *Journal of Applied Genetics*, *47*(1), 23–28. https://doi.org/10.1007/BF03194595

Sketchley, R. (2019). Phytophthora ramorum in larch UK Situation Report. In *Forestry Commision England* (Issue May). https://www.forestry.gov.uk/pdf/PramorumSituationReport31Oct2017.pdf/$FILE/PramorumSituationReport31Oct2017.pdf

Slavicek, J. M., & Knight, K. S. (2012). Generation of American elm trees with tolerance to Dutch elm disease through controlled crosses and selection. In R. A. Sniezko, A. D. Yanchuk, J. T. Kliejunas, K. M. Palmieri, J. M. Alexander, & S. J. Frankel (Eds.), *Proceedings of the 4th International Workshop on Genetics of Host-Parasite Interactions in Forestry. Gen. Tech. Rep. PSW-GTR-240* (pp. 342–346). Pacific Southwest Research Station, Forest Service, U.S. Department of Agriculture.

Slawson, D., Blackburn, J., & Bennett, L. (2007). An Update on Phytophthora ramorum in European Nurseries. *Proceedings of the Sudden Oak Death Third Science Symposium. Gen. Tech. Rep. PSW-GTR-214*, 31–34. https://doi.org/https://doi.org/10.2737/PSW-GTR-214

Slobodník, B. (2002). Pollination success and full seed percentage in European larch (Larix decidua MILL.). *Journal of Forest Science*, *48*(6), 271–280. https://doi.org/10.17221/11884-jfs

Sniezko, R. A. (2006). Resistance breeding against nonnative pathogens in forest trees-current successes in North America. *Canadian Journal of Plant Pathology*, *28*. https://doi.org/10.1080/07060660609507384

Sniezko, R. A., Johnson, J. S., Reeser, P., Kegley, A., Hansen, E. M., Sutton, W., & Savin, D. P. (2020). Genetic resistance to Phytophthora lateralis in Port-Orford-cedar (Chamaecyparis lawsoniana) – Basic building blocks for a resistance program . *Plants, People, Planet*, *2*(1), 69–83. https://doi.org/10.1002/ppp3.10081

Soga, M., Evans, M. J., Tsuchiya, K., & Fukano, Y. (2021). A room with a green view: the importance of nearby nature for mental health during the COVID-19 pandemic. *Ecological Applications*, *31*(2), 1–10. https://doi.org/10.1002/eap.2248

Sollars, E. S. A., Harper, A. L., Kelly, L. J., Sambles, C. M., Ramirez-Gonzalez, R. H., Swarbreck, D., Kaithakottil, G., Cooper, E. D., Uauy, C., Havlickova, L., Worswick, G., Studholme, D. J., Zohren, J., Salmon, D. L., Clavijo, B. J., Li, Y., He, Z., Fellgett, A., Mckinney, L. V., … Buggs, R. J. A. (2017). Genome sequence and genetic diversity of European ash trees. *Nature Publishing Group*, *541*. https://doi.org/10.1038/nature20786

Stocks, J. J., Buggs, R. J. A., & Lee, S. J. (2017). A first assessment of Fraxinus excelsior (common ash) susceptibility to Hymenoscyphus fraxineus (ash dieback) throughout the British Isles. *Scientific Reports*, *7*(1), 1–7. https://doi.org/10.1038/s41598-017-16706-6

Strable, J., Yen, J., Scanlon, M., & Sylvester, A. (2020). Toluidine Blue O Staining of Paraffin-Sectioned Maize Tissue. *Bio-101*, *e3612*. https://doi.org/10.21769/bioprotoc.3612

Sturrock, R. N., Frankel, S. J., Brown, A. V., Hennon, P. E., Kliejunas, J. T., Lewis, K. J., Worrall, J. J., & Woods, A. J. (2011). Climate change and forest diseases. *Plant Pathology*, *60*(1), 133–149. https://doi.org/10.1111/j.1365-3059.2010.02406.x

Suo, B., Chen, Q., Wu, W., Wu, D., Tian, M., Jie, Y., Zhang, B., & Wen, J. (2016). Chemotactic responses of Phytophthora sojae zoospores to amino acids and sugars in root exudates. *Journal of General Plant Pathology*, *82*(3), 142–148. https://doi.org/10.1007/s10327-016-0651-1

Tabbush, P. (2010). *Cultural Values of Trees, Woods and Forests*. https://www.forestresearch.gov.uk/documents/1412/Cultural_value_woods_full_report_March2010.pdf

Tang, Y., Masaaki, H., & Wenxuan, L. (2016). ggfortify: Unified Interface to Visualize Statistical Result of Popular R Packages. *The R Journal*, *8*(2). https://journal.r-project.org/

The Woodland Trust. (2017). *The economic benefits of woodland. A report for the Woodland Trust prepared by Europe Economics.* https://www.woodlandtrust.org.uk/media/1732/economic-benefits-of-woodland.pdf

Tjosvold, S. A., Chambers, D. L., Davidson, J. M., & Rizzo, D. M. (2006). Incidence of Phytophthora ramorum Inoculum Found in Soil Collected from a Hiking Trail and Hikers' Shoes in a California Park. *Sudden Oak Death, Second Science Symposium: The State of Our Knowledge*. http://cesantacruz.ucdavis.edu/files/51736.pdf

Tomlinson, J. A., Boonham, N., Hughes, K. J. D., Griffin, R. L., & Barker, I. (2005). On-site DNA extraction and real-time PCR for detection of Phytophthora ramorum in the field. *Applied and Environmental Microbiology*, *71*(11), 6702–6710. https://doi.org/10.1128/AEM.71.11.6702-6710.2005

Trujillo-Moya, C., Ganthaler, A., Stöggl, W., Kranner, I., Schüler, S., Ertl, R., Schlosser, S., George, J.-P., & Mayr, S. (2020). RNA-Seq and secondary metabolite analyses reveal a putative defence-transcriptome in Norway spruce (Picea abies) against needle bladder rust (Chrysomyxa rhododendri) infection. *BMC Genomics*, *21*(1), 336. https://doi.org/10.1186/s12864-020-6587-z

Václavík, T., Kanaskie, A., Hansen, E. M., Ohmann, J. L., & Meentemeyer, R. K. (2010). Predicting potential and actual distribution of sudden oak death in Oregon: Prioritizing landscape contexts for early detection and eradication of disease outbreaks. *Forest Ecology and Management*, *260*, 1026–1035. https://doi.org/10.1016/j.foreco.2010.06.026

van der Vossen, E., Sikkema, A., Hekkert, B. te L., Gros, J., Stevens, P., Muskens, M., Wouters, D., Pereira, A., Stiekema, W., & Allefs, S. (2003). An ancient *R* gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. *The Plant Journal*, *36*(6), 867–882.

https://doi.org/10.1046/j.1365-313X.2003.01934.x

Van Ghelder, C., Parent, G. J., Rigault, P., Prunier, J., Giguère, I., Caron, S., Stival Sena, J., Deslauriers, A., Bousquet, J., Esmenjaud, D., & Mackay, J. J. (2019). The large repertoire of conifer NLR resistance genes includes drought responsive and highly diversified RNLs. *Scientific Reports*, *9*(1), 1–13. https://doi.org/10.1038/s41598-019-47950-7

van Loon, L. C., Rep, M., & Pieterse, C. M. J. (2006). Significance of Inducible Defense-related Proteins in Infected Plants. *Annual Review of Phytopathology*, *44*(1), 135–162. https://doi.org/10.1146/annurev.phyto.44.070505.143425

Van Poucke, K., Franceschini, S., Webber, J. F., Vercauteren, A., Turner, J. A., McCracken, A. R., Heungens, K., & Brasier, C. M. (2012). Discovery of a fourth evolutionary lineage of Phytophthora ramorum: EU2. *Fungal Biology*, *116*(11), 1178–1191. https://doi.org/10.1016/j.funbio.2012.09.003

Vercauteren, A., De Dobbelaere, I., Grünwald, N. J., Bonants, P., Van Bockstaele, E., Maes, M., & Heungens, K. (2010). Clonal expansion of the Belgian Phytophthora ramorum populations based on new microsatellite markers. *Molecular Ecology*, *19*(1), 92–107. https://doi.org/10.1111/j.1365-294X.2009.04443.x

Warren, R. L., Keeling, C. I., Yuen, M. M. Saint, Raymond, A., Taylor, G. A., Vandervalk, B. P., Mohamadi, H., Paulino, D., Chiu, R., Jackman, S. D., Robertson, G., Yang, C., Boyle, B., Hoffmann, M., Weigel, D., Nelson, D. R., Ritland, C., Isabel, N., Jaquish, B., … Bohlmann, J. (2015). Improved white spruce (Picea glauca) genome assemblies and annotation of large gene families of conifer terpenoid and phenolic defense metabolism. *Plant Journal*, *83*(2), 189–212. https://doi.org/10.1111/tpj.12886

Wasternack, C., & Parthier, B. (1997). Jasmonate-signalled plant gene expression. *Trends in Plant Science*, *2*(8), 302–307. https://doi.org/https://doi.org/10.1016/S1360-1385(97)89952-9

Webber, J. F. (2017). *Advice on Replanting Sites Affected by Phytophthora ramorum*. https://www.forestresearch.gov.uk/documents/5469/Advice_on_replanting_sites_felled_due_to_Phytophthora_2017.pdf

Webber, J. F. (2007). Status of Phytophthora ramorum and P. kernoviae in Europe. *Proceedings of the Sudden Oak Death Third Science Symposium. Gen. Tech. Rep. PSW-GTR-214*, 19–26. https://doi.org/https://doi.org/10.2737/PSW-GTR-214

Webber, J. F., Mullett, M., & Brasier, C. M. (2010). Dieback and mortality of plantation Japanese larch (Larix kaempferi) associated with infection by Phytophthora ramorum. *New Disease Reports*, *22*(19). https://doi.org/10.5197/j.2044-0588.2010.022.019

Webber, J. F., & Rose, J. (2007). Dissemination of Aerial and Root Infecting Phytophthoras by Human Vectors. *Proceedings of the Sudden Oak Death Third Science Symposium. Gen. Tech. Rep. PSW-GTR-214*, 195–198. https://doi.org/https://doi.org/10.2737/PSW-GTR-214

Webber, J. F., Turner, J., & Jennings, P. (2010). *Report on research undertaken between October 2009 to March 2010, on Phytophthora ramorum incited dieback of larch (Larix kaempferi)*.

Wei, X. X., & Wang, X. Q. (2003). Phylogenetic split of Larix: Evidence from paternally inherited cpDNA trnT-trnF region. *Plant Systematics and Evolution*, *239*(1–2), 67–77. https://doi.org/10.1007/s00606-002-0264-3

Werres, S., & De Merlier, D. (2003). First Detection of Phytophthora ramorum Mating Type A2 in Europe. *Plant Disease*, *87*(10), 1266–1266. https://doi.org/10.1094/pdis.2003.87.10.1266c

Werres, S., Marwitz, R., Man In't veld, W. A., De Cock, A. W. A. M., Bonants, P. J. M., De Weerdt, M., Themann, K., Ilieva, E., & Baayen, R. P. (2001). Phytophthora ramorum sp. nov., a new pathogen on Rhododendron and Viburnum. *Mycological Research*, *105*(10), 1155–1165. https://doi.org/10.1016/S0953-7562(08)61986-3

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag. https://ggplot2.tidyverse.org

Widmer, T. L. (2009). Infective Potential of Sporangia and Zoospores of Phytophthora ramorum. *Plant Disease*, *93*(1), 30–35. https://doi.org/10.1094/PDIS-93-1-0030

Witzell, J., & Martín, J. A. (2008). Phenolic metabolites in the resistance of northern forest trees to pathogens - Past experiences and future prospects. *Canadian Journal of Forest Research*, *38*(11), 2711–2727. https://doi.org/10.1139/X08-112

Worrall, D., Holroyd, G. H., Moore, J. P., Glowacz, M., Croft, P., Taylor, J. E., Paul, N. D., & Roberts, M. R. (2012). Treating seeds with activators of plant defence generates long-lasting priming of resistance to pests and pathogens. *New Phytologist*, *193*(3), 770–778. https://doi.org/10.1111/j.1469-8137.2011.03987.x

Yang, J. K., Tong, Z. J., Fang, D. H., Chen, X. J., Zhang, K. Q., & Xiao, B. G. (2017). Transcriptomic profile of tobacco in response to Phytophthora nicotianae infection. *Scientific Reports*, *7*(1), 1–7. https://doi.org/10.1038/s41598-017-00481-5

Yang, X., Tyler, B. M., & Hong, C. (2017). An expanded phylogeny for the genus Phytophthora. *IMA Fungus*, *8*(2), 355–384. https://doi.org/10.5598/imafungus.2017.08.02.09

Yde-Andersen, A. (1979). Host spectrum, host morphology and geographic distribution of larch canker, Lachnellula willkommii: A literature review. *European Journal of Forest Pathology*, *9*(3–4), 211–219. https://doi.org/10.1111/j.1439-0329.1979.tb00680.x

Zhang, Liu, L., Wang, X., Vossen, J., Li, G., Li, T., Zheng, Z., Gao, J., Guo, Y., Visser, R. G. F., Li, J., Bai, Y., & Du, Y. (2014). The Ph-3 gene from Solanum pimpinellifolium encodes CC-NBS-LRR protein conferring resistance to Phytophthora infestans. *Theoretical and Applied Genetics*, *127*(6), 1353–1364. https://doi.org/10.1007/s00122-014-2303-1

Zhang, X., Abrahan, C., Colquhoun, T. A., & Liu, C.-J. (2017). A Proteolytic Regulator Controlling Chalcone Synthase Stability and Flavonoid Biosynthesis in Arabidopsis. *The Plant Cell*, *29*(5), 1157–1174. https://doi.org/10.1105/tpc.16.00855

Zonneveld, B. J. M. (2012). Conifer genome sizes of 172 species, covering 64 of 67 genera, range from 8 to 72 picogram. *Nordic Journal of Botany*, *30*(4), 490–502. https://doi.org/10.1111/j.1756-1051.2012.01516.x