

The epidemiology of *Phytophthora ramorum* associated with *Larix* in the UK

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Abstract

Phytophthora ramorum is the cause of Sudden Oak Death in the USA and also infects many ornamental shrub species in both North America and Europe. In addition, it is now causing widespread disease in UK commercial larch plantations. The epidemiology of *P. ramorum* including sporulation potential, pathogenicity, ability to persist and the changing threat posed by the recently discovered EU2 lineage of the pathogen were investigated.

Results indicated that all three species of commercially grown larch in the UK are not only able to support prolific sporulation of *P. ramorum* (approximately 960 sporangia per cm²) but are similarly vulnerable to bark colonisation, resulting in extensive dieback and mortality. Sporulation of *P. ramorum* on larch foliage exceeded that of all known sporulating hosts, including Californian bay laurel (approximately 77 sporangia per cm²), which drives epidemics in North America. Sporulation potential on foliage and larch bark susceptibility varied significantly over the growing season and with genotype. Sporulation on larch foliage was highest in summer but lower in spring and autumn. In contrast, susceptibility of larch bark was highest in spring and decreased in late summer. Asymptomatic infection of foliage occurred early in the year with symptom development mainly seen on old foliage and late in the year. *Phytophthora ramorum* also persisted in the litter layer but not the soil for up to two years after removal of infected Japanese larch in south-east England. In comparison with the widespread EU1 lineage, the EU2 had a faster grow rate over 2.5-29°C and was significantly more pathogenic to Japanese larch, European larch and English oak bark. However, isolates of the EU2 lineage produced significantly lower inoculum loads than the EU1 suggesting a trade-off between pathogenicity and sporulation.

The implications of the aggressive nature of *P. ramorum* infection on commercially grown larch, its persistence on infested sites and the new established adaptively different EU2 lineage are discussed in relation to the future of disease management in the UK.

Author's Declaration of Originality

I hereby declare that the work presented in this thesis is my own. Information from external sources is fully referenced within.

Anna R. Harris

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*This thesis is dedicated to my parents
for their endless love, support and encouragement.*

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Chapter one - Introduction

1.1 Research project background

The stimulus for this research project is to understand the impact that recently arrived *Phytophthora ramorum* could have on commercially grown larch (*Larix*) species in the UK.

1.1.1 Forestry in the UK

The UK is an ideal place to practice forestry due to its mild winters, plentiful rainfall, fertile soil and hill-sheltered topography (Hart, 1994). As such it delivers growth rates three times those of Sweden for conifer species, while growth of broadleaved trees exceeds those in mainland Europe (Hart, 1994). Woodland covers a total of 3,097,000 hectares in the UK, making up 13% of the total land coverage, with broadleaved species comprising 1,487,000 hectares and conifer species 1,610,000 hectares (Forestry Commission, 2012a). Forestry industries make a significant contribution to the UK economy, generating over £7 billion annually to UK GDP (Forestry Commission, 2012a), but the UK is also amongst Europe's largest wood products importers. Around 81% of UK domestic demand for wood is met by imports, amounting to a value of around £6.8 billion in 2011 (Forestry Commission, 2012a).

The use of non-native conifer species in the UK dates back to the 16th century with the introduction of Norway spruce (*Picea abies*) (Samuel, 2007). Most conifers in Britain are not native, but have been introduced for commercial forestry. Out of the 66 native tree species in the UK (FAO, 2006) Scots pine (*Pinus sylvestris*) is the only native conifer to be grown commercially for timber (Forestry Commission, 2012c). The most frequently grown conifer species consist of spruce (*Picea*), pine (*Pinus*) and larch (*Larix*) making up 32%, 17% and 6% of the total woodland area of Britain respectively. In Britain, Japanese, hybrid and European larch are grown commercially, collectively covering 134,000 hectares (approximately 47,000 ha or 4.3% in England; 23,000ha or 8% in Wales and 65,000 ha or 5.1% in Scotland) (Forestry Commission, 2012a). Larch species are fast growing, deciduous trees that produce durable wood with high mechanical strength and decay resistance. They are an important conifer species and used not only for timber production, but also for landscape, biodiversity and recreational purposes.

1.1.2 Invasive non-native species and the global plant trade

Over the past century invasive non-native species have become an increasing threat not only to biodiversity and ecosystem services, but also to economic development and human well-being. Invasive non-native species cost the British economy an estimated £1.7 billion each year (Williams *et al.*, 2010). Growing international trade in exotic plant species has made a significant contribution to the spread of invasive species worldwide. Exotic and invasive plant pathogens pose a significant threat to the sustainability of the world's forests, costing an estimated £1.4 million annually to forestry in Great Britain alone; although since this report was published (Williams *et al.*, 2010) the impact of *Phytophthora* is likely to have significantly increased the cost due to increased statutory felling, burning and monitoring (Forestry Commission, 2012a). By definition, plant pathogens are biotic agents that cause disease in plants usually by disturbing the metabolism of cells through enzymes, toxins, growth regulators and by absorbing foodstuff from the host plant cells (Agrios, 2005). They reduce plant productivity and can result in death, causing significant socioeconomic and ecological impacts. In the last decade many previously unknown invasive *Phytophthora* pathogens with the potential to cause serious damage to the world's forests, natural ecosystems, gardens and plant collections have been spread via the international plant trade (Brasier, 2006). Once introduced, invasive species including many *Phytophthora* species prove impossible to eradicate (Brasier, 2008).

Species of the aptly named genus *Phytophthora* (from the Greek: *phyto* 'plant' and *phthora* 'destruction'; 'plant destroyer') are arguably the most destructive group of plant pathogens. They are known for their ability to cause widespread disease epidemics which can result in enormous economic losses to agriculture and forestry (Johnson *et al.*, 1997; Suslow, 2005; Dart & Chastagner, 2007) as well as causing extensive environmental damage to natural ecosystems (Shearer *et al.*, 2007). The destructive potential of *Phytophthora* diseases can be illustrated by late blight (*P. infestans*), which was responsible for the Irish potato famine and *Phytophthora* dieback (*P. cinnamomi*) in Australia which threatens entire native ecosystems, reducing the density & diversity of native vegetation (Reiter, Weste & Guest, 2004).

Phytophthora ramorum is a recently discovered invasive pathogen of forests and natural ecosystems (Werres *et al.*, 2001). It is the causal agent of 'Sudden Oak Death' (SOD), resulting in widespread mortality of tanoaks (*Notholithocarpus densiflorus*), coastal live oaks (*Quercus agrifolia*) and Californian black oaks (*Q. kelloggii*) in predominantly central and northern California and in southern Oregon (Rizzo *et al.*, 2002b; Rizzo, Garbelotto & Hansen, 2005). Affected oaks were first observed in California in 1995, but the cause, a new

species of *Phytophthora* was only recognised in California in 2000 and in Oregon in 2001. *Phytophthora ramorum* was formally identified in Europe in 2001 (Werres *et al.*, 2001), although it had been found in the nursery trade in the early 1990s. In 2002, emergency phytosanitary measures were implemented by the EU to prevent spread of *P. ramorum* within the European Community (Commission Decision, (2002/757/EC)) focusing mainly on the ornamental nursery trade of rhododendrons. Nevertheless, it spread rapidly and in 2003 was found infecting woodland trees in southwest England. Between 2003 and 2009, tree infections were rare in comparison to those in California, with only approximately 100 trees affected; mainly native beech (*Fagus sylvatica*) and non-native oaks; southern red oak (*Quercus falcata*), Turkey oak (*Q. cerris*) and holm oak (*Q. ilex*). All were growing close to infected rhododendron. Between 2004 and 2007, research groups from all over Europe joined in the European Union FP7 project Risk Analysis for *Phytophthora ramorum* (RAPRA), which included epidemiological studies and development of risk management strategies and contingency plans to try and combat disease spread (Sansford *et al.*, 2008). Within the project, 105 species of tree and understory plants from the UK were tested as potential hosts of the pathogen and ranked according to susceptibility. Testing focused on broadleaf tree species within the *Fagaceae* although commercially important conifer species were tested from the genera *Pinus* and *Picea* including North American species commonly also grown in Europe and the UK. However, species of *Larix* grown in Europe (Japanese larch (*Larix kaempferi*), European larch (*L. decidua*) and hybrid larch (*Larix x eurolepis*)) were not included. Then in August 2009, widespread areas of Japanese larch in the West Country were found with symptoms of branch death and extensive crown dieback, of which *P. ramorum* was found to be the causal agent (Brasier & Webber, 2010).

Phytophthora ramorum disease epidemics in California are estimated to have resulted in the death of over a million tanoak and coastal live oak (Frankel, 2011). In Marin County (an area heavily affected) tanoak has been virtually eliminated from the forest stands, where it was once dominant (Frankel, 2011). In addition to the ecological impacts, *P. ramorum* is estimated to have cost the nursery industry in the USA \$100 - \$300 million and reduced the values of properties in affected urban areas (Kliejunas, 2010). Economic costs to the forestry industry have been relatively minor, as host mortality occurs primarily in non-timber species. However, between 2001-2008 eradication treatments on 971 hectares of affected forest in Curry County, Oregon cost \$4.3 million (Kanaskie *et al.*, 2010).

In light of known impacts of *P. ramorum* epidemics in North America, a greater knowledge of the epidemiology of *P. ramorum* on larch trees is required if effective management of the

disease is to be achieved in the UK. *Phytophthora ramorum* is also a regulated quarantine pest bringing certain statutory requirements for disease control.

Epidemiology is the study of a 'disease triangle' describing the interactions between the pathogen, host and environment, through which disease is manifest. Extensive research has been carried out on the epidemiology of *P. ramorum* in US forests and nurseries giving a good understanding of many critical factors in the biology of this pathogen. Genetic differences in the populations of *P. ramorum* in the UK and North America have been analysed and three separate near clonal lineages (EU1, NA1 and NA2) distinguished. Factors such as climate, host availability and woodland composition also affect disease development but differ considerably between countries and continents requiring research to explore the epidemiology of the disease in the UK. The development of improved understanding to develop effective management strategies is critical in the light of the spread of *P. ramorum* to larch.

1.2 Review of *Phytophthora ramorum*, the organism

1.2.1 Classification

Although commonly referred to as a fungus, the genus *Phytophthora* is classified in the Oomycetes which fall within the kingdom Chromalveolata, phylum Heterokontophyta (Cavalier-Smith & Chao, 2006). Oomycetes, commonly called 'water moulds', were long regarded as fungi due to their filamentous growth form and saprophytic/parasitic lifestyles. They are however diploid organisms and have cell walls composed of cellulose and β -1,3-glucan polymers, in contrast to fungi which have either haploid or dikaryotic hyphae with chitin-containing cell walls. As such, oomycetes are more closely related to brown algae (Chromista) than true fungi.

To date (August 2014) there are 116 formally identified *Phytophthora* species and an additional 21 new species awaiting description (*Phytophthora* database - <http://www.Phytophthoradb.org> [Accessed online, 2014]). In recent years advances in molecular analysis have enabled phylogenetic classification of the genus *Phytophthora*, which divides it into 10 separate clades, placing *P. ramorum* in clade 8c, along with the closely related tree-damaging *Phytophthora*, *P. lateralis* (Blair *et al.*, 2008; Grünwald *et al.*, 2011; Kroon *et al.*, 2012).

1.2.2 Host Range

Phytophthora ramorum is considered a generalist pathogen, lacking host specificity (Huberli & Garbelotto, 2012). Its known host range in Europe and North America is extensive, including a range of important shrubs, herbaceous plants, ferns and trees (including hardwoods and conifers), of both ornamental and environmental significance (Kliejunas, 2010). At present, natural hosts (those that have been found naturally infected in the field) appear in 41 plant families, within 82 genera (Fera, 2012b). In the UK 56 plant species have been found naturally infected 'outdoors', and in UK nurseries 27 plant species have been found to be hosts (Fera, 2012b). In addition to natural hosts, laboratory research determining *P. ramorum*'s potential host range has led to an extensive database of potential hosts which were identified as part of EU project RAPRA (<http://rapra.csl.gov.uk/>), many of which have since been found naturally infected in the field.

Infection can take the form of leaf necrosis and/or shoot dieback. For other hosts the bark is infected producing bleeding lesions or bark cankers. In the UK tree species found with

bleeding bark cankers include; *Fagus sylvatica* (beech), *Quercus petraea* (sessile oak), *Q. falcata* (southern red oak), *Q. acuta* (evergreen oak), *Aesculus hippocastanum* (horse chestnut), *Nothofagus obliqua* (noble beech), *Chamaecyparis lawsoniana* (Lawson cypress) (Brasier & Webber, 2012) and *Acer pseudoplatanus* (sycamore). Other tree species display only foliar or dieback symptoms such as; *Q. ilex* (holm oak), *Castanopsis orthocantha* (castanopsis), *Fraxinus excelsior* (ash), *Michelia doltsopa* (Michalia), *Drimys winteri* (winter's bark), *Acer laevigatum* (evergreen maple), *Cornus kousa x capitata*, *Eucalyptus haemastoma* (scribbly gum), *Osmanthus delavayi* (Delavay osmanthus) and *Schima wallichii* (Chinese guger) as well as several species of *Magnolia* (Sansford & Woodhall, 2007). Some tree species found in the UK can display both foliar and bole infections including *Q. cerris* (turkey oak), *Castanea sativa* (sweet chestnut), *Cinnamomum camphora* (camphor tree), (Sansford & Woodhall, 2007; Forestry Commission, 2012b) and *Larix kaempferi* (Japanese larch) (Forestry Commission, 2012b).

In the USA, Californian bay laurel (*Umbellularia californica*) and tanoak (*Notholithocarpus densiflorus*) are major sporulating hosts driving the disease epidemics of *P. ramorum* (Davidson *et al.*, 2005; Davidson, Patterson & Rizzo, 2008), whereas in the UK the major sporulating and epidemiologically important host has been the invasive understory plant *Rhododendron ponticum* (Brasier & Kirk, 2004). Many tree infections are associated with proximity to infected rhododendrons (Brasier & Jung, 2006). Within the nursery trade, key hosts on which the pathogen has been found repeatedly and which are thus involved in the movement of the pathogen throughout the nursery trade include: *Rhododendron*, *Viburnum*, *Pieris*, *Syringa* and *Camellia* species (Grünwald, Goss & Press, 2008).

Tree species from the family *Fagaceae* (especially species of *Quercus* and *Fagus*) have been considered to be most at risk from lethal stem cankers when in close proximity to sporulating hosts such as *R. ponticum* (Sansford & Woodhall, 2007; Sansford *et al.*, 2008). In the USA major tree hosts from the genus *Quercus*, in particular coastal live oak (*Q. agrifolia*), California black oak (*Q. kelloggii*) and tanoaks (*N. densiflorus*) are considered the most susceptible (Davidson *et al.*, 2003). Unlike tanoaks, *Quercus* species are considered dead end hosts being unable to support foliar infections or sporulation. Nevertheless *P. ramorum* has been responsible for the rapid decline of native coastal live oak (*Q. agrifolia*) woodlands in California (Rizzo *et al.*, 2002a). In Europe, until the recent discovery of *P. ramorum* affecting larch trees in the UK, tree infections had been comparatively rare with mainly native beech and non-native oak growing close to rhododendron becoming infected.

Due to the diverse array of plants apart from tree species that can act as hosts, a large number of environmental and economic habitats are at risk in Europe. These include; woodland (managed, semi-natural or natural), heathland, maquis (macchia) shrubland, and managed gardens (including those of heritage value), parks and public greens (Sansford *et al.*, 2008). Heathland in northern Europe is thought to be at significant risk as many are composed of species from the genera *Calluna* and *Vaccinium* (Sansford *et al.*, 2008). The latter in particular has been shown to be particularly susceptible in laboratory testing and is a significant sporulating hosts (Sansford *et al.*, 2008). Further, *V. myrtillus* has been found as a natural host at a number of locations in the wild (Fera, 2009) and on open heathland in November 2010 in the UK (Fera, 2012b).

1.2.3 Symptoms

Phytophthora ramorum is an aerial pathogen, generally attacking above ground parts of plants. It causes an array of different disease symptoms on its many hosts ranging from minor foliar lesions to stem cankers and tree death depending on the host and part of the plant that is infected. Two disease types have typically been recognised: ‘ramorum bleeding canker’, (also known as Sudden Oak Death (SOD) in the North America) and ‘ramorum blight’ (Hansen *et al.*, 2002). Ramorum bleeding canker is characterised by potentially lethal, bleeding bark lesions on the trunk of the host. Ramorum blight refers to foliar and stem infections which cause dieback in green non-lignified tissues (Grünwald, Goss & Press, 2008; Hansen *et al.*, 2002). Ramorum blight tends to be associated with ornamental trees, woody shrubs and understory plants, whilst ramorum bleeding canker is typically associated with forest and garden trees (Grünwald, Goss & Press, 2008) [Table 1.1]. Hosts may display only one disease type; such as holm oak (*Q. ilex*) in the UK which has only been observed in nature with foliar symptoms, although laboratory tests have demonstrated the susceptibility of its bark to the pathogen (Sansford *et al.*, 2008). Alternately, some hosts can exhibit more than one disease type, for example in North America all aerial parts of tanoak (*N. densiflora*) can be affected by the pathogen, displaying both bark cankers along with leaf and shoot infections (Davidson *et al.*, 2003).

1.2.3.1 Rhododendron

Infected plants exhibit Ramorum blight. Stems with dieback develop a characteristic brown-black discolouration, which typically originates at the tip and extends down the stem. If it spreads into the main stem base it causes plant death (Davidson *et al.*, 2003). Foliar infection is often visible as necrosis along the mid-vein of the leaf [Figure 1.1]; alternatively

distinct leaf spots/ lesions can be observed (Davidson *et al.*, 2003). No symptoms have been observed in the roots despite isolation of the pathogen from these tissues (Colburn, Sechler & Shishkoff, 2005; Fichtner *et al.*, 2011a).



Figure 1.1 – Symptoms of *P. ramorum* on rhododendron; necrosis along mid-vein of the leaf migrating down into the stem (Picture provided by Forestry Commission).

Table 1.1- Associated diseases and symptoms of *P. ramorum* infections modified from (Grünwald, Goss & Press, 2008)

Disease	Symptoms	Host Categories	Typical Host	Geographical environment
Ramorum bleeding canker or SOD	Stem cankers; bleeding cankers, discolouration of bark	Forest trees; garden trees	European beech, Japanese larch & others, Coast live oak, tanoak.	European gardens & forests, North American Forests.
Ramorum blight	Foliar and twig blight; tip and shoot dieback; leaf blight	Ornamental trees and woody shrubs; forest understory plants	California bay laurel, <i>Viburnum</i> , rhododendron, <i>Vaccinium</i> , Douglas fir, coast redwood, yew, tanoak & others	European nurseries and gardens, North American nurseries and forests.

1.2.3.2 *Quercus* species

The most consistent and diagnostic symptoms on infected trees are cankers that develop before crown symptoms become evident (Rizzo *et al.*, 2002a). Cankers develop on the bark with a brown/black discolouration and ooze a dark coloured fluid. They are typically 1-2 m above ground but can be up to 20 m high, over 2 m in length and do not extend into the

roots below the soil line (Rizzo *et al.*, 2002a). In North America infected trees can show gradual leaf loss and death, whilst others undergo a sudden browning of the crown followed by death. Death is due to the pathogen girdling the whole stem (Rizzo *et al.*, 2002a).

1.2.3.3 Japanese larch

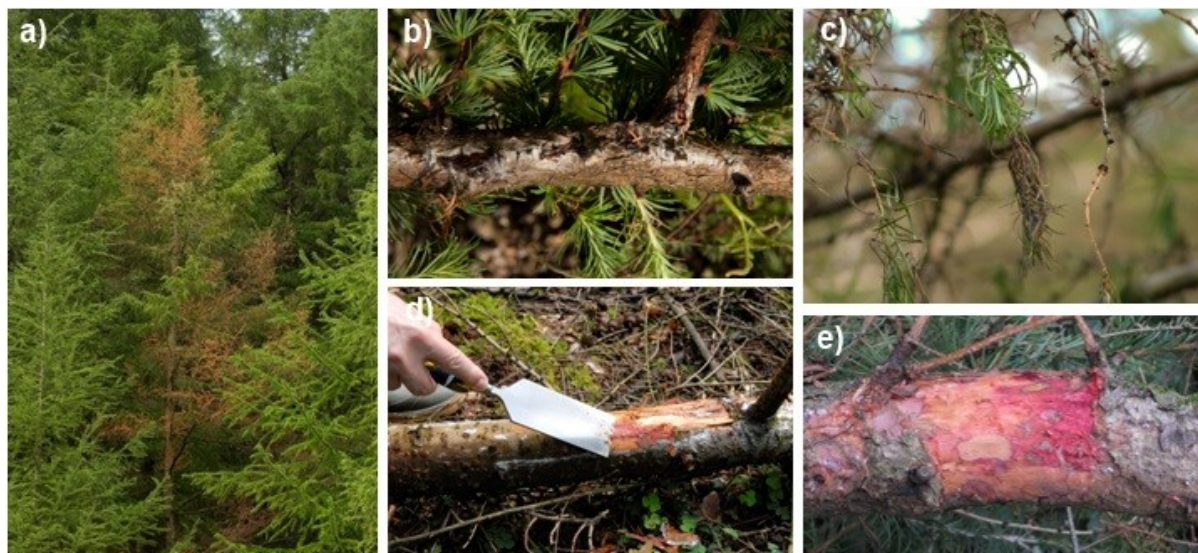


Figure 1.2 - Symptoms of Sudden Larch Death: a) Crown dieback, b) resin bleed on a branch, c) necrotic needles and premature needles loss, d) old rusty brown lesion in the phloem, e) pink necrotic bark tissue (Pictures provided by Forestry Commission).

Infected trees exhibit two main forms of symptoms; foliar symptoms consisting of aborted bud flush, withering and senescence of shoot tips, needle necrosis with black-purple-brown discolouration, especially to the tips and premature needle loss. Abundant resin bleeds on trunks, branches and side shoots along with extensive dieback of branches and sometimes the whole crown [Figure 1.2]. Phloem lesions under resinous outer bark, exhibit necrotic tissue which can be bright pink to maroon-red at the lesion margins (Webber, Mullett & Brasier, 2010), turning to a rusty brown/cinnamon colour in older lesions (Webber, Mullett & Brasier, 2010; Webber, Turner & Jennings, 2010). Foliar symptoms are not detectable during the winter, due to the deciduous nature of larch trees, however, cankers are visible all year round, although the resin bleeding becomes less visible once it dries and hardens (Forestry Commission, 2012b).

1.2.4 Geographic distribution

To date the native range of *P. ramorum* is unknown. Clues to its origin taken from its biology and temperature-growth relationship suggest it is adapted to temperate climates (Brasier *et al.*, 2004). The distribution of *P. ramorum*'s mating type hint at an exotic origin. As a

heterothallic species it has two mating types, A1 and A2, and it is assumed that both mating types would have evolved in the same place for sexual reproduction to occur (Sansford *et al.*, 2008). However, the A1 mating type has been found almost exclusively in Europe and the A2 mating type has only been found in North America. Its recent discovery in the UK also suggests that it was recently introduced (Grünwald *et al.*, 2012). The pathogen's wide host range within Europe and America provides limited information on its origin. Rhododendrons may not be its native host, as they tend to be susceptible to many *Phytophthora* species (Erwin & Ribeiro, 1996) within the nursery trade and may instead act as carriers (Brasier *et al.*, 2004).

Phytophthora ramorum is speculated to have originated from forested areas in eastern Asia (Brasier *et al.*, 2004) in regions such as southern Japan, southern Korea and eastern China (Kluza *et al.*, 2007). This area of Asia is a centre of diversity for many genera of plants that are *P. ramorum* hosts (Goheen, Kubisiak & Zhao, 2005). Yunnan province in eastern China in particular stands out as a possible site of origin due to its climatic regime, diverse forest vegetation types containing many unique species, and because it is a popular location for plant collectors (Brasier *et al.*, 2004). Thus it is possible that *P. ramorum* has been unknowingly transported on commercial or privately collected ornamental plants. In 2004 a survey for this pathogen was carried out in several regions of the Yunnan province on sites associated with *Quercus*, *Larix* and *Notholithocarpus* species with understory rhododendron. Dieback symptoms similar to those associated with *P. ramorum* were observed, however, DNA assays to identify the pathogen were inconclusive (Goheen, Kubisiak & Zhao, 2005).

1.2.4.1 Europe

Phytophthora ramorum is thought to have been present in Europe as early as 1993 on nursery plants of rhododendron in Germany and the Netherlands, however, at this point it had not been described (Werres *et al.*, 2001; California Oak Mortality Task Force, 2011). In 2000 Clive Brasier of the GB Forestry Commission Research Agency noted similarities between a new *Phytophthora* species associated with the dying oak trees in California and the infected rhododendron species found in Germany and the Netherlands (California Oak Mortality Task Force, 2011) which was formally identified as *P. ramorum* in 2001 by Werres *et al.* (2001). Ramorum disease was first detected in England in 2002 on *Viburnum tinus* in a nursery setting (Lane *et al.*, 2003). With the realisation that *P. ramorum* was the cause of Sudden Oak Death, emergency legislation was put into place, requiring all EU member states to conduct surveys for the pathogen (Commission Decision, (2002/757/EC); Sansford

et al., 2008), and over the next few years *P. ramorum* was detected in 20 EU countries and three non EU countries [Table 1.2].

Table 1.2 – Distribution of *P. ramorum* within Europe (Tsopelas *et al.*, 2011; Kliejunas, 2010; Sansford *et al.*, 2008).

		Nurseries	Gardens, Parks, Woodlands	Eradicated
EU	Belgium	*	*	
	Czech Republic	*		*
	Denmark	*	*	
	Estonia	*		
	Finland	*		
	France	*	*	
	Germany	*	*	
	Greece	*		
	Ireland	*	*	
	Italy	*		
	Latvia	*		
	Lithuania	*		
	Luxembourg	*	*	
	the Netherlands	*	*	
	Poland	*		
	Portugal	*		
	Slovenia	*	*	
	Spain (including Mallorca)	*	*	
	Sweden	*		
	UK (all countries including Channel Islands)	*	*	
Non-EU	Norway	*	*	
	Switzerland	*	*	
	Serbia	*	*	

Within Europe *P. ramorum* is subject to an eradication/containment programme. Eradication has been successful in the Czech Republic, where it was found on imported viburnum plants in 2003 (Běhalová, 2006). Prior to the discovery of ramorum disease on larch in the UK, the pathogen was predominantly detected on non-tree hosts of ornamental plants grown in containers in nurseries and retail garden centres (Sansford *et al.*, 2008). In England and Wales, 558 nursery outbreaks were recorded at 475 sites, between 2002 - 2007, with *P. ramorum* being successfully eradicated from 459 of them. In contrast but over the same period, 185 outbreaks of the disease were recorded across 166 sites in natural and semi-

natural environments, but *P. ramorum* was eradicated from only 60 sites (Sansford & Woodhall, 2007).

1.2.4.2 North America

The disease Sudden Oak Death was observed in 1995 on native plants in coastal evergreen forests, in 14 neighbouring California counties (Marin, Santa Cruz, Sonoma, Napa, San Mateo, Monterey, Santa Clara, Mendocino, Solano, Alameda, Contra Costa, San Francisco, Lake, and Humboldt) (Kliejunas, 2010) although not ascribed to *P. ramorum* until 2000. In Oregon the disease was located via aerial surveys in Curry County in 2001 (Goheen *et al.*, 2002). Eradication was not considered feasible in Californian forests, but attempts were made to eradicate the pathogen in Oregon due to its limited distribution in nine forest sites covering 16 ha near the town of Brookings. Eradication treatments were carried out from 2001 to 2008, on 971 ha of forest, costing \$4.3 million (Kanaskie *et al.*, 2010). Complete eradication has been unsuccessful, but it has successfully limited the spread of the disease (Kanaskie *et al.*, 2010).

The connection between infected rhododendron and SOD was only discovered later in North America in contrast to findings in Europe (Kanaskie *et al.*, 2010). *Phytophthora ramorum* was found infecting rhododendron container plants in a Santa Cruz nursery in 2001 (California Oak Mortality Task Force, 2008) although the epidemic on ornamentals did not become apparent until 2003 when 20 nurseries tested positive for the pathogen (Kliejunas, 2010) in California, Oregon, Washington and British Columbia (California Oak Mortality Task Force, 2011). The outbreak of *P. ramorum* in North American nurseries peaked in 2004, with 177 UDSA APHIS positive confirmations of the pathogen in 22 states. The pathogen is still subject to eradication and containment measures in nurseries but continues to be detected although the numbers of positive findings have been decreasing, with only 21 US nurseries, in four states (California, Oregon, Washington and South Carolina) testing positive for its presence in 2011 (California Oak Mortality Task Force, 2011).

Phytophthora ramorum has only been documented as present in Canadian nurseries (California Oak Mortality Task Force, 2011). It was first detected in a British Columbia nursery in June 2003 on potted rhododendrons (California Oak Mortality Task Force, 2011) followed by additional detections in other nurseries in 2004 and 2005.

To date there is no record of *P. ramorum* in Central America, South America, Caribbean, Asia, Africa and Oceania.

1.2.5 Genetic Diversity

The presence of *P. ramorum* in both Europe and North America was initially thought to be due to human mediated transport, predominantly through the nursery trade. Analysis of *P. ramorum* isolates from different geographic ranges have used a combination of AFLP, microsatellite analysis, sequencing and morphological analysis (Kroon *et al.*, 2004; Ivors *et al.*, 2006; Prospero *et al.*, 2007; Mascheretti *et al.*, 2008; Martin, 2008; Mascheretti *et al.*, 2009). As a result, three distinct clades are recognised, representing distinct evolutionary lineages (Goss, Carbone & Grünwald, 2009; Grünwald *et al.*, 2009). Each lineage is informally designated as the NA1, NA2 and EU1 after their initial outbreak location and order of appearance (Grünwald *et al.*, 2009). Molecular evidence suggests that they are near clonal in their areas of introduction, having diverged from their sexually reproducing ancestors 165,000-500,000 years ago (Goss, Carbone & Grünwald, 2009). Most recently, genetic analysis of *P. ramorum* isolates within Europe has revealed that apart from the EU1 lineage, a second European lineage (EU2) exists [Figure 1.3] (Van Poucke *et al.*, 2012).

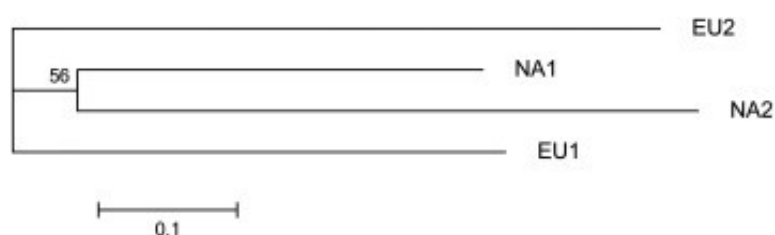


Figure 1.3 - Neighbour-joining tree of the four *Phytophthora ramorum* lineages (EU1, EU2, NA1, NA2) based on microsatellite data of 16 loci. Numbers on branches represent bootstrap support values. The tree is drawn to scale, with branch lengths proportional to the genetic distance (Van Poucke *et al.*, 2012).

Current evidence indicates that *P. ramorum* appears to have been introduced from unknown origins at least five times independently, being introduced to America at least three times and twice into Europe [Figure 1.4] (Grünwald *et al.*, 2012; Van Poucke *et al.*, 2012).

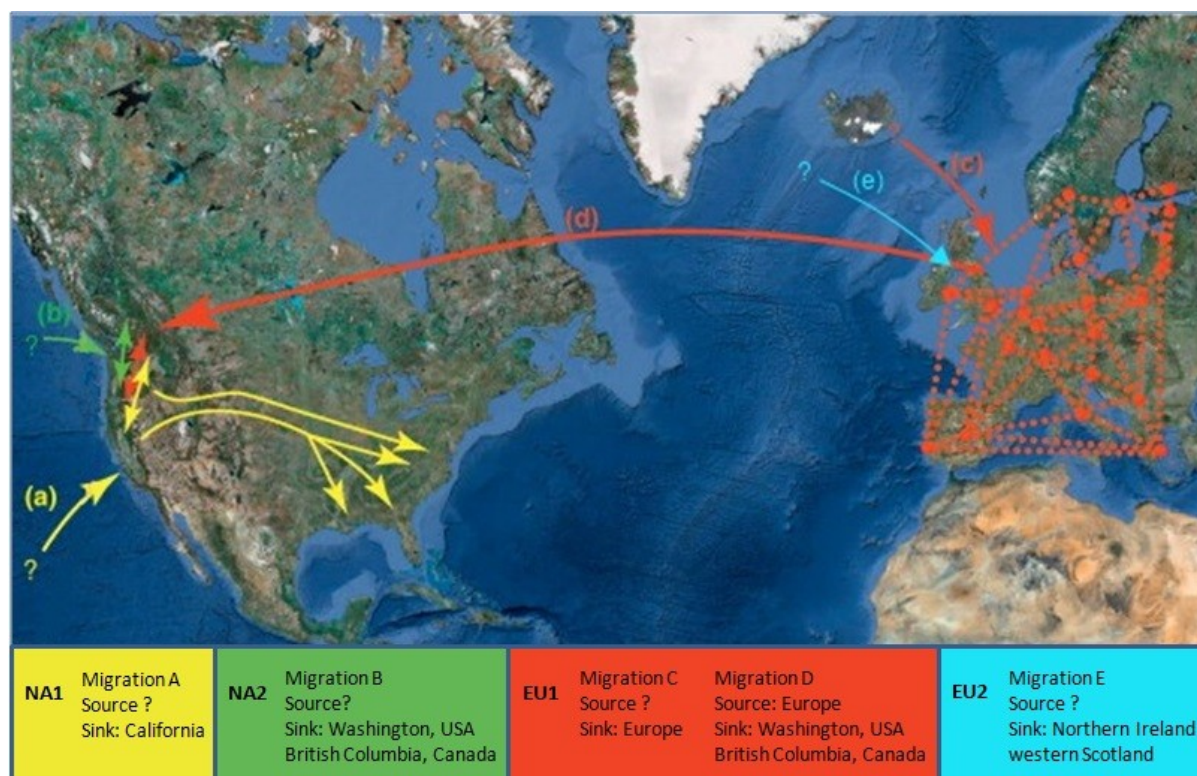


Figure 1.4 - Scenarios depicting the five intercontinental migrations of *P. ramorum* supported by population genetic and evolutionary studies modified from Grünwald *et al.* (2012).

1.2.5.1 The lineages

EU1 – is the most prevalent lineage found in Europe on trees, in gardens and nurseries. It is thought to have been spread through movement of infected ornamentals to North America as isolates of this lineage have been found in Californian nurseries, northern Oregon, Washington (Grünwald *et al.*, 2008), British Columbia and Canada (Goss *et al.*, 2011).

EU2 – has been found recently Japanese larch plantations in Northern Ireland and south west Scotland (Van Poucke *et al.*, 2012).

NA1 – is the clonal lineage responsible for natural infestations in California and Oregon forests and some nurseries in North America.

NA2 - has been isolated from only a few nurseries in California and Washington State (Ivors *et al.*, 2006), but is more common in nurseries in Canada (Grünwald *et al.*, 2012).

Mitochondrial sequencing has shown EU1 and NA genomes to be identical in gene order, encoding for the same suite of genes. They differ in that the NA mitochondrial genome has 13 single nucleotide polymorphisms (SNP) and is longer, by 180 bp (NA genome = 39, 494

bp, EU1 genome = 39,314 bp) (Martin, 2008). Genome analysis on EU2 lineage has yet to be reported.

1.2.6 Morphology

Phytophthora ramorum has a distinctive set of morphological traits that can be observed in culture on certain media; such as carrot piece agar (CPA), V8, PARP medium or corn meal agar (CMA) (Parke & Lucas, 2008).

1.2.6.1 Colony patterns

Colony patterns are dependent on both culture media and isolate, and show great variability. European isolates showed no distinct colony pattern when grown on CPA, but many produce weak concentric circles, even when incubated in the dark (Werres *et al.*, 2001; Werres & Kaminski, 2005) and weak rosette patterns, with a growth rate of 3 mm per day (Anon, 2010). Isolates from North America show a greater variability in colony morphology than European when grown on CPA, with faster growing isolates similar to European and slow growing isolates showing flattened colony edges, more compact growth with sectoring (Werres & Kaminski, 2005). When grown on V8 agar colonies produce pronounced concentric rings, compared to those on CMA (Werres *et al.*, 2001). When grown on cherry decoction agar (CHA) the colonies grow with oppressed aerial mycelium and have weak rosette like patterns (Werres *et al.*, 2001). Variability of colony morphology on agar media is not restricted to *P. ramorum* but is common throughout the genus. Thus, the usefulness of colony morphology as an identification aid beyond a supplementary purpose is questionable (Erwin & Ribeiro, 1996; Widmer, 2009).

1.2.6.2 Vegetative growth

Phytophthora ramorum exhibits vegetative filamentous growth, consisting of nodose highly branched, non-septate hyphae that form a dendritic pattern (Erwin & Ribeiro, 1996; Cave, Randall-Schadel & Redlin, 2008) [Figure 1.5]. Growth in culture can occur between 2° and 26-30°C, with optimum growth at ca. 20°C for most isolates (Werres *et al.*, 2001). The hyphae of *P. ramorum* are 5-8 µm wide (Werres *et al.*, 2001) and branch almost at right angles (Erwin & Ribeiro, 1996; Cave, Randall-Schadel & Redlin, 2008).

1.2.6.3 Asexual reproductive structures

Phytophthora ramorum is characterised by the abundant production of large chlamydospores and semi-papillate, caducous sporangia [Figure 1.5] (Werres *et al.*, 2001). Chlamydospores produced *in vitro* are hyaline but turn a golden brown with age. Their reported size varies in the literature, ranging from 40-80 μm (Rizzo *et al.*, 2002a) and 20-79 μm (Werres *et al.*, 2001). They are formed predominantly terminally on short pedicels and thus can be distinguished from *P. lateralis* which forms chlamydospores (as the name suggests) laterally. Sporangia of *P. ramorum* are notably more elongate than their closest relatives (Parke & Lucas, 2008), and form on short pedicels in a sympodial fashion, with an average length of 43.6 μm (20-79 μm) and average width of 23.9 μm (12-40 μm) (Werres & Kaminski, 2005).



Figure 1.5 - Structures of *P. ramorum* grown on CA a) hyphae; b) sporangia releasing zoospores; c) sporangia and chlamydospore; d) chlamydospore (scale bar = 20 μm).

1.2.6.4 Sexual reproductive structures

Phytophthora ramorum is a heterothallic *Phytophthora*, requiring compatible mating types (A1 & A2) for sexual reproduction to occur, but it is bisexual and able to produce both oogonia and antheridia on a single thallus [Figure 1.6]. Oospores are produced both terminally and laterally. They are smooth and spherical in shape, ranging in size from 25-35 μm in diameter (Brasier & Kirk, 2004). Antheridia are amphigynous (the oogonium grows through the antheridium) and are mainly rounded to barrel-shaped, with an average length of 15 μm (Brasier & Kirk, 2004). Oospore size ranges from 22.5 – 27.5 μm in diameter (Brasier & Kirk, 2004), however they have not been reported in nature and have only been observed under laboratory conditions (Brasier & Kirk, 2004).



Figure 1.6 - Oogonia and amphigynous antheridia of *Phytophthora ramorum* produced in intraspecific pairings on CA medium (Brasier & Kirk, 2004).

1.2.7 Life History

The *Phytophthora ramorum* life cycle follows the basic steps of any plant disease: inoculum production, dispersal of inoculum, establishment of infection (entry into host), colonisation (invasion), symptom development (interaction of the pathogen with the host) and survival (Kliejunas, 2010). Like other aerially infecting *Phytophthora* species, *P. ramorum* spreads through the production of asexual sporangia and zoospores. Survival structures such as chlamydospores and sexually produced oospores may also contribute to the pathogen's distribution but are primarily thought to be involved in ensuring its persistence in the ecosystem (Parke & Lucas, 2008).

1.2.7.1 Hyphae

Hyphae invade host tissue initially establishing a biotrophic relationship within living cells before switching to a more destructive role gaining nutrition necrotrophically by killing the cells and feeding on them. *Phytophthora ramorum* can also grow as saprophyte but is thought to be a poor competitor against other microorganisms (Sansford *et al.*, 2008). Hyphae have been detected in the pith, primary and secondary xylem, cambium, phloem and roots of rhododendron (Parke & Lewis, 2007) as well as being found in leaves. Primarily a foliar pathogen, *P. ramorum* is likely to overwinter as hyphae or chlamydospores in stems and leaves on the plant or in abscised leaves on the soil surface, in leaf litter, or in the shallow depths of soil (Tooley, Browning & Berner, 2008). The persistence of hyphae in host tissue is, however, not fully understood. It has been speculated that hyphal aggregates produced under the epidermis on several Mediterranean plants may protect the pathogen from dry atmosphere and/ or solar radiation and may serve as an over-summering survival structure (Moralejo *et al.*, 2006; Browning *et al.*, 2008). Hyphal colonies have considerable cold tolerance to freezing temperatures; exposure to -5°C for 24 h has no impact on its survival (Browning *et al.*, 2008). Hyphae can survive exposure to temperatures of 30-32°C

for 8 h. They are also able to survive exposure with no deleterious effects to a diurnal cycle of temperature and humidity ranging from 27.5°C and 63.8% RH to 14.9°C and 92.2% RH (Browning *et al.*, 2008).

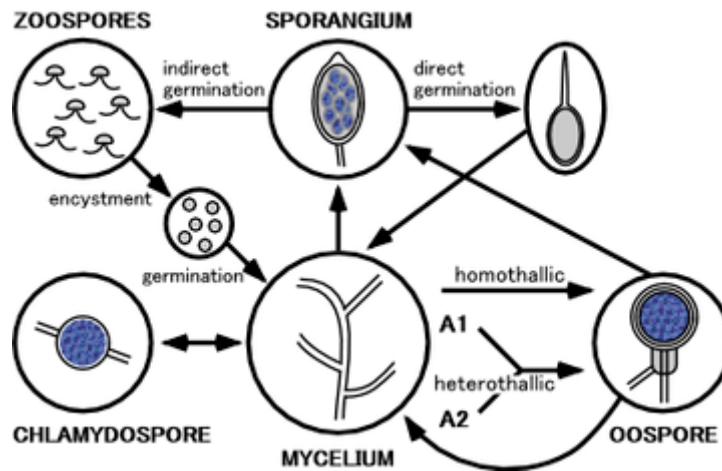


Figure 1.7 – Life history of *Phytophthora ramorum* modified from Erwin & Riberio (1996).

1.2.7.2 Sporangia

Sporangia are the most common and characteristic propagule of *Phytophthora* species (Erwin & Ribeiro, 1996). Their primary function is dispersal (Sansford *et al.*, 2008) and as such, sporangia production drives disease epidemics as with other aerially dispersed species of *Phytophthora* (e.g. *P. capsici*, *P. palmivora* and *P. infestans*). Sporangia of *P. ramorum* are caducous which allows them to become easily detached from the sporangiophore by rain splash. Once detached, wind and rain may spread them within forest canopies. Sporangia germinate either directly to produce mycelia (which can go on to produce further sporangia) or indirectly by releasing zoospores [Figure 1.7 and Figure 1.8] depending on environmental conditions. Zoospore production typically occurs around 15–20°C, whilst the threshold for direct germination is 25°C (Davidson *et al.*, 2005). Direct germination of *P. ramorum* sporangia, however, is not considered to be as effective in causing necrosis (a measure of disease severity) as an equivalent number of zoospores (Widmer, 2009). Humidity has the greatest effect on sporangial production and zoospore germination, both require a minimum of 6–12 h of free standing water for germination (Garbelotto *et al.*, 2003).



Figure 1.8 - *Phytophthora ramorum* sporangia releasing zoospores (Picture provided by Forestry Commission).

Sporangia are formed on specialised hyphae (sporangiophores) which rupture the leaf's surface or poke through stomata. They are readily produced on foliage and twig cankers of certain hosts (Davidson *et al.*, 2005; Rizzo, Garbelotto & Hansen, 2005) and have also been observed on the roots of some hosts (Shishkoff, 2011). In nature spore production varies yearly as well as seasonally (Davidson *et al.*, 2005; Davidson *et al.*, 2005; Rizzo, Garbelotto & Hansen, 2005; Kanaskie *et al.*, 2010). In California, high levels of inoculum can be detected during and after both winter and warm spring rains, but inoculum is undetectable during the hot dry summer (Davidson *et al.*, 2005). In Oregon however, inoculum is produced all year round, even during long periods without rain due to the availability of water in the form of fog that surrounds tree canopies (Hansen *et al.*, 2008). Inoculum is however, most readily detected following spring rains in both California and Oregon (Rizzo, Garbelotto & Hansen, 2005; Davidson *et al.*, 2005; Davidson, Patterson & Rizzo, 2008; Hansen *et al.*, 2008).

1.2.7.3 Zoospores

Whilst sporangia are the main structure for dispersal, zoospores are thought to be the primary structure for infectivity (Davidson *et al.*, 2005; Rizzo, Garbelotto & Hansen, 2005; Widmer, 2009). Zoospores of *P. ramorum* are reniform, biflagellate, motile spores that form through the cleavage of the sporangial cytoplasm (Hardham & Hyde, 1997). They are released from sporangia under cool, moist conditions (Garbelotto *et al.*, 2003; Davidson *et al.*, 2005). Upon release, zoospores detect host tissue by chemical, electrical and physical cues (Tyler, 2002). Encystment occurs upon contact with a host or can be induced artificially by agitation (Erwin & Ribeiro, 1996). Zoospores of this pathogen have been observed encysting, germinating and penetrating hosts through stems (Brasier & Brown, 2008), roots (Parke & Lewis, 2007) and foliage (Denman *et al.*, 2005; Denman *et al.*, 2008). Infections via

zoospores are considered the most important pathway in the disease cycle. *Phytophthora ramorum* sporangia can each release an average of 32 zoospores (Widmer, 2009). Moralejo and Descals (2011) however suggest that cysts may be more adapted to long distance dispersal than sporangia based on size comparisons between the two structures and therefore may be the most important agent in the disease cycle.

Phytophthora ramorum displays monomorphic diplanetism and microcyclic sporulation, in that cysts may germinate directly forming a emerging tube or indirectly by releasing secondary zoospores (Moralejo & Descals, 2011). Secondary zoospores do not differ morphologically and can infect hosts; however symptom expression is delayed and infectivity is lower than for primary zoospores (Moralejo & Descals, 2011). Zoospores have been observed being released in abundance from sporangia on infected bay laurel leaves under natural field conditions during rain (Davidson *et al.*, 2005).

As zoospores are membrane-bound and lack a cell wall, they are considered extremely susceptible to desiccation, UV radiation and other environmental extremes (Erwin & Ribeiro, 1996) and are not well adapted for persistence. Cysts do, however, display asynchronous germination which may aid in the persistence of the pathogen lineage over time under unpredictable environmental conditions and in avoidance of self-competition for plant resources (Moralejo & Descals, 2011)

1.2.7.4 Chlamydospores

Chlamydospores are large, thick walled, asexually produced spores that play an important role in the persistence of the pathogen. They have been observed growing both in and on an array of different host plant tissue; leaf surfaces (Davidson *et al.*, 2005), bark phloem and xylem tissue (Lewis & Parke, 2006; Parke & Lewis, 2007) and roots (Shishkoff, 2008) as well as being found in forest litter and soil (Fichtner, Lynch & Rizzo, 2007) and potting media (Linderman & Davis, 2006). Unlike hyphae which can grow both intra and intercellularly, chlamydospores only form intercellularly (Pogoda & Werres, 2004).

Chlamydospores germinate to produce hyphae and sporangia [Figure 1.7]. In low nutrient conditions they germinate producing germ tubes and sporangia which release zoospores to infect host tissues. In nutrient rich environments chlamydospores germinate to form germ tubes which develop into hyphae and grow vegetatively. Unlike sporangia, chlamydospores are not readily detached from mycelium and are therefore thought to be relatively insignificant in increasing secondary inoculum in polycyclic epidemics (Widmer, 2009).

1.2.7.5 Oospores

Oospores are considered to be the most persistent propagules produced by *Phytophthora* species (Erwin & Ribeiro, 1996). Gametangia and oospores of *P. ramorum* have been produced *in vitro* through both inter-specific and intra-specific pairings on agar (Brasier & Kirk, 2004) and in rhododendron twigs (Zielke & Werres, 2003). Within Europe there are no reports of A2 isolates, apart from a case in a Belgian nursery in 2002 and 2003 (De Merlier, Chandelier & Cavelier, 2003; Werres & De Merlier, 2003) which is thought to be the result of somatic recombination (Vercauteren *et al.*, 2011). As virtually all known isolates of *P. ramorum* in Europe (both EU1 and EU2) are of the A1 mating type, and there is limited chance of sexual recombination. *In vitro* production of gametangia and germination of oospores is reported to be low and inconsistent (Brasier, 2003; Brasier & Kirk, 2004; Boutet *et al.*, 2010), with a high proportion of genetic disorders during the mating (Boutet *et al.*, 2010) and a high percentage of abnormal development (Zielke & Werres, 2003; Boutet *et al.*, 2010) and abortion of oospores (Werres *et al.*, 2007) when compared to other heterothallic *Phytophthoras*. As such there has been much speculation over the functionality of the breeding system of this pathogen (Brasier & Kirk, 2004; Brasier, 2003). There are several theories to explain the dysfunction within the *P. ramorum* sexual cycle; 1) the pathogen may have a strong developmental shift from the sexual to the asexual cycle triggered by environmental conditions, which has evolved as an adaptation to tree canopy lifestyle. 2) the sexual cycle may no longer be genetically functional due to separate evolutionary adaptations in breeding strategies. 3) *In vitro* conditions may not provide a suitable environment for the production of gametangia (Brasier & Kirk, 2004).

A great deal of research has focused on the potential for oospore production in this pathogen, as sexual reproduction serves to reassort genetic diversity thereby fixing adaptations and novel mutations within the species. Oospore production as a result of genetic crossing poses a serious risk of producing new genotypes of the pathogen. The main threat is that compatible mating types will produce progeny with increased pathogenic fitness and aggressiveness thus overcoming plant resistance. Such an occurrence has happened in Europe with the introduction of the A2 mating type of *P. infestans* (Drenth, Tas & Govers, 1994).

1.2.8 Disease transmission

Transmission pathways of *P. ramorum* are consistent with other aerial *Phytophthora* species and are summarised in [Figure 1.9].

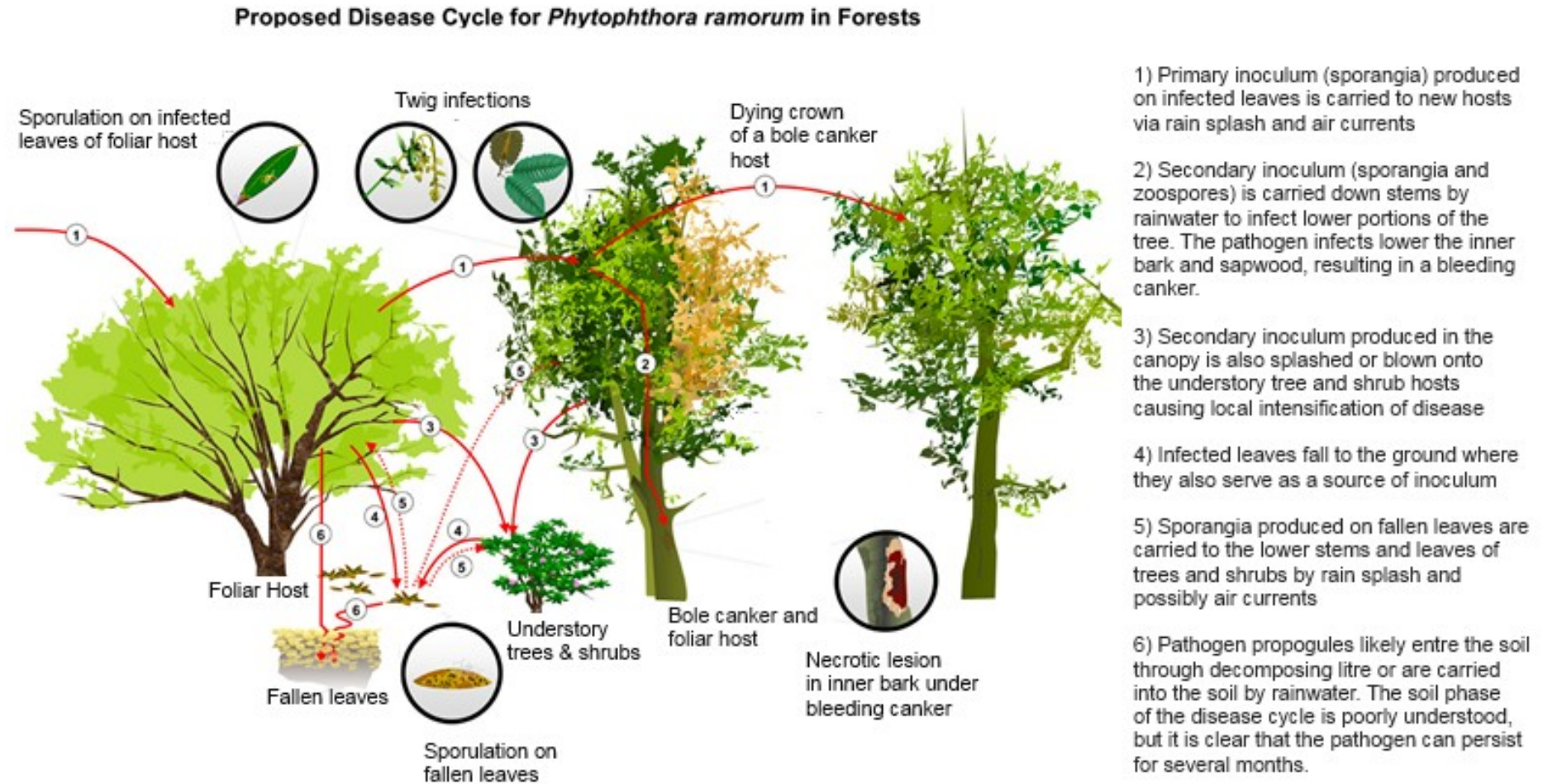


Figure 1.9 — Proposed disease cycle for *Phytophthora ramorum* in forests (Parke & Lucas, 2008).

Local dispersal of inoculum typically occurs through rain splash on infected leaves, dispersing caducous sporangia 5-10 m from the inoculum source (Davidson *et al.*, 2005; Moralejo *et al.*, 2006; Moralejo, Garcia Munoz & Descals, 2006). The pathogen is spread around the forest through repeated local dispersal of secondary inoculum from one neighbouring host to another (Sansford & Woodhall, 2007). The potential for long distance dispersal has been suggested by Aylor (2009), and *P. ramorum* has been recorded as dispersing over 4 km in Oregon Tanoak forests with more than half of all new infections being found more than 100 m, and 79% 300 m from the inoculum source (Hansen *et al.*, 2008). The mode of transmission over these distances is thought to be wind driven rain, with high winds associated with turbulent air blowing raindrops carrying spores over long distances and depositing sporangia or zoospores on new hosts, thereby creating new infection centres (Davidson *et al.*, 2005; Hansen *et al.*, 2008). Wind dispersal of *P. ramorum* without rain has not yet been demonstrated (Davidson & Shaw, 2003). It has been speculated that the recent discovery of EU2 isolates in south west Scotland could be due to prevailing winds blowing 'sporangial clouds' from heavily sporulating EU2 isolates on larch plantations in Northern Ireland across the Irish Sea to the adjacent area of Scotland (Van Poucke *et al.*, 2012).

Phytophthora ramorum can be detected in watercourses and has been recovered in a forested and riparian site 4 and 6 km downstream from infected forest (Davidson *et al.*, 2005). Its potential to disperse via watercourses in nature is debated in the literature. Hansen *et al.* (2008) suggest that watercourses are not an important source of distribution, having found no link between streams and the distribution of newly infected trees in Oregon. However, *P. ramorum* zoospores can survive up to 6 months in diH₂O in the laboratory (J. Davidson, unpublished data in (Davidson *et al.*, 2005) and can maintain a high population even at pH11 over 14 days and thus spread rapidly via irrigation systems (Kong *et al.*, 2012). In the nursery industry irrigation water is a major route of spread (Tjosvold & Chambers, 2008; Chastagner *et al.*, 2010). Viable propagules have been recovered annually in a stream in Washington State despite the nursery, which was the source of inoculum, later testing negative for *P. ramorum* (Chastagner *et al.*, 2010). Additional evidence which supports watercourses as a means of dispersal can be found in Mississippi. Here streamside vegetation tested positive for *P. ramorum* after exposure to contaminated stream water during high stream flows (Chastagner *et al.*, 2010). Moralejo and Descals (2011) speculate that diplanetism and microcyclic sporulation in the pathogen may provide a second opportunity for host infection and may increase the chance of dispersal over long distances in moving water. The relevance of watercourses in the epidemiology of this pathogen is still not fully understood, with the need to address inoculum threshold levels for successful

infection. Natural levels of inoculum in streams have been found to range between 0.0 - 2.5 infective propagules/ pearbait/ litre (Tjosvold & Chambers, 2008), but whether this is sufficient to initiate infection is unknown. Watercourses could be important reservoirs of inoculum as well as pathways for spread. Monitoring of watercourses show typically higher concentrations of propagules detected in stream water in the winter and spring in North America, coinciding with higher levels of inoculum present in forests (Tjosvold & Chambers, 2008).

Propagules of *P. ramorum* are frequently detected in soils (Davidson & Shaw, 2003; Davidson *et al.*, 2005; Fichtner, Lynch & Rizzo, 2007; Hansen *et al.*, 2008) which suggests that *P. ramorum* has a soil phase within its life cycle. The pathogen is able to survive and produce chlamydospores in leaves within forest soils in dry conditions, providing an inoculum reservoir for the onset of more favourable wetter conditions (Fichtner, Lynch & Rizzo, 2007). Soil and leaf litter may serve as a hospitable environment shielding the pathogen from environmental extremes (Fichtner, Lynch & Rizzo, 2006). Contaminated soil may serve as a source of primary inoculum for foliar infections by splash dispersal during rain events (Fichtner, Lynch & Rizzo, 2009). It may also serve as an indirect source of inoculum leading to infection of susceptible host seedlings via intermediate contamination of leaf litter as observed experimentally on Californian bay laurel seedlings (Davidson *et al.*, 2005). This suggests that soil may act as a source of inoculum, although this is not yet proven. In nature *P. ramorum* has been isolated from roots of tanoaks in California (Parke *et al.*, 2006) and rhododendrons in unmanaged woodlands in the UK (Fichtner *et al.*, 2011a).

Human movement of infected soil is another means of local as well as long distance dispersal. *Phytophthora ramorum* has been recovered from the soil of hikers' shoes (Webber & Rose, 2008) and along hikers' trails (Tjosvold *et al.*, 2002) with the probability of disease occurrence (across its geographic range in California) increasing as human population increases (Cushman & Meentemeyer, 2008). The pathogen can also be spread by humans in potting media (potting mix, soil and sand) (Shishkoff, 2007). *Phytophthora ramorum* can survive up to 6 months in potting media when introduced as sporangia and 12 months as chlamydospores, and as such can be disseminated geographically without being detected visually (Linderman & Davis, 2006). The pathogen has also been recovered from rootless forest substrates in European climatic conditions 33 months after inoculation (Vercauteren *et al.*, 2013).

The nursery trade has played a major role in the dispersal of *P. ramorum* throughout Europe (Brasier & Jung, 2006; Davidson & Shaw, 2003) and America (Frankel, 2008), with all three

lineages of *P. ramorum* having been identified in US nurseries (Ivors *et al.*, 2006). An example of this can be seen in the introduction of the pathogen into Majorca, which was traced back to a shipment of infected rhododendrons (Moralejo & Werres, 2002). In 2004 the spread of the pathogen in the US was linked to a nationwide nursery supplier in California, which had shipped potentially infected material to 783 garden centres in 39 states in a single year (Stokstad, 2004). In addition, anecdotal evidence suggests the pathogen has been introduced to regions of forest adjacent to landscaped areas with infected rhododendron in Northern California (Sansford & Woodhall, 2007). Similarly, in Cornwall evidence suggests that *P. ramorum* spread from planted rhododendrons present in infected nurseries onto trees (Brasier & Jung, 2006). Despite emergency phytosanitary measures *P. ramorum* continues to be detected on EU commercially-traded plants (Slawson, Blackburn & Bennett, 2007). Long distance spread may occur through the movement of infected foliage and stems (Davidson & Shaw, 2003), growing media (Linderman & Davis, 2006) and possibly even bark (Brown & Brasier, 2007). In addition to long distance movement of infected material, *P. ramorum* propagules have been detected in both standing and flowing water in and around nurseries (Chastagner *et al.*, 2010) with the potential to spread into local areas.

Other suggested modes for dispersal include insect and bird vectors, however to date no evidence supporting this have been found (Hansen *et al.*, 2008). Experimental studies on grey garden slugs (*Deroceras reticulatum*) have demonstrated that when fed on foliage infested with chlamydospores they were able to infect rhododendron and tanoak leaves and tanoak logs through their faeces (Parke *et al.*, 2008).

1.2.9 Host infection

1.2.9.1 Infectivity

Infection by zoospores of susceptible host plants typically occurs where water accumulates, for example at leaf tips. *Phytophthora ramorum* requires as little as 1 h dew period to infect attached *Rhododendron* leaves, 4 h to infect more than 10 percent of the leaves and periods of 24 - 48 h to achieve maximum infection (Tooley *et al.*, 2009). Maximum levels of zoospore germination occur at 100 percent humidity (Kessel, Werres & Webber, 2007). Infectivity in *Rhododendron ponticum* 'Cunningham's white' can occur in dew chambers in temperatures ranging from 10 - 31°C (Tooley *et al.*, 2009). Sporangium formation and zoospore release takes place over a wide range of temperature *in vitro*, ranging from 5 - 25°C, with highest numbers released at 15 - 20 °C (Davidson *et al.*, 2005). The optimum temperature for germination and infection rate is however, dependent on humidity. Infection rates for

Californian bay laurel leaves averaged 92% at 18°C, 50% at 12°C and 37% at 30°C in a study by (Garbelotto *et al.*, 2003). Detached leaf studies have shown that infection can occur on some species at concentrations as low as 10 sporangia/ ml (Tooley, Browning & Leighity, 2013).

Infection by the pathogen usually takes place through natural openings on the host plant e.g. stomata, lenticels (Florance, 2002) as well as wounds (Defra, 2005a; Lewis & Parke, 2006). One study found infection to occur on leaf surfaces in rhododendron only where stomata were present (Defra, 2005b). Conversely, examination of inoculated mature rhododendron leaves, by Lewis and Parke (2006), demonstrated that *P. ramorum* did not require stomata for leaf infection; instead the germ tubes were able to penetrate the leaf surface directly through the leaf cuticle. Infection potential can vary according to host, with some hosts requiring wounding for infection, and others not (Defra, 2005a). Germinating zoospores are also capable of penetrating the bark of mature trees (Florance, 2006; Brown & Brasier, 2007), although infection occurs most readily through thinner bark. Interestingly many sapling hosts are less susceptible to direct bark infections than mature trees, only becoming infected when wounded (Defra, 2005b). Increased susceptibility observed in mature host trees, could be due to the thickness and fissures of the bark. A study by (Swiecki & Bernhardt, 2006) highlighted a positive correlation between bark thickness and the risk of developing cankers associated with *P. ramorum* on coastal live oak. Bark thickness increases with stem diameter but in growing trees it rapidly expands creating fissures so it could potentially be a weaker barrier in mature trees compared to thin-stemmed saplings. A higher frequency of infection has also been observed in thin-barked tree species (American red oak, sweet chestnut, European beech) (Webber, 2004). Histological studies have also shown once the bark is penetrated the hyphae move along rays penetrating deeper into the host (Florance, 2006).

1.2.9.2 Susceptibility

Host susceptibility can vary under different inoculum pressures, with higher zoospore concentrations generally resulting in more symptoms (Hansen, Parke & Sutton, 2005). Host age can also affect susceptibility, as already mentioned in relation to bark infection (Defra, 2005b) while in detached-leaf dip assays, mature leaves of tanoak and California bay laurel were less susceptible than young leaves (Hansen, Parke & Sutton, 2005). In rhododendron species and cultivars, young leaves were more resistant than mature leaves (De Dobbelaere, Heungens & Maeas, 2006). Changing susceptibility with age in rhododendron species is thought to be due to resistance operating at the level of leaf penetration, as

wounding the leaves made all ages susceptible. This resistance has been shown to be associated with leaf hydrophobicity, which may prevent zoospores reaching the leaf surface, or by the number of leaf hairs, which may form barriers preventing penetration (De Dobbelaere, Heungens & Maes, 2008; De Dobbelaere *et al.*, 2010). Denman *et al.* (2005) however, found leaf susceptibility to *P. ramorum* in broadleaf trees was increased in younger leaves compared to older leaves.

Susceptibility also varies with temperature and season. Exposure of rhododendrons to high temperatures prior to inoculation resulted in decreased susceptibility, possibly associated with stomatal closure preventing zoospores gaining entry (De Dobbelaere, Heungens & Maes, 2008). In the field, susceptibility of rhododendron is significantly lower during late autumn and winter in California (De Dobbelaere, Heungens & Maes, 2008). In the UK, similar seasonal variation has been observed in the susceptibility of host tree log inoculations with other *Phytophthora* species, with the highest susceptibility levels in the summer and autumn and apparent resistance in early spring (Brasier & Kirk, 2001).

1.2.9.3 Colonisation

Phytophthora ramorum has been observed colonising an array of different host tissues although only a few host species have been examined in detail. Hyphae have been located in the cortex, phloem, xylem and pith of necrotic zones in rhododendron twigs (Pogoda & Werres, 2004). It has also been observed in the roots of rhododendrons (Parke & Lewis, 2007; Fichtner *et al.*, 2011a; Shishkoff, 2011; Vercauteren *et al.*, 2013), tanoaks (Parke *et al.*, 2006), Californian bay laurel (Shishkoff, 2008) and numerous other host species (see Shishkoff, 2008; Shishkoff, 2011).

Colonisation of different tissues can vary according not only to host species, but also to host age. A study by Lewis and Parke (2006) revealed that the pathogen was able to spread from the initial point of infection through the leaf tissues of a young rhododendron to the petiole and into the stems, moving both up and down, and into adjacent leaves. However, in mature leaves infection remained within the leaves without advancing into petioles and stems.

Hyphae have been observed in abundance in the sapwood and xylem vessel tissues in tanoaks (Parke *et al.*, 2007), and in pith cells and both primary and secondary xylem of rhododendron stems (Lewis & Parke, 2006). Analysing samples taken from a range of broadleaf tree species (*Acer pseudoplatanus*, *F. sylvatica*, *Q. acuta*, *Q. cerris*, *Q. petraea* and *Schima argentea*) Brown and Brasier (2007) typically found that colonisation within the xylem occurred beneath phloem lesions, with the pathogen able to spread in the xylem

tissues ahead of phloem lesions in *Q. cerris*. The xylem provides a potential 'superhighway' for rapid spread of the pathogen vertically through the plant and may provide a link between foliar and twig infections and bole cankers (Brown & Brasier, 2007; Parke *et al.*, 2007).

Invasion of the xylem tissues reduces transpiration (Parke *et al.*, 2007; Collins *et al.*, 2009) and may contribute to crown mortality which previously was thought to be a result of the pathogen impeding transport of photosynthate via necrosis of the phloem (Parke *et al.*, 2007). Chlamydospores have also been observed in the xylem (Parke *et al.*, 2007), cortical parenchyma (Pogoda & Werres, 2004; Parke *et al.*, 2007) and roots of hosts (Shishkoff, 2008).

1.3 Ramorum disease on larch in the UK

1.3.1 *Phytophthora ramorum* discovered on larch

In August 2009, widespread areas of Japanese larch (*Larix kaempferi*) in the West Country were found with symptoms of branch death and extensive crown dieback [Figure 1.10] (Webber, Mullett & Brasier, 2010; Webber, Turner & Jennings, 2010). During investigations into the cause of the symptoms on the larch at an affected site in the Glynn Valley, *Rhododendron ponticum* bushes displaying symptoms typical of *P. ramorum* infection were found next to juvenile (young thicket stage) larch trees with foliar symptoms. Samples from both the larch and rhododendron were taken from the site and confirmed to be caused by *P. ramorum*. Later, bark samples from beech (*Fagus sylvatica*) and foliage from sweet chestnut (*Castanea sativa*) taken in an area with sparse rhododendron cover were also found to be positive for *P. ramorum*.



Figure 1.10 - *Phytophthora ramorum* affecting larch trees in Plym Forest South West England (Picture provided by Forestry Commission)

At the same time, in Plym Forest near Plymouth, a mature beech with resinous bark lesions was found growing next to a plantation of juvenile Japanese larch with extensive dieback. Bark samples taken from the beech tested positive for *P. ramorum*. Surprisingly, no rhododendron was found growing under or close to beech or larch trees. Tests on bark samples taken from the main trunk of a larch tree with necrotic phloem also tested positive

for *P. ramorum*. Additionally, the foliage of three sweet chestnut trees on the same site but some distance away from the beech tree and rhododendrons but next to symptomatic mature larch, again tested positive for *P. ramorum*. Further sampling at this site revealed resinous lesions on the stems and branches of western hemlock (*Tsuga heterophylla*) trees from which *P. ramorum* was also isolated (Webber, Turner & Jennings, 2010).

A similar situation was observed on a site in Canonteign, west of Exeter with *P. ramorum* being isolated from the bark of mature larch and sweet chestnut trees with stem lesions and crown dieback. Once again, coverage of rhododendron on the site was sparse and *P. ramorum* infection on these plants was sporadic.

By September 2009, surveys of the Peninsula Forest District had found *P. ramorum* infecting rhododendrons throughout the area. Nevertheless, these infections were patchy and frequently distant from symptomatic trees (Webber, Turner & Jennings, 2010), suggesting that the epidemic on larch was being supported by an alternative significantly sporulating host. An observation supporting this theory was the way in which the majority of *P. ramorum*-incited bleeds on broadleaf species were found high up the trunks, suggesting the inoculum was coming from above (i.e. tree canopies) and not from below (i.e. the rhododendron understory) (Webber, 2011). As sweet chestnut was known to be able to support sporulation *in vitro* (Denman *et al.*, 2006) it was considered as a possible source of inoculum. However, the finding of necrotic larch foliage raised questions about the epidemiological significance of larch as a host and whether it could support sporulation.

The ability of Japanese larch to support significant levels of sporulation was later confirmed by Webber *et al.* (2010). They demonstrated that the needles of Japanese larch could support production of exceptionally high numbers of sporangia, with counts of up to 2,685 sporangia on some needles. Immediate biosecurity procedures were put into place to reduce the risk of infection spreading and limit further production of spores. The measures included removal of rhododendron in the understory of infected larch as well as felling of infected larch sites. Additionally, national surveys involving both aerial and land-based assessments were undertaken to determine the distribution of *P. ramorum* in larch plantations. By the end of 2010, *P. ramorum* had been detected on Japanese larch not only in south west England but in Wales, western Scotland, Northern Ireland, The Isle of Man and the Republic of Ireland (Forestry Commission, 2012b). It had also been found infecting Lawson cypress (*Chamaecyparis lawsoniana*) trees in Scotland (Brasier & Webber, 2012).

In 2011, further outbreaks on Japanese larch were identified in Derbyshire (the Peak District), Lancashire and in Cumbria in north west England, along with the island of Mull in

western Scotland [Figure 1.11]. The number and size of outbreaks confirmed by Forest Research were however, found to be significantly lower than those recorded in the previous two years (2009 and 2010), and many of the new sites were close to previously identified infected sites (Forestry Commission, 2012b). The apparent reduction in the spread of the disease is speculated to be the result of dry weather in the summer of 2010, resulting in limited sporulation (a process that requires high humidity).

In March 2011, European larch (*Larix decidua*) was confirmed as a natural host and by September 2011 hybrid larch (*L. eurolepis*) was also found infected in the field. A further finding of naturally infected Sitka spruce (*Picea sitchensis*) in the Republic of Ireland also raised concerns as this is the most important commercial conifer species in the UK. It had been hoped that European larch would not be susceptible and could continue to be grown commercially in Britain in place of Japanese larch. Previous laboratory testing of Sitka spruce for the European RAPRA project categorised this species as moderately susceptible to *P. ramorum* on wounded bark, however, unwounded bark was ranked low in susceptibility (Sansford *et al.*, 2008). The naturally infected Sitka spruce tree was, however, a small single tree and had been exposed to a high inoculum pressure, growing below the canopy of a large infected rhododendron. No other Sitka spruces on the site were infected. In light of this, and taking into consideration that only a handful of samples sent for diagnosis since 2010 have been found to be positive for *P. ramorum*, it is considered that *P. ramorum* does not pose a significant threat to Sitka spruce crops in Britain (Forestry Commission, 2012b).

The disease epidemic appeared to be slowing down by 2012, with only 1.1 thousand hectares of larch requiring felling compared to 2.3 thousand hectares in 2011 (Forestry Commission, 2012a).

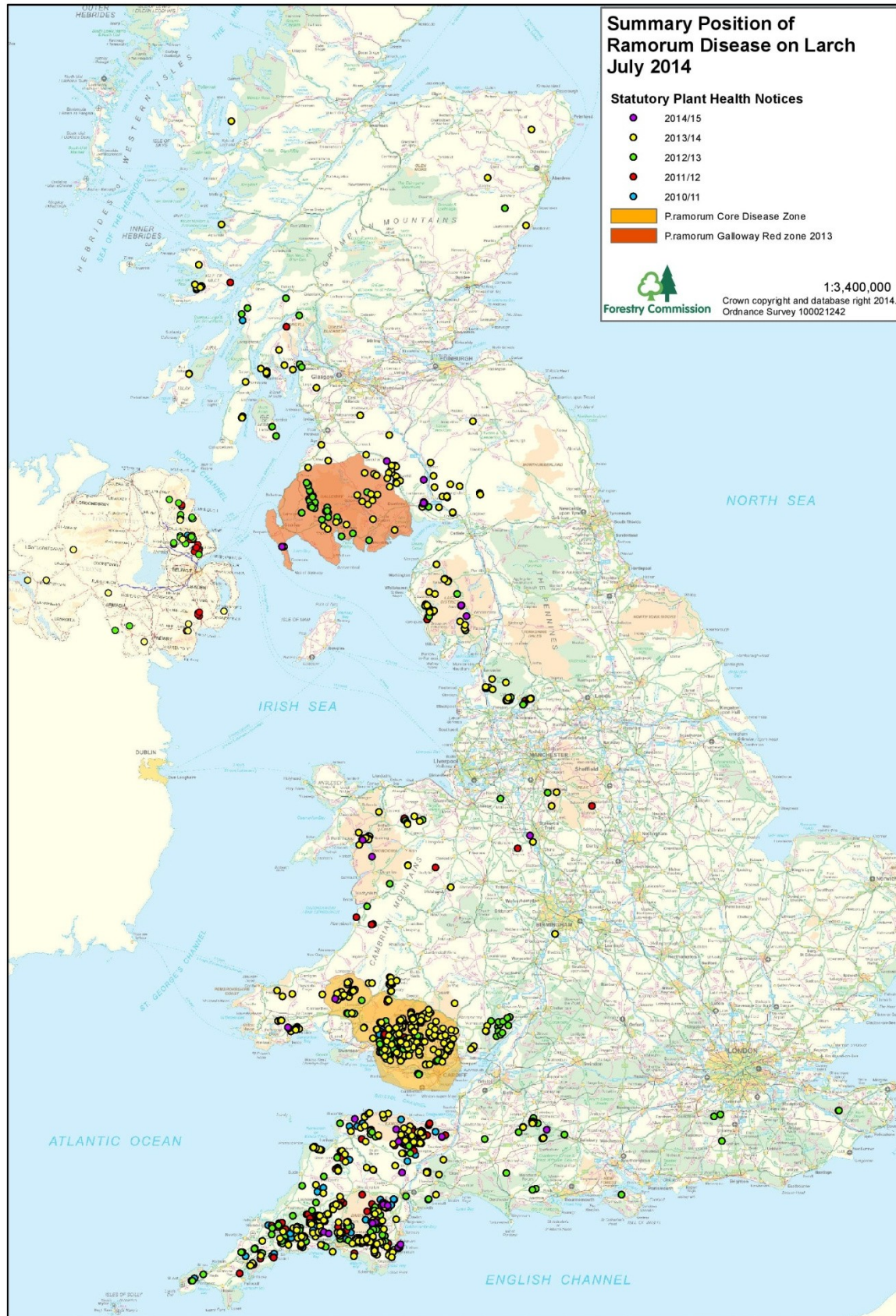


Figure 1.11 – Summary position of Ramorum disease on larch (Forestry Commission, 2014). Statutory plant health notices issued; blue dots 2009-10, red dots 2010-2011, green dots 2011-2012, yellow dots 2012-2013, purple dots 2013-2014.

Nevertheless, 2013 saw a resurgence of the disease with increased areas of felling, particularly in western Scotland and Wales [Table 1.3]. This increased intensity of the disease was probably the result of weather condition in 2012 (second wettest on record (Met Office, 2012b)) which was highly conducive for sporulation and dispersal via rain splash.

By the end of 2013 over 10,000 hectares has been felled or were under notice to fell, which is estimated to be more than 3 million trees (Forest Research, unpublished data). Such rapid spread of *P. ramorum* in larch has many parallels with its spread on tanoaks in North America.

Table 1.3 – Area (ha) of felling within sites issued with Statutory Plant Health Notices due to *Phytophthora ramorum* (Forest Research, unpublished data)

Year	England	Wales	Scotland	Northern Ireland	United Kingdom
2010	1,107.3	876.6	1.6	283.0	2,331.0
2011	492.8	545.0	69.6	71.4	1,178.8
2012	494.5	1,280.0	362.4	150.8	2,287.7
2013	798.1	3,265.0*	285.0*	494.2	4,842.3
Total	2,955.2	5,966.6	718.6	999.4	10,639.9

* Additional 468 ha and 6,000 ha of larch suspected to be infected with *P. ramorum* not under a SPHN in Wales and Scotland respectively

1.3.2 Quarantine and diagnostics

Phytophthora ramorum is a quarantine organism listed under EU Emergency Measures (Commission Decision, (2002/757/EC)), therefore, its presence or suspected presence in Britain must be notified to authorities. Forestry Commission Plant Health Inspectors have the power to serve statutory Plant Health Notices, which are enforceable by law, on woodland owners of infected or suspected infected stands. Notices require the felling of sporulating host trees, along with other disease management measures.

Laboratory diagnosis of *P. ramorum* in bark and foliage samples from larch has proved challenging, with approximately two-thirds of all symptomatic material tested by direct isolation returning inconclusive results (Webber, 2011). It is speculated this may be due to the high level of tannins present in larch bark (Aaron, 1982) and such compounds are known to reduce growth and sporulation of *P. ramorum* (Manter *et al.*, 2010b). As a consequence, diagnosis initially relied on several additional indicators. This included visual characteristic symptoms on the trees, field tests on bark and foliage using lateral flow devices (LFDs)

which can detect the presence of *Phytophthora* to genus level; infection on nearby *Rhododendron ponticum* in the forest; and detection of *P. ramorum* in larch litter from the forest floor (Forestry Commission, 2012b). All these indicators, even if the pathogen could not be isolated from symptomatic bark or foliage samples, resulted in a suspect site being served a Plant Health Notice. As the epidemic has persisted however more reliable and rapid diagnostic methods have been developed using molecular techniques to detect the pathogens DNA in suspect bark samples. Such methods have proved to be highly sensitive compared to both direct isolation and identification using LFDs (Hunter *et al.*, 2014).

1.3.3 Research to date

Since the discovery of *P. ramorum* on larch, Fera and Forest Research have focused their research primarily on the extent to which *P. ramorum* poses a threat to forestry and the timber industry and the potential for spread throughout the UK. Dendrochronological assessments concluded that whilst it remains possible that abiotic factors such as the winter cold damage of late 2008/ early 2009 exacerbated bark killing by the pathogen, it is more likely that *P. ramorum* colonisation of bark, causing multiple stem and branch infection, was the singular cause for the extensive dieback in Japanese larch in 2009 (Webber, Turner & Jennings, 2010). Tests on the ability of the pathogen to over-winter in buds of infected Japanese larch trees proved negative, whilst larch litter shed in 2009 in infected plantations tested positive, suggesting that the pathogen is likely to persist on infected sites post removal of infected trees (Webber, Turner & Jennings, 2010). Field-based bark susceptibility tests have proved inconclusive due to low levels of inoculum in the field during the trial (Webber, Turner & Jennings, 2010), however *P. ramorum* can be isolated from bark samples in the lab (Webber, Turner & Jennings, 2010).

Research into the important biosecurity question of whether *P. ramorum* can be found in the sapwood of infected trees, as well as on the foliage and bark, has concluded that whilst the pathogen could be isolated from the sapwood in approximately 25 per cent of samples tested, infections were superficial, only penetrating 1-2 mm into the sapwood below bark lesions (Webber, Turner & Jennings, 2010). Therefore, if logs harvested from infected stands are subjected to appropriate biosecurity protocols by removing and burning the bark and outermost layers of sapwood, entry into the wood chain is unlikely. As such, larch wood from infected sites free from bark and cambium has no restrictions on its use and movement (Forestry Commission, 2010).

Monitoring of spores using rain traps in infected areas of Plym and Largin wood consistently resulted in detection of high levels of spores between October and December 2009, with the maximum number detected being 100 spores/ mL and 4,000 spores/ mL at Largin and Plym respectively, at the beginning of November (Webber, Turner & Jennings, 2010). A drop in the level of spores detected in rain traps following this coincided with end of needle fall from the trees. Results also showed that the frequency of spores trapped declined with distance from infected areas, although, spores were detected up to 1 km from diseased larch sites (Webber, Turner & Jennings, 2010).

Field assessment of mature 30 m tall European beech (*F. sylvatica*) growing within a mixed species block next to a compartment of *P. ramorum*-infected Japanese larch of a similar height provided further evidence of Japanese larch supporting high levels of sporulation in the field. Multiple bleeding cankers caused by *P. ramorum* occurred 7 - 11 m above ground on the beech trees in a 10 km wide zone neighbouring the infected larch (Webber, Turner & Jennings, 2010). As distance increased from the infected larch, the number and height of bleeding cankers decreased. Beech trees with bleeding cankers were found up to 90 m away from the infected larch (Webber, Turner & Jennings, 2010). This finding coincides with aerial dispersal distances in Oregon from sporulating host trees; these are typically around 100 m but 'jumps' of up to 3 km have been recorded (Rizzo, Garbelotto & Hansen, 2005). In contrast, inoculum dispersal from rhododendron, which is an understory shrub, occurs over relatively short distance. Aerial dispersal has reported for up to 50 m with the inoculum being capable of causing lesions on the stems of host plants most often only 2 m distance (Brasier & Jung, 2006). The dispersal of *P. ramorum* from the canopy of mature larch trees could pose a more serious threat to susceptible trees and habitats, as the increase in the height of inoculum source is likely to increase the risk of spread over longer distances.

1.4 Project Aims and Objectives

The overall aim of this research is to enhance our understanding of 'ramorum' disease on larch in the UK and thus facilitate effective management of the pathogen in forest plantations. Current uncertainties include the lack of information on the relative susceptibility of commercially grown larch in the UK to *P. ramorum*, and the ability of the pathogen to sporulate on larch foliage. Such data could be used in conjunction with data from the US on likely distance of aerial spread of the pathogen from sporulating hosts to determine risk of pathogen spread and its impact. Results will also be used to make informed recommendations of which species, other than Japanese larch, that could continue to be grown commercially in Britain or those at risk that may be less viable as forestry crops given the presence and potential spread of *P. ramorum*. There is also a need to establish whether any genetic or adaptive changes have occurred in the *P. ramorum* population since the epidemic spread to larch. Knowledge is also needed of the persistence of the pathogen on infected sites to establish how quickly cleared infected sites can be restocked with larch or indeed with any other susceptible tree species.

To achieve this aim, the following research objectives were formulated:

Objective 1: To evaluate the potential of the foliage of larch species used in the UK forestry (Japanese larch (*L. kaempferi*), European larch (*L. decidua*) and hybrid larch (*L. eurolepis*)), to support sporulation by *Phytophthora ramorum* and therefore act as a source of inoculum for other known susceptible tree hosts in the absence of any infected *Rhododendron*. Also to compare sporulation potential on larch against other major sporulation hosts such as California bay laurel (*Umbellularia California*), sweet chestnut (*Castanea sativa*) and bilberry (*Vaccinium myrtillus*).

Objective 2: To determine if the sporulation potential of larch foliage varies with season, and with *P. ramorum* genotype and lineage.

Objective 3: To evaluate the bark susceptibility of Japanese, European and hybrid larch to *P. ramorum* and comparative susceptibility. Additionally, use this method as a tool to explore variation in the pathogen to look for adaptive differences.

Objective 4: To determine how readily *P. ramorum* can persist in the litter of infected larch sites after felling, and if the degree of sporulation on foliage can be related to levels of site contamination.

Objective 5: To determine the pathogenic potential of the new European lineage of *P. ramorum* against key tree species to establish if it shows adaptive differences. Also to compare temperature-growth responses and pathogenicity on *Rhododendron* of key genotypes to reveal any differences in pathogen behaviour.

Chapter two - Materials and general methods

2.1 Introduction

This chapter outlines the source of *Phytophthora ramorum* isolates, their maintenance and the growth media used in experiments during this study. Additionally, methods which were used across a series of experiments within this study are described.

2.2 Quarantine procedures

Phytophthora ramorum is a quarantine organism listed under EU emergency measures (Commission Decision 2002/757/EC, as amended) and as such this study was required to comply with plant health legislation. All experiments were carried out in licensed quarantine laboratories at Forest Research, Alice Holt under the conditions specified in license PHL 297/6608(07/2011). All plant material, cultures and media were autoclaved after experimentation prior to disposal. Re-usable equipment was surface sterilised by washing in bleach and spraying with IMS before autoclaving.

2.3 Sterilisation methods

To avoid contamination of cultures aseptic conditions were maintained throughout all experiments. All agar media were sterilised by autoclaving (details specified below). Culturing utensils were sterilised by heating in a flame, cooling in methylated spirit (meths) and evaporating off the remaining meths. Reusable plastics were sterilised by washing in bleach, drying and spraying with meths.

In molecular studies, pipette tips and microcentrifuge tubes (MCT) were sterilized by autoclaving and then oven dried. Glassware was washed in 0.4 M hydrochloric acid (HCl) prior to sterilisation via autoclaving. Utensils were sterilised by dipping in 4% HCl, rinsing in sterile distilled water (SDW), heating in a flame, cooling in meths and evaporating off the remaining meths. Iron ball bearings were sterilised by soaking in 4% HCl for three minutes and rinsing twice in SDW.

2.4 Source of *Phytophthora ramorum* cultures

A range of isolates of *Phytophthora ramorum* were used in this study. All isolates came from the Forest Research Phytophthora culture collection held at Alice Holt. The majority of UK isolates within this collection were obtained through surveys carried out as part Defra's Phytophthora programme.

2.5 Culturing

2.5.1 Maintenance of cultures

The Forest Research Phytophthora culture is stored on Carrot Agar (CA) slopes in 7 ml plastic specimen tubes under paraffin oil, incubated at 15°C in the dark. Cultures in regular use during this study were grown on nine centimetre diameter disposable Petri dishes on CA and incubated at 20°C in darkness. These cultures were then sub-cultured every two to three weeks.

2.5.2 Artificial growth medium

2.5.2.1 Carrot Agar (2%)

Carrot agar was used for general maintenance of cultures, sporangia and zoospore production and measuring linear growth. Methods were taken from Brasier (1967) as follows: 200 g of organic carrots were washed and macerated in a blender with 500 ml of tap water. The carrot water mix was then filtered through muslin (squeezed to extract all the juice) into one litre media bottles containing 15 g of technical agar (Oxoid® n°3). A further 500 ml of tap water was added to make the carrot juice up to one litre and gently shaken by hand to mix. Media bottles were autoclaved twice for ten minutes at 121°C/15 lb psi.

2.5.2.2 Modified synthetic mucor agar

Modified synthetic mucor agar (SMA) is a *Phytophthora* selective medium which is based on the synthetic mucor agar of Elliot *et al.* (1966) with the addition of antibiotics (Brasier *et al.*, 2005). It was used in all isolation experiments (bark, baits & plant foliage) throughout this study. SMA was prepared 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 (Oxoid®

n°3), 0.5 g potassium dihydrogen orthophosphate, 0.5 ml 4% benomyl hydrochloride solution and 1000 ml distilled water were mixed in one litre media bottle. The pH was adjusted to 6.5 with 1 M sodium hydroxide. After autoclaving at 121°C/15 lb psi for 15 minutes the agar was cooled and 0.4 ml of a 2.5% suspension of pimaricin and 3 ml of a 1% w/v solution of rifamycin SV was added. After pouring, plates were stored in the fridge and used within two weeks.

2.5.2.3 V8 agar (2%)

V8 agar was used in growth rate experiments in chapters six. Methods for V8 agar preparation were taken from Franceschini *et al.* (2014) and were prepared as follows: 200ml V8 Juice®, 6 g calcium carbonate, 20 g technical agar (Oxoid® n°3) and 800 ml distilled water were mixed and autoclaved at 121°C/15 lb psi for 15 minutes. Unlike other V8 agar methods no centrifugation was carried out.

2.5.2.4 Potato dextrose agar (2%)

Potato dextrose agar (PDA) was used in growth rate experiments in chapter six. It was prepared by suspending 39 g of Oxoid® PDA powder in one litre of distilled water and autoclaved at 121°C/15 lb psi for 15 minutes.

2.6 Isolation

Isolation methods were used throughout this study. All experiments involved direct isolation from bark or plant foliage in order to satisfy Koch's Postulates. Indirect isolation via baiting was used in chapter five to isolate the pathogen from soil and litter samples. All resulting colonies formed on SMA were sub-cultured on to CA for identification (see section 2.8).

2.6.1 Direct

Bark samples were prepared by removing the periderm to reveal the phloem lesion, which when infected with *P. ramorum* is typically cinnamon brown in colour with red/pink margins. The latter were avoided as it is difficult to isolate *P. ramorum* from this tissue (Webber, Turner & Jennings, 2010) [Plate 2.1]. Small fragments (~4 x 4 x 0.5 mm²) were cut from the leading edge of the lesion using a round bladed scalpel and plated onto SMA, with approximately 15 fragments per 9 cm Petri dish. Foliage samples were prepared by cutting fragments from lesion edges of symptomatic plant foliage. On non-symptomatic foliage,

fragments were cut from multiple regions on the leaves and placed on SMA. Larch needles were simply placed on SMA. Plates were incubated at 20°C in the dark and checked after 3-6 days.

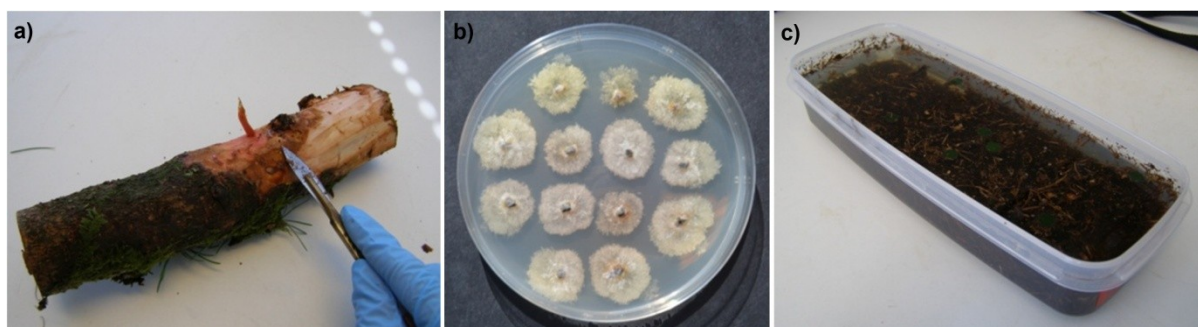


Plate 2.1 - Isolating *P. ramorum*; a) sampling from bark lesion; b) colonies growing from bark chips on SMA; c) baiting using rhododendron leaf discs

2.6.2 Baiting

Soil and litter samples were placed in sterile plastic containers and SDW was added to moisten [Plate 2.1c]. After 24 h the samples were flooded with SDW making sure the water level was at least 1.5 cm above the litter. Once the sample had settled, any remaining floating material was moved to one side and ten rhododendron leaf discs were placed on the surface of the water, abaxial facing down. Samples were incubated in natural light in an air-conditioned room at 18-20°C. After 5-7 days the leaf discs were removed and surface sterilised in 60% ethanol for 30 seconds and rinsed in SDW. The baits were then dried on sterile paper towels, plated onto SMA and incubated in the dark at 20°C for 3-6 days.

2.7 DNA extraction

Phytophthora ramorum DNA was extracted from plant material in all experimental chapters. Genomic DNA was extracted from *P. ramorum* mycelium and all plant material using Thermo Scientific KingFisher Plant DNA kits (Rev. 1.1, Cat. No. N11996). Methods for DNA extraction from bark samples were modified from protocols developed by Forest Research and FERA (Hunter *et al.*, 2014).

2.7.1 Plant foliage

Approximately 20-50 mg of sample material was cut into small pieces (~3 mm x 3 mm) and placed in 2.0 ml screw-cap MCTs along with an iron ball bearing and an initial quantity of 70

µl of lysis buffer solution (containing RNase A (0.25 mg/ml)). The samples were homogenised by grinding the material in a Retsch Mixer Mill MM200 (beadmill) for 3 minutes at a frequency of 30 (1/s). A further 500 µl of the lysis buffer solution was added to the resultant mixture which was then vortexed to distribute the ground material. The samples were then incubated for 30 minutes at 56°C and centrifuged for 20 minutes at 6,000 x g. The supernatant (400 µl) was transferred to a Thermo Scientific Microtiter deep well 96 plate and the DNA purified using the KingFisher™ Flex as per manufactures instructions for the KingFisher Plant DNA Kit. The purified DNA was then stored at -20°C until required.

2.7.2 Bark

Genomic DNA was extracted from bark samples using a method modified from that used for extracting DNA from plant foliage [section 2.7.1]. Approximately 100-200 mg of infected wood and bark fragments were cut into small pieces (~ 3 mm × 3 mm x 0.5 mm) and placed in 2.0 ml screw cap MCTs. An initial quantity of 400 µl of CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl amended with 1% Na₂SO₃ and 2% Polyvinylpyrrolidone-40). Two iron ball bearings were added to the tubes, one before the addition of sample material and one after. The samples were homogenised by grinding the material in the beadmill for 4 minutes at a frequency of 30 (1/s). After 4 minutes the samples were checked and run for a further 4 minutes on the beadmill if the samples were insufficiently macerated. A further 500 µl of CTAB extraction buffer was added and samples vortexed to distribute the ground material. As with the plant foliage methods the samples were then incubated for 30 minutes at 56°C and then centrifuged for 20 minutes at 6,000 x g. The supernatant (400 µl) was then transferred to a Thermo Scientific Microtiter deep well 96 plate and the DNA purified using the KingFisher™ Flex as per manufactures instructions for the KingFisher Plant DNA Kit. The purified DNA was then stored at -20°C until required.

2.8 Identification

2.8.1 Culturing

2.8.1.1 Identification of *P. ramorum*

Isolates for identification were placed on CA and incubated at 18-20°C under continuous daylight (60W Daylight bulbs, suspended 30 cm above the plates). After 14 days the presence of *P. ramorum* was determined based on morphological characteristics [Plate 2.2];

primarily the large chlamydospores formed on short pedicels and semi-papillate, caducous sporangia (Werres *et al.*, 2001).

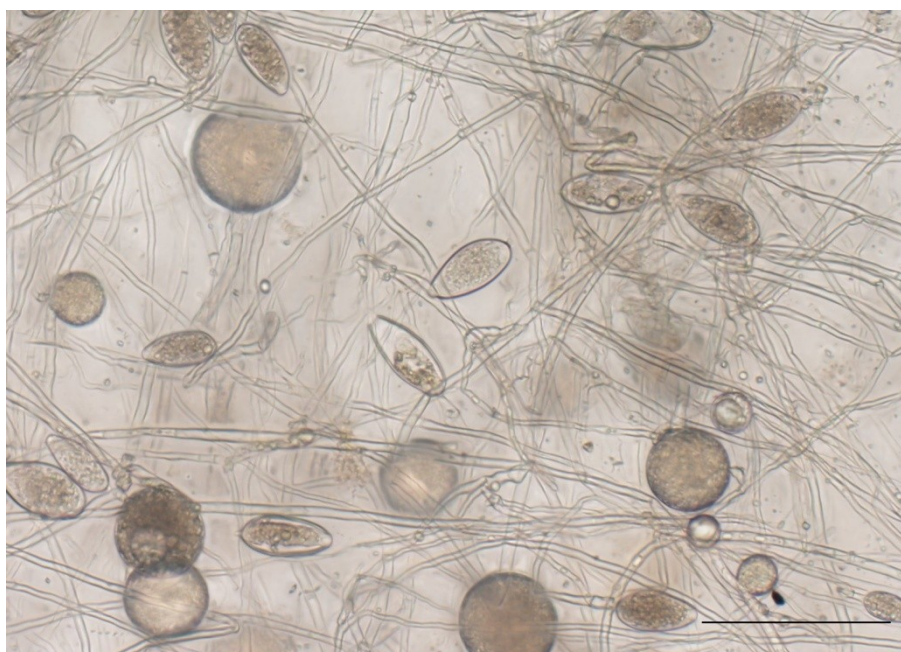


Plate 2.2 - *Phytophthora ramorum* culture growing on CA after 14 days (scale bar = 100 μ m).

2.8.1.2 Identification of EU1 and EU2 isolates

Cultural methods used in this study to discriminate between EU1 and EU2 lineages of *P. ramorum* were developed by Franceschini *et al.* (2014). Lineage was determined by colony development on 2 % V8 agar [section 2.5.2.3] incubated in the dark at 25°C and 28°C. At 25°C isolates from EU2 lineage can be distinguished from EU1 isolates due to their faster growth rate (approximately 3.84 ± 0.03 and 2.92 ± 0.29 mm/day⁻¹ respectively (Franceschini *et al.*, 2014)). At 28°C isolates were identified as EU1 on the basis of “dense-woolly” colonies with irregular to lobed margins” whilst EU2 isolates produced “finely striated colonies with uniform or sharp colony margins and slightly “floccose centres” (Franceschini *et al.*, 2014). Test isolates were compared to four standards of each lineage [Table 2.1].

Table 2.1 - *Phytophthora ramorum* isolates used as standard in the study. The lineage of all isolates was confirmed via colony development and restriction fragment length polymorphism test, except for isolate P2111 for which lineage was designated only by colony development (Franceschini *et al.*, 2014).

Lineage	Isolate	Region	Host	Date Isolated
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EU1	P1367	N/A	<i>Viburnum bodnantense</i>	N/A
EU1	P1376	Sussex, England	<i>V. tinus</i>	April 2002
EU1	P1577	Germany	<i>Rhododendron catawbiense</i>	2002
EU1	P1578	UK	<i>R. grandiflora</i>	N/A
EU2	P2111	Down, Northern Ireland	<i>Quercus rubra</i>	November 2007
EU2	P2460	Antrim, Northern Ireland	<i>Larix kaempferi</i>	August 2010
EU2	P2461	Antrim, Northern Ireland	<i>Larix</i> sp.	August 2010
EU2	P2462	Antrim, Northern Ireland	<i>Larix</i> sp.	August 2010

2.8.2 Real time polymerase chain reaction (PCR)

Methods for the molecular diagnosis of the presence of *P. ramorum* in plant material in this study used a modified version of those developed by Forest Research and Fera (Hunter *et al.*, 2014) using real-time PCR assays based on TaqMan chemistry. This method relied upon the oligonucleotide primers Pram-114F, Pram-190R and molecular Pram probe (Hughes *et al.*, 2006; Tomlinson *et al.*, 2005) for detection of *P. ramorum*, targeting the internal transcribed spacer 1 region (ITS 1) of the nuclear ribosomal (nr) RNA. Internal controls targeting the cytochrome oxidase (COX) gene were used to verify efficient DNA extraction and absence of PCR inhibitors. The internal controls consisted of the plant primers COX F, COX RW and the COX probe (Hughes *et al.*, 2006) [Table 2.2]. Real-time PCR reactions were run on a LightCycler® 480 II using 384-well reaction plates. Reactions consisted of 1x LightCycler® 480 Probes Master (Roche Applied Science), 0.3 µM of each Pram primer, 0.1 µM Pram probe, 0.2 µM of each COX primer and 0.1 µM COX probe, 2.5 µl template DNA and PCR-grade water to a final volume of 10 µl. Reactions were carried out under the following conditions: 95°C for 10 minutes pre-incubation period; followed by 40 cycles at 95°C for 15 seconds for denaturation, primer annealing took place at 60°C for one minute followed by 72°C for one second for primer extension and cooling at 40°C for 1 second. DNA of *P. ramorum* was used in each run as a positive control and PCR grade water was used as a negative control. DNA extracts were tested in duplicate.

Crossing point (Cp), the point at which fluorescence of the sample rises above the background ‘noise’ was calculated using the second derivative maximum method available in the LightCycler® 480 II detection software. The mean Cp value and standard deviation were calculated for each set of duplicate samples. Cut-off values for both the COX and ITS

tests established by Forest Research diagnostic laboratory were used. Positive Cp values for the *P. ramorum* ITS rDNA operon were those with a Cp value of 28.5, while positive Cp values for the COX gene region were below a Cp value of 31.5. For a positive detection of *P. ramorum*, positive Cp values for both the COX region and ITS region were required. Samples that failed the COX reaction were repeated along with ‘suspicious’ Cp values (28.51 - 30.00 Cp) for the ITS region.

Table 2.2 - Characteristics of primers and TaqMan probes

Primer or probe	Sequence (5' - 3')	Reporter (5') ^a	Quencher (3') ^a
Pram-114F	TCATGGCGAGCGCTTGA		
Pram-190R	AGTATATTCAGTATTTAGGAATGGGTTTAAAAAGT		
Pram probe	TTCGGGTCTGAGCTAGTAG	FAM	BHQ1
COX F	CGTCGCATTCCAGATTATCCA		
COX RW	CAACTACGGATATATAAGRRCRRRAACTG		
COX probe	AGGGCATTCCATCCAGCGTAAGCA	JOE	BHQ1

^a FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1; JOE, 6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein

2.9 Log inoculation with mycelial plugs

Log inoculation methods adapter from Brasier and Kirk (2001) were used to assess the pathogenicity of *P. ramorum* isolates in chapter four and six.

2.9.1 Log preparation

Logs 130 cm long x 30-35 cm diameter were cut from trees 36 hours before the start of the experiment, from stems of mature, healthy (free from obvious lesions, bark damage and die back) trees. Trees with low hanging branches were avoided as the branches create knots in the bark and result in distorted lesion when inoculated with a pathogen, making lesion assessment difficult. Two logs were cut from each tree starting one metre up the stem from the base. After cutting, the ends and side branch wounds were sealed with Iso-flex[®] (non-phytotoxic bitumen sealant) to prevent moisture loss and fungal infection. Two bands were marked out 10 cm from each end of the log in case fungal ingress from the log ends occurred. A further three bands were marked out (with PVC tape), one around the middle of

the log and the other two equidistant between the middle band and each end band [Figure 2.1]. Inoculation points were marked out along the three inner bands, each 10cm apart and staggered around the circumference between each band, to prevent lesions merging together.

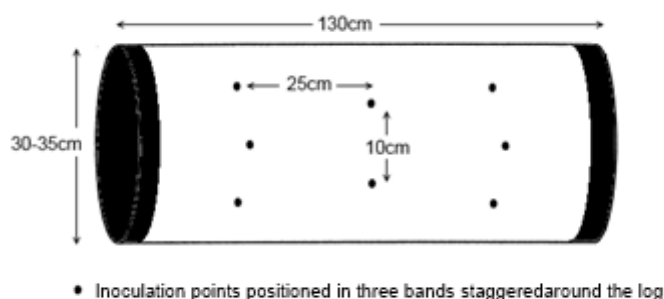


Figure 2.1 - Position of inoculation points and log set up. Adapted from Brasier & Kirk (2001).

2.9.2 Inoculation and incubation

At each inoculation point, a 5 mm diameter hole was cut through the bark to the wood surface using a sterilised cork borer. A 5 mm CA agar plug taken from the margin of an actively growing colony of *Phytophthora ramorum* was cut and inserted, mycelium side down into the hole. Two drops of SDW were added and the bark disk replaced in the hole. The wound was then covered with cotton wool soaked in SDW and a 10 x 10 cm piece of aluminium foil was placed over the top and secured with PVC tape which was stapled in place. Once all inoculation points were completed the log was sprayed with SDW and each inoculation band was wrapped in Parafilm® to help retain moisture throughout the experiment. Each log was placed in a tube of polythene, sprayed with SDW once more and then sealed at each end. The logs were stood upright and incubated at 18-20°C in an air-conditioned room.

One 'tester' log of each tree species was set-up in addition to the experimental logs for each experiment to allow checks on the progression of lesion development at the end of the desired incubation period, whilst maintaining replication, as sampling was destructive. If the lesions of tester logs were found to be small but still actively growing (edges of the lesions were 'fuzzy', indicating that the pathogen was still advancing in the tissues (Susan Kirk, personal communication) and at no risk of running into one another. Then the remaining logs were incubated further.

2.9.3 Sampling

The logs were destructively sampled by removing the periderm over the inoculation points to expose the phloem using a drawknife [Plate 2.3]. Upon exposure and prior to oxidative staining, lesions around the inoculation points were photographed and the outer edge and distinct coloured regions of the lesions were outlined in marker pen. Outlines were traced onto tracing paper and scanned on an Epson Perfection 1660 dpi photo scanner. The area of each lesion was calculated using Image J 1.45s (National Institutes of Health) or APS Assess 2.0 software.

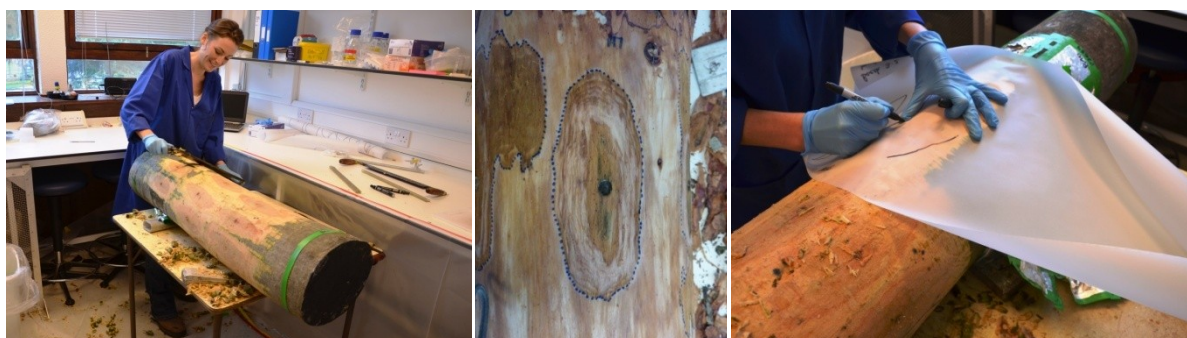


Plate 2.3 - Sampling lesions by a) removing periderm with a draw knife; b) marking the edge of the lesion; c) tracing the lesion.

2.9.4 Re-isolation

Fifteen small pieces of tissue were excised from the leading edges of the lesions and re-isolated using methods described in section 2.6.1 to fulfil Koch's postulates.

2.10 Larch sapling stems inoculation

Stems of larch saplings approximately two meters tall were cut in half, side branches were removed, each stem labelled according to the tree and region of the stem it came from e.g. top or bottom. A stem section from each tree was randomly allocated to the experimental temperature treatment.

2.10.1 Inoculation, incubation & sampling

Methods used to inoculate mature larch bark were adapted in order to inoculate the stems. Each stem had three inoculation points; one in the middle and one positioned equally spaced between the middle inoculation point and the end of the stem either side of the middle point. Inoculation points were made on opposite sides of the stem to prevent lesions from running into one another. One stem from each tree was inoculated with one isolate of each lineage and a CA plug which acted as a control. Isolates of each lineage were randomly allocated to a stem and inoculation position. Bark was removed at each inoculation point using a 2.5 mm diameter cork border and a 2.5 mm CA agar, plug taken from the margin of an actively growing colony of *P. ramorum*, was cut and inserted mycelium side down [Plate 2.4.a] The wound was then covered with cotton wool soaked in SDW and a piece of aluminium foil was wrapped around the inoculation point and secured with Parafilm®, which also aided in moisture retention [Plate 2.4.b]. Stems were placed in a plastic container standing upright and covered with a polythene bag (the inside of which had been sprayed with SDW) and were secured in place with an elastic band [Plate 2.4.c]. Stems were incubated at the desired temperature in the dark for seven days.



Plate 2.4 - Larch sapling stems a) inoculation technique, b) stem set up, c) incubation.

The stems were destructively sampled by removing the periderm over the inoculation points to expose the phloem, using a round bladed scalpel. Upon exposure, lesions were photographed and outlines were traced onto tracing paper and scanned on an Epson Perfection 1660dpi photo scanner. The area of each lesion was calculated using APS Assess 2.0 software.

2.11 Sporulation potential and foliage susceptibility

Foliage from selected hosts was tested for susceptibility (determined by symptom development/expression) and sporulation potential. Susceptibility testing used a detached-

leaf-dip assay adapted from earlier methods devised by (Parke, Linderman & Hansen, 2002; Denman *et al.*, 2005). Assessment of sporulation potential was based on methods adapted from (Fichtner *et al.*, 2012). See section 2.10 for details.

2.11.1 Inoculum production

Isolates were cultured on CA and incubated at 18-20°C under continuous daylight (60W Daylight™ bulb suspended 30 cm above the plates) for 14 days to produce sporangia. Zoospores were collected by flooding each plate with 5 mL of SDW, rubbing the surface of the culture with a sterile disposable spreader to dislodge the sporangia and collecting the liquid. Enough plates were processed to generate 50 mL of spore suspension. To induce zoospore release, the sporangia suspensions were placed in a refrigerator at 7°C in the dark for 1 h, then returned to room temperature (20°C) for a further 75 min (Parke, Linderman & Hansen, 2002). The resulting zoospore suspension for each isolate was then poured into a separate sterile glass beaker, leaving the sediment containing residual pieces of mycelium, chlamydospores and empty sporangia behind thereby generating a pure zoospore suspension. Zoospores were not separated from the sediment using a filter as this can agitate the zoospores causing them to encyst (Ahonsi *et al.*, 2010). Zoospore counts were obtained by taking a 300 µl aliquot of each zoospore suspension and placing it in a separate 1 mL microtube and agitating it for 90 seconds on a vortex mixer, initiating zoospore encystment to enable zoospore counts using a haemocytometer. The concentration of each suspension was adjusted to the desired concentration. Fifty millilitres of zoospore suspension for each isolate was also used to inoculate plant material. In all experiments SDW was used as a negative control.

2.11.2 Inoculation and incubation

The apical tip of each leaf or shoot was dipped into the zoospore suspension up to the 4 cm mark and gently swirled for 30 seconds. Plant material was dipped in a random order to minimise bias as foliage dipped at the start could be exposed to slightly higher zoospore concentrations than those dipped at the end. Once inoculated, each larch shoot was placed upright in a sterile tube (150 ml volume containing SDW) with foil lids in a test tube rack. The racks were then placed within polyethylene bags held open by connected straws to prevent the plant material from touching the sides.

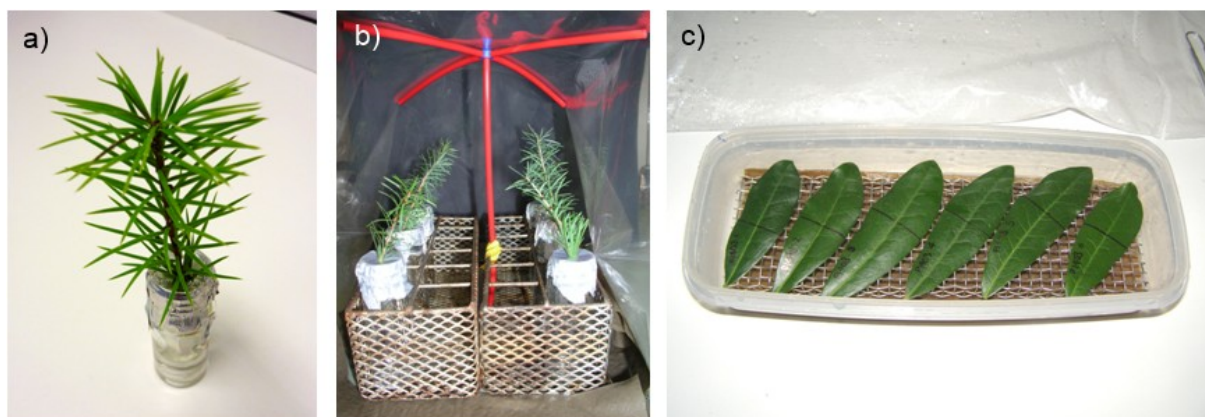


Plate 2.5 – Setup of; a) individual larch shoots, b) larch shoots damp incubation chamber, c) rhododendron leaves in incubation chamber used for broadleaved hosts

The bottom of each bag was lined with wet paper towels to create a humid incubation chamber and the inside surfaces were sprayed with SDW prior to placing the shoots inside [Plate 2.5]. Inoculated leaf material was placed on a raised sterile metal grid within plastic sandwich boxes, and as before wet paper towels were added under the metal grids to create a humid incubation chamber. The entire box was then enclosed in a clean Petri dish bag, the inside of which had been sprayed with SDW [Plate 2.5]. All inoculated material was incubated at 18-20°C near a window in a room with 8 h cool white fluorescent light for seven days.

2.11.3 Spore counts

On day seven of incubation, six needles were removed – approximately one in every five needles from the apex of long shoots and when using short shoots one from each fascicle. Each needle was mounted on a glass slide, abaxial downwards, with 10 µl cotton blue (5%) in lactophenol and topped with a glass cover slip (18 x 18 mm). Using this method spores could be seen and counted on the adaxial side of needles using a compound microscope. Spores were counted on the adaxial surface, as sporangia formed on this surface are potentially more epidemiologically significant than those formed on the abaxial surface, as rain-splash dispersal would be most likely from this side (Moralejo, Garcia Munoz & Descals, 2006). Counts were made along the length of the needle and in the surrounding cotton blue solution, as many sporangia were knocked off the needles during the mounting process.

This method could not be used for the other foliar hosts (with the exception of bilberry) as the tissue was too thick to allow visualisation of the sporangia. Instead, the area of leaf surface inoculated and any visible lesion beyond this area was gently scraped with a

rounded scalpel blade to free all sporangia from the leaf's abaxial surface. A 200 μ L drop of SDW was then placed on the inoculated area to suspend the scalpel scrapings and the droplet then transferred to a 1 mL microtube along with 5 μ L of cotton blue (5%) in lactophenol. Microtubes were kept in cold storage until they were prepared for counting. Preparation involved centrifuging the microtubes for 10 minutes at 6,000 x g and pipetting off the liquid. The remaining spore pellet was resuspended in 20 μ L of SDW followed by agitation for 30 seconds using a vortex machine. Slides were then prepared by dispensing two 10 μ L drops onto a glass slide and covering each with a glass cover slip. The total number of spores was then counted using a compound microscope.

2.11.4 Host foliage susceptibility / *P. ramorum* pathogenicity

For the broadleaf species, susceptibility was determined by the degree of visible symptoms (necrosis). Leaves were scanned on an Epson Perfection 1660 photo scanner at 1660 dpi resolution after the sporangia had been removed. The leaf surface area and the area of necrosis (lesion area) was calculated using Image J software (National Institutes of Health).

The lesion area for infected larch needles was calculated as the surface area of the needle as the presence/absence of necrotic lesions could not be reliably defined. Images of the needles were taken by scanning the prepared slides, to avoid knocking any sporangia off the surface of the needles. To estimate symptom expression on the larch hosts all the needles on each shoot were removed, counted and categorised according to their observed symptoms; blackened, brown/banded, chlorotic and green (Webber, Mullett & Brasier, 2010).

2.12 *In vitro* growth response

2.12.1 Radial growth rate

Radial growth rate tests were carried out by taking a 5 mm plug from the edge of actively growing colonies and placing them onto newly dispensed 20 ml agar plates which had been pre-marked with two lines perpendicular to one another. The plugs were placed in the middle of the plate where the two lines crossed [Figure 2.2]. Plates were incubated in the dark at the desired temperature and checked every 24h for colony development. When mycelium could be observed growing onto the agar from the plug on all sides, four marks were placed on the perpendicular lines where the colony edge touched. Plates were then further incubated until the colonies filled the plates but were not touching the edge. The plates were again marked

along the two lines where the colony edge touched. The radial growth rate was calculated by taking measurements along the four lines, between the first and second marks. The means of these measurements were then divided by the number of days of incubation between the first and the second marking of the plates to provide a mean radial increment rate per day

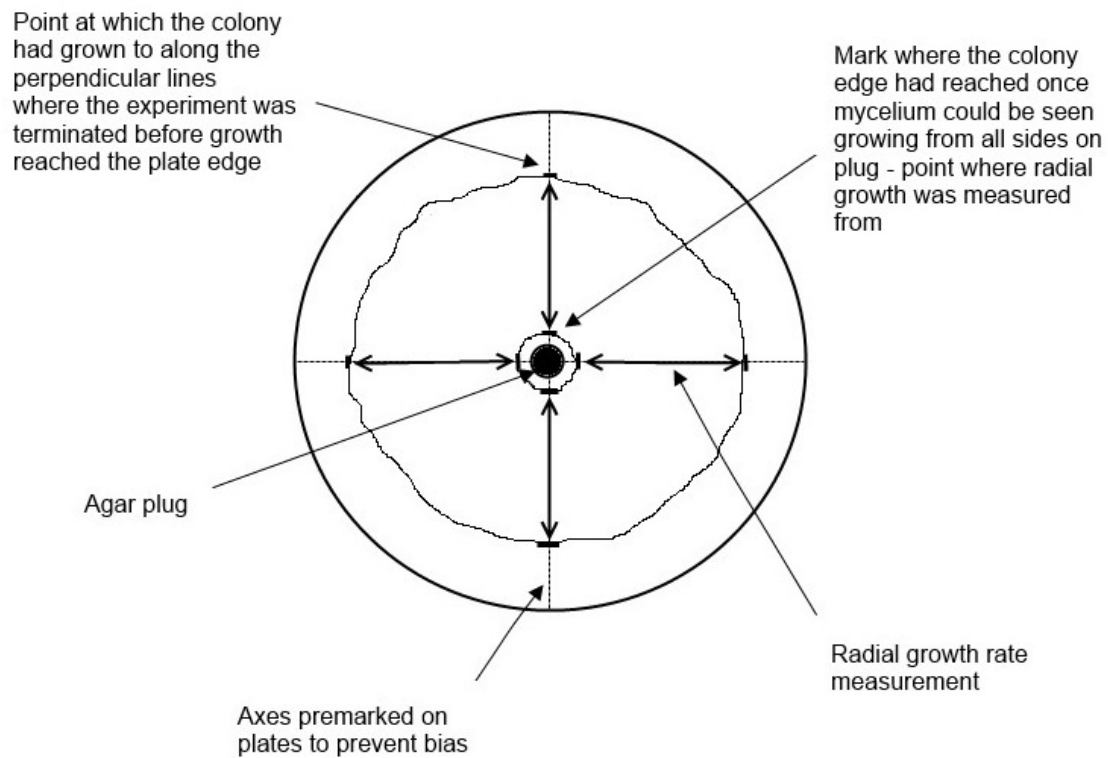


Figure 2.2 – Diagram of radial growth measurement set-up.

Chapter three - Sporulation potential of *Phytophthora ramorum* on UK *Larix* species

3.1 Introduction

The 2009 discovery of *Phytophthora ramorum* causing widespread dieback and mortality of mature and juvenile Japanese larch (*Larix kaempferi*) in plantations across south-west England signalled a major shift in the pathogen's epidemiological behaviour (Brasier & Webber, 2010). Prior to this, tree infections in Britain had been comparatively rare, with only approximately 100 tree infections over a six year period, mainly on native beech and non-native oak growing close to rhododendron. The discovery of *P. ramorum* on Japanese larch marked the first time the pathogen had been found causing widespread infections in Europe. It was also the first time *P. ramorum* had been found killing commercially important conifers anywhere in the world (Forestry Commission, 2011; Forestry Commission, 2012b). Even more significantly, in addition to causing lethal stem infections *P. ramorum* was found to sporulate abundantly on larch foliage, producing up to 2,685 sporangia per needle in laboratory studies (Webber, Mullett & Brasier, 2010).

Since 2009, 'Sudden larch death' has resulted in the felling of over three million trees, covering 10,000 hectares, between 2009 and 2013 in the UK (Forest Research, unpublished data). This rapid spread has many parallels with the disease epidemic on native tanoaks in North America. The ability of Japanese larch to support sporulation of *P. ramorum* is of major concern to forestry and the natural environment. Sporulating hosts play a crucial role in *P. ramorum* disease epidemics, acting as key sources of inoculum (Davidson *et al.*, 2005; Davidson, Patterson & Rizzo, 2008). Prior to the outbreak on larch in the UK, the ramorum epidemics in North America were considered much more damaging compared to those in Europe, with more than a million trees killed by the disease in North America (Parke & Lucas, 2008), and despite the fact that European isolates of the pathogen have been demonstrated to be more aggressive than North American isolates (Brasier, Kirk & Rose, 2006). Sporulation potential and host species prevalence in woodlands were thought to be critical factors in explaining the difference in tree mortality observed between Europe and North America (Sansford *et al.*, 2008). In North America, Californian bay laurel (*Umbellularia californica*) and tanoaks (*Notholithocarpus densiflorus*) are major sporulating hosts of *P. ramorum* (Davidson *et al.*, 2005; Davidson, Patterson & Rizzo, 2008), whereas in the UK until 2009 the major sporulating host was rhododendron, which typically produces around 8

sporangia per cm² of infected leaf tissue in contrast to over 200 sporangia per cm² from Californian bay laurel (Defra, 2005b).

In light of the damage occurring in North America and the discovery of *P. ramorum* sporulating on Japanese larch there was concern that the disease may become widespread and damaging in the UK. Also, higher inoculum loads from Japanese larch could lead to tree species previously not considered vulnerable to the disease, including European and hybrid larch becoming infected. This situation has occurred in the Republic of Ireland where Sitka spruce (*Picea sitchensis*) was found to be infected when growing in a heavily *P. ramorum* infected area (Forestry Commission, 2012b).

With no economically feasible and genus-specific treatment for *P. ramorum* infections, felling of all infected larch and some associated trees is currently taking place throughout the private sector and on public forest estate land to minimise further disease spread (Forestry Commission, 2014a). So far only Japanese larch has been shown to be a major sporulation host (Webber, Mullett & Brasier, 2010), so comparisons of the ability of all three commonly grown larch species (Japanese, European (*L. decidua*) and hybrid (*L. × eurolepis* (synonym: *L. marschlinsii*)) to support sporulation are required to determine the threat *P. ramorum* poses to the wider larch industry in the UK. There is also uncertainty about whether or not sporulation occurs on larch needles throughout the year and, if so, when sporulation is most prolific, although field observations suggest it may be linked to needle abscission (J. Webber, personal communication). This information would be valuable for disease management as felling of infected trees before the optimal time for sporulation could help limit the spread of the pathogen. The potential of larch to support sporulation compared with other major sporulating hosts in Europe (rhododendron (*Rhododendron ponticum*), common bilberry (*Vaccinium myrtillus*) and sweet chestnut (*Castanea sativa*)) and North America Californian bay laurel (*Umbellularia californica*), is also unknown and the information is required for models to predict disease spread. These data could then be compared with information from North America on the likely distance of aerial spread of the pathogen from sporulating hosts, as well as being used to provide recommendations on whether larch species other than Japanese larch could continue to be grown commercially in Britain.

The main aim of this study was to investigate the sporulation of *P. ramorum* on larch foliage. Two experiments were undertaken to achieve this. The objectives of the first experiment were to (i) determine whether the foliage of European and hybrid larch were susceptible to *P. ramorum* and could support sporulation, (ii) compare the levels of susceptibility and sporulation on a number of potential and known major sporulating hosts (Japanese larch,

European larch, hybrid larch, rhododendron, California bay laurel, sweet chestnut and Bilberry), and (iii) to assess the effect of season on susceptibility and sporulation potential on these host species. The second experiment also investigated variation between *P. ramorum* isolates by assessing sporangial and chlamydospore production of a number of different isolates of the pathogen to assess whether the pathogen is adapting to larch foliage in the UK.

3.2 Materials and Methods

The relative sporulation potential of *P. ramorum* on Japanese, European and hybrid larch needles was assessed and compared to other hosts known to support sporulation using methods adapted from (Fichtner *et al.*, 2012). Susceptibility testing used a detached-leaf-dip assay adapted from earlier methods devised by (Parke, Linderman & Hansen, 2002; Denman *et al.*, 2005).

3.2.1 Plant material

The host susceptibility and sporulation potential of *P. ramorum* on four known broadleaved foliar hosts; Californian bay laurel, common rhododendron, sweet chestnut and common bilberry was compared with that of Japanese, European and hybrid larch foliage collected at different times of year (experiment one). Shoots of Japanese and European larch and rhododendron leaves alone were used to study the variation in sporulation potential of *P. ramorum* isolates (experiment two).

Fully expanded, unblemished, size-matched leaves were collected from the broadleaved hosts grown in the Forest Research intensive nursery. For the larch species used in experiment one, the current year's long shoots, approximately 15 cm long and bearing individual needles were selected from mature healthy trees, in October and August grown in trial grounds at Alice Holt Research Station. In May, long shoots are unavailable as they are yet to develop, therefore young, short shoots with rosettes of needles were used. Long shoots from Japanese and European larch trees approximately 10 years old, grown in the Forest Research intensive nursery were used in experiment two.

The cut shoots were placed in SDW overnight prior to use. Broadleaved host material was collected the morning of inoculation. Immediately prior to inoculation leaves were rinsed in SDW and placed on sterile paper towels to air dry. The leaves and shoots were marked 4

cm from the tip using a permanent marker pen to indicate the inoculation area. Each isolate was tested on six leaves/shoots which came from three different plants/trees (i.e. two leave/shoots from each individual plant).

3.2.2 Isolates

In experiment one, two isolates of *P. ramorum* cultured from recently diseased trees were used. Both were EU1 lineage and A1 mating type. In experiment two, the sporulation potential of six *P. ramorum* isolates on larch and rhododendron foliage were assessed. Four of the isolates were EU1 lineage and A1 mating type and two were NA1 lineage and A2 mating type. Details of the isolates are in Table 3.1 below.

Table 3.1- *Phytophthora ramorum* isolates used in experiments

Lineage	Isolate	Region	Host	Sample	Isolated	Experiment
EU1	BOC07	Cornwall, UK	<i>Larix decidua</i>	Bark	Feb 2011	2
EU1	BRC01	Devon, UK	<i>L. kaempferi</i>	Foliage	May 2010	1 & 2
EU1	P1376	Sussex, UK	<i>Viburnum tinus</i>	Foliage	Apr 2002	2
EU1	P2540	Lancashire, UK	<i>L. kaempferi</i>	Bark	Sep 2011	2
EU1	PLY72	Plymouth, UK	<i>Pseudotsuga menziesii</i>	Litter	Aug 2010	1
NA2	P1349	North America	<i>Rhododendron</i>	Foliage	Dec 2000	2
NA2	P1403	North America	<i>Vaccinium ovatum</i>	Foliage	2002	2

3.2.3 Inoculum production, inoculation and incubation

Inoculum of *P. ramorum* isolates was produced as described in section 2.10.1. Host foliage was inoculated using zoospore suspensions at a concentration of 3×10^5 zoospores mL⁻¹ and incubated as described in section 2.11.1.

3.2.4 Sporulation potential

Different methods were used to assess sporulation on larch needles and broadleaf hosts (see section 2.11.3). In experiment one, only sporangia were counted, whilst in experiment two both chlamydospores and sporangia were counted.

3.2.5 Susceptibility

For the broadleaf species in experiment one and rhododendron in experiment two, susceptibility was determined by the degree of symptom expressed in the form of lesion size compared to the area of leaf surface inoculated (see section 2.11.4). For larch hosts susceptibility was established by calculating the proportion of needles per shoot belonging to four cumulative categories of symptom development determined by needle colour (see section 2.11.4). Needle counts included the needles mounted on slides for sporangial counts and those used in the reisolation of the pathogen.

3.2.6 Reisolation of pathogen

At the end of the experiment, ten pieces of plant tissue per leaf or ten needles per shoot were plated onto SMA to re-isolate the pathogen and confirm that symptoms had been caused by *P. ramorum* (see section 2.6.1). Where *P. ramorum* could not be re-isolated from larch foliage after seven days for experiment two, needles were taken off SMA, transferred to microtubes and stored at -80°C until they were prepared for DNA extractions (see section 2.7.1) and the pathogen detected via real time PCR (see section 2.8.2).

3.2.7 Experimental design and statistical analysis

3.2.7.1 Host susceptibility and sporulation potential of *P. ramorum* on larch foliage at different times of year

This study was carried out in October 2010 prior to needle and leaf abscission on larch and sweet chestnut, and repeated in May 2011 when larch trees had recently flushed with needles which were still soft, and again in August 2011 during optimum growth.

A total of seven foliar hosts were tested in the study during October 2010; three larch species and four broadleaf sporulating hosts of *P. ramorum*. For the same assessments in May and August 2011 bilberry was not included due to time limitations. Two isolates of *P. ramorum* were tested against these hosts. Data on the following parameters were recorded for all hosts:

Parameter 1. – Comparisons of sporulation potential based on the density of sporangia produced on all hosts (to allow comparisons between hosts the number of sporangia was expressed per cm² of needle).

Parameter 2. - Comparisons of sporulation density recorded at the three different times (i.e. October, May, August).

Parameter 3. – Assessment of susceptibility of hosts based on the percentage of inoculated surface area that developed necrosis on California bay laurel, sweet chestnut, rhododendron and bilberry, and based on percentage of inoculated needles within different categories of necrosis on larch species.

Parameter 4. – Evaluation of reisolation success, based on positive or negative reisolation of *P. ramorum* from each host at different times of the year

For parameters 1 and 2 - sporulation potential of different hosts and different times of year - an experimental unit comprised of one leaf per combination of host, isolate and month for the broadleaf hosts. For the larch species six needles were taken from six shoots. Statistical design, therefore, consisted of six replicates for broadleaved hosts and six replicates of larch shoots, the latter having a subset of six needle replicates (n=36) from three plants of each tree/plant species (n=3). The data were analysed using GenStat (13th Edition). A restricted likelihood model (REML) variance components analysis was fitted to the data. Isolate, host genotype (i.e. plant/tree 1, 2 or 3 of each host), shoot and needle were treated as random effects in the model-building. Fisher's unprotected least significant difference test was carried out on the data to draw out significant differences between hosts and months.

For parameter 3 - host susceptibility - an experimental unit for both the larch and non-larch host-testing consisted of one leaf per combination of host, isolate and month, replicated six times. A generalised linear model with a binomial error structure and logit function was fitted to the non-larch host data to test for differences in the lesion development of the two isolates of *P. ramorum* on different hosts and times of year. Data for larch susceptibility consisted of the number of needles from a shoot that fell into four scaled symptom categories; green, chlorotic, brown banded and blackened, with blackened needles being the most severe symptom. Comparisons of larch symptoms were made between control shoots (dipped in SDW) and inoculated shoots (dipped in *P. ramorum* zoospore suspensions) for all species and months. Analysis was carried out by separating the data into the nine sets of combinations (larch species x time of year) and then for each set combining the two poorest condition levels (blackened and brown needles) as no blackened needles were observed for some combinations and applying a cumulative mixed effect ordinal response regression in

SAS® 9.4. Comparisons were then made between the treated shoots comparing the effects of month and larch species on symptom development. Analysis was restricted to treated needles and an ordinal regression model fitted to the needle symptom.

For parameter 4 - reisolation success - the experimental design consisted of ten reisolation attempts from each host replicated six times. A generalised linear mixed model with a binomial error structure and logit function was fitted to the data to test for differences in reisolation success between isolates, hosts and times of year. Host genotype and leaf number were treated as random effects in the model-building. Fisher's unprotected least significant difference test was carried out on the data to draw out significant differences between hosts and months.

3.2.7.2 Variation in sporulation potential between isolates of *Phytophthora ramorum*

This study was carried out in October 2011. Three hosts were used for this study, two larch species and rhododendron. Hybrid larch was not included due to time limitations. Six isolates of *P. ramorum* were tested against these hosts, four EU1 UK isolates and two NA1 isolates.

Parameter 1. – Comparisons of sporulation potential of isolates of *P. ramorum* based on the density of sporangia and chlamydospores produced on all hosts.

Parameter 2. – Assessment of the relationship between production of chlamydospores and sporangia on each host for each isolate.

Parameter 3. - Assessment of susceptibility of hosts based on the percentage of inoculated surface area that developed necrosis on rhododendron leaves.

Parameter 4. – Assessment of reisolation success of isolates based on positive or negative reisolation of *P. ramorum* from each host at different times of year.

For parameter 1 - comparison of *P. ramorum* isolate sporulation potential - the experimental design consisted of six replicates for rhododendron leaves and six replicates for larch shoots taken from three trees/ plant of each species, with the latter having a subset of five needle replicates on each shoot. The data were log transformed and analysed using GenStat (13th Edition). A restricted likelihood model (REML) variance components analysis was fitted to the data to test differences in total propagule production (combined counts of sporangia and

chlamydospores) between isolates and hosts. Host genotype, shoot and needle were treated as random effects in the model-building. Fisher's unprotected least significant difference test was carried out on the data to draw out significant differences between isolates and hosts.

For parameter 2 - assessing the relationship between sporangia and chlamydospore production - Spearman rank correlation analysis was performed on the data to determine whether the production of both types of spores was correlated or whether there was a trade-off.

For parameter 3 - an experimental unit comprised of six rhododendron leaves per combination isolate. A generalised linear model with a binomial error structure and logit function was fitted to the non-larch host data to test for differences between the *P. ramorum* isolate lesion development.

For parameter 4 - reisolation success - experimental design consisted of ten reisolation attempts from each host replicated six times for each isolate. A generalised linear mixed model with a binomial error structure and logit function was fitted to the data to test for differences in reisolation success between isolates and hosts. Host genotype was treated as a random effect in the model-building. Fisher's unprotected least significant difference test was carried out on the data to draw out significant differences between isolates and hosts.

3.3 Results

3.3.1 Sporulation potential of *P. ramorum* on larch foliage at different times of year

Sporulation occurred on the surface of all hosts' foliage [Plate 3.1] and at each time of year the tests were undertaken, but varied with season and host. Bilberry was only tested in October 2010 and so was excluded from the statistical analysis. Chlamydospores were infrequently observed, so counts focused on sporangia.

Analysis showed that host species had a significant effect on the sporulation potential of *P. ramorum* ($F(5, 5.6) = 7.17, p < 0.05$). Japanese larch shoots consistently supported the highest levels of sporulation, with a count of 4,341 sporangia on one needle in August. The highest numbers of sporangia associated with European and hybrid larch needles were 1,688 and 1,493 sporangia respectively, also in August. However, model predictions found

that the overall the ability of larch species to support sporulation of *P. ramorum* did not differ significantly from each other or from sweet chestnut probably because even with single shoots the number of sporangia could differ hugely between individual needles [Figure 3.1]. The ability of all three larch species to support high levels of sporulation was, however, significantly greater than that of rhododendron and Californian bay laurel [see Figure 3.1a for LSD between hosts sporulation potential]. Japanese larch supported, on average, 16 and 22 times more sporulation than rhododendron and Californian bay laurel respectively. The level of sporulation supported by sweet chestnut fell between larch and other non-larch hosts and did not differ significantly from either in the model predictions [Figure 3.1a].

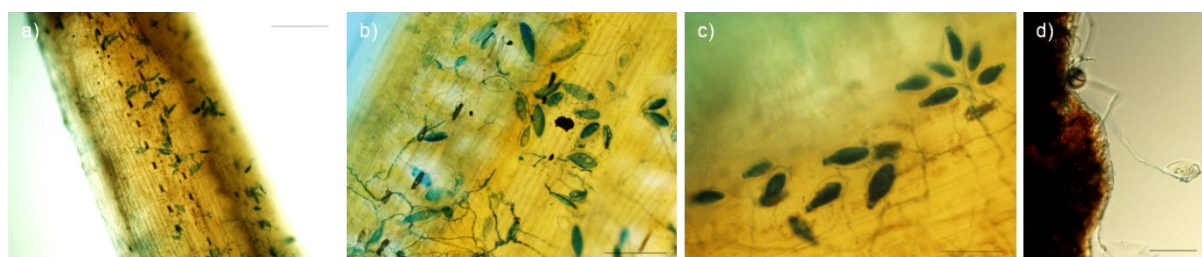


Plate 3.1 - Sporangia of isolate PLY72 growing on the surface of hybrid larch needles in; a) October; b) May; c) August, and d) on bilberry in October (scale bar; a) =100 µm, b-d) =50 µm).

Statistical analysis also indicated that time of year had a highly significant effect on sporulation ($F(2, 10.5) = 46.22, p < 0.001$). Most sporulation took place on the August collected material for all hosts. There was no significant effect of a host x time of year interaction ($F(10, 11.3) = 2.24, p = 0.09$) on the pathogen's sporulation potential. Levels of sporulation were at their lowest on non-larch hosts during May, but collective sporulation on all hosts did not differ significantly between October and May, but was significantly greater during August [Figure 3.1]. Overall the model found sporulation to be 83% and 87% higher in August when compared to October and May respectively.

Japanese larch had the highest mean sporulation potential in all three months, increasing 23-fold from 187 to 4315 sporangia per cm² between October and August, followed by European larch. *Phytophthora ramorum* produced fewer sporangia on hybrid larch in all months compared to Japanese and European larch. It had the lowest sporulation potential in May and October on rhododendron leaves producing 1.5 and 8.3 sporangia per cm² respectively, whilst it was in August that *P. ramorum* produced the least sporangia on Californian bay laurel (127 sporangia per cm²). The difference between larch and non-larch hosts' sporulation potential was lowest in October and highest in August.

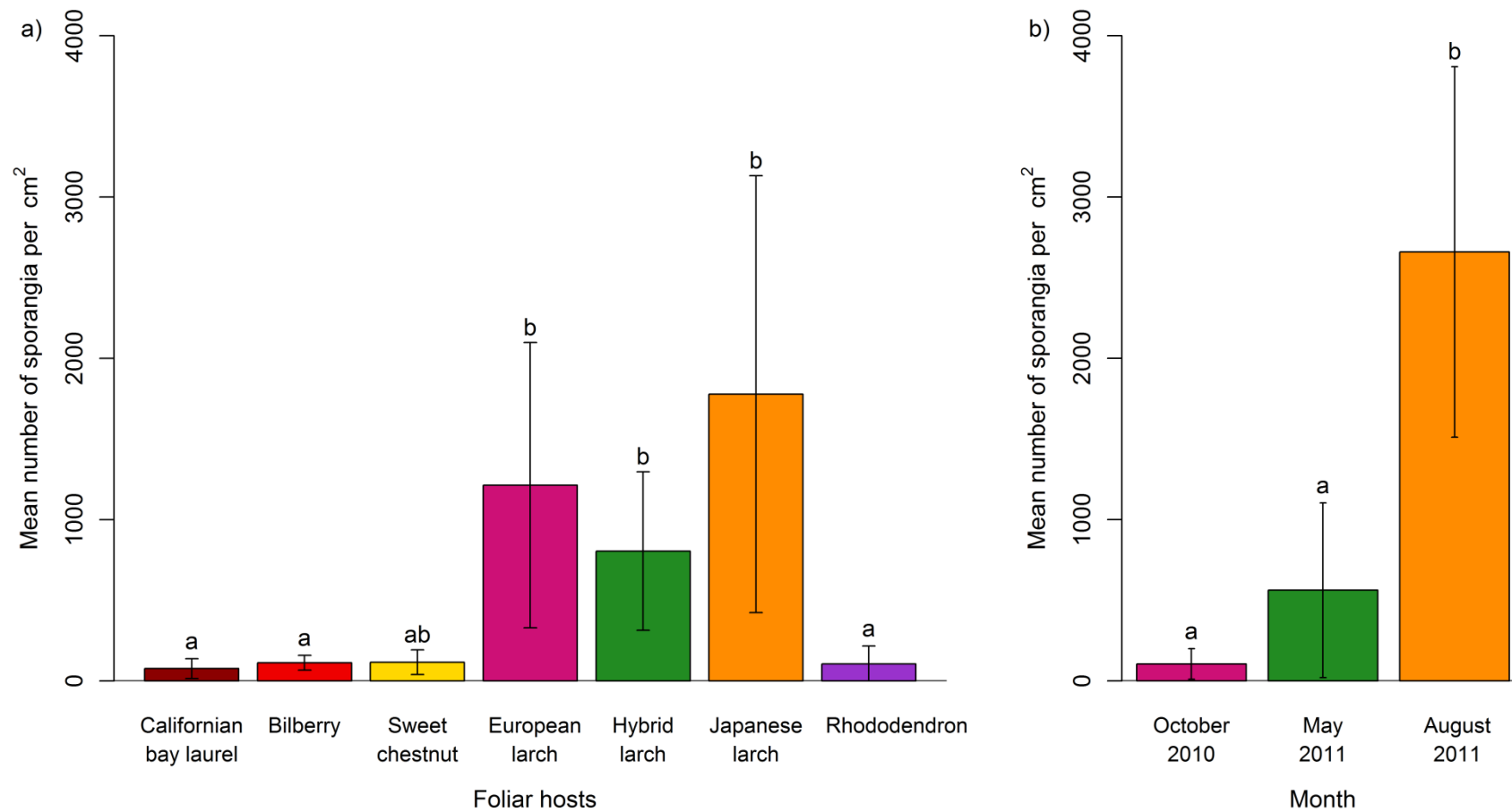


Figure 3.1 - Mean sporulation potential of *P. ramorum*; a) on different host foliage (n = 3); b) at different times of year (n = 10, 12 and 12 for October, May and August respectively). Error bars represent the standard error of the mean. Different letters indicate differences among treatments determined by Fisher's unprotected least significant difference test at $P < 0.05$.

Overall, the most prolific sporulation occurred on larch regardless of time of year the tests were undertaken.

3.3.2 *Phytophthora ramorum* symptom development on host foliage

Symptom development occurred predominantly on older foliage for all hosts tested. There were also striking differences between the conifer (larch) hosts and the broadleaved hosts.

3.3.2.1 Larch foliage

For the larch species, symptom development was most pronounced on European larch and least apparent on Japanese larch, with intermediate symptom expression on hybrid larch but overall few symptoms developed on inoculated larch shoots [Figure 3.2]. Indeed, symptoms did not differ significantly between the control shoots and inoculated shoots of Japanese larch in May and August 2011, although for all other combinations inoculated needles did develop some symptoms [Table 3.2]. Analysis of effect of season and species on the inoculated shoot symptoms indicated that they both had a significant effect on symptom development ($F(2, 9456) = 67.28, p < 0.0001$ and $F(2, 6) = 31.40, p < 0.0007$ respectively), but did not interact ($F(4, 9456) = 2.33, p = 0.054$). Symptom development was least obvious in May followed by August, and most apparent in October [Table 3.3].

Table 3.2 - Cumulative mixed effect ordinal response regression results for comparisons of needle symptoms between inoculated and control shoots.

Host	Month	<i>t</i> value	d.f.	<i>P</i> value
European larch	October 2010	2.59	557	0.0099
Hybrid larch	October 2010	7.87	959	< 0.0001
Japanese larch	October 2010	6.09	1163	< 0.0001
European larch	May 2011	6.74	3146	< 0.0001
Hybrid larch	May 2011	-7.89	2796	< 0.0001
Japanese larch	May 2011	1.12	2890	0.2616
European larch	August 2011	9.93	782	< 0.0001
Hybrid larch	August 2011	8.12	704	< 0.0001
Japanese larch	August 2011	0.98	940	0.3295

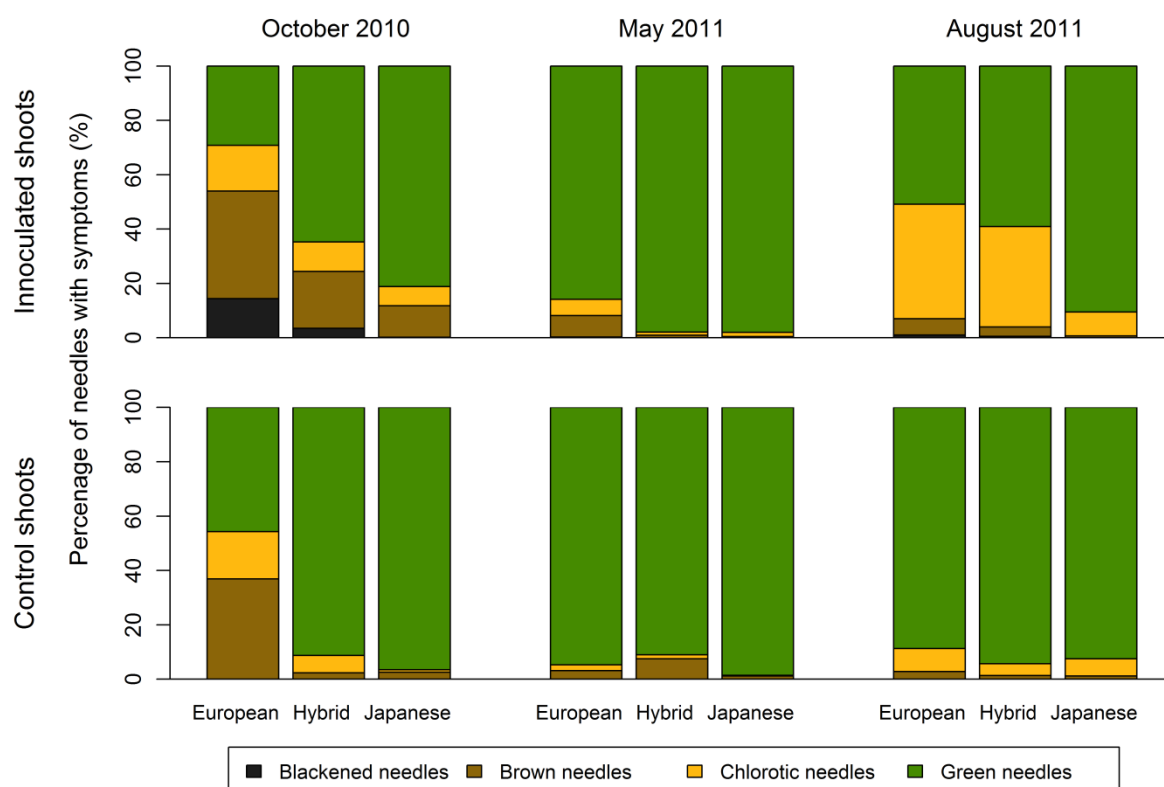


Figure 3.2 - Symptom development of *P. ramorum* infection on larch needles based on the mean percentage of needles per shoot categorised by tissue colour following inoculation and incubation for seven days (n = 48 for inoculated shoots (n=24 for each isolate), n = 24 for control shoots).

Table 3.3 - Cumulative mixed effect ordinal response regression results for comparisons of inoculated needle symptoms.

Interaction	t value	d.f.	P value
October v May	-11.44	9456	<.0001
October v August	-3.96	9456	<.0001
May v August	7.49	9456	<.0001
European larch v hybrid larch	-4.79	6	0.0030
European larch v Japanese larch	-7.86	6	0.0002
Japanese larch v hybrid larch	-3.39	6	0.0148

3.3.2.2 Non-larch foliage

Symptom development on non-larch foliage was the most pronounced on bilberry, followed by sweet chestnut and rhododendron [Plate 3.2 & Figure 3.3]. Only small amounts of necrosis were apparent on bay laurel, with small dotted lesions developing in clusters, differing markedly from the one or two large lesions observed on the other non-larch hosts [Plate 3.2].

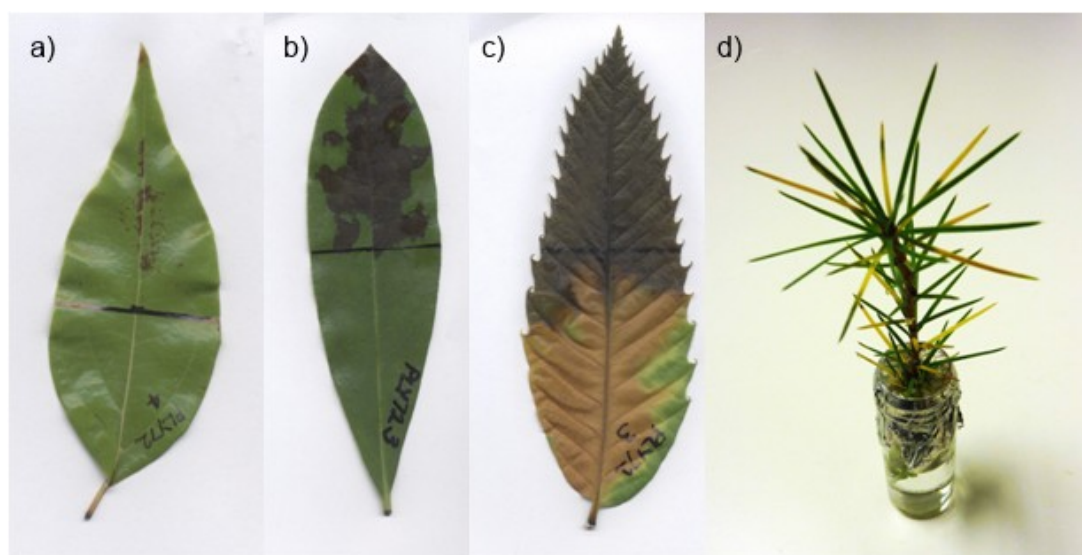


Plate 3.2 - Typical disease symptoms of isolate PLY72 observed on hosts; a) bay laurel, b) rhododendron, c) sweet chestnut, d) European larch after seven days incubation.

As before, statistical analysis indicated that both time of year and host had a significant effect ($p = 0.001$) on symptom development. The model also revealed that time of year and host interacted significantly ($p = 0.001$), as infections of *P. ramorum* produced different sized lesions at different times of the year on different hosts ($p = 0.001$) [Figure 3.3]. For example, on rhododendron symptom expression increased as the age of the leaf and time of year progressed. For California bay laurel, symptoms were greatest in October but were much more limited in May and August.

The two isolates of *P. ramorum* incited similar symptoms on each host, however they produced significantly different lesion sizes to each other at different times of the year ($p = 0.001$) (For estimates of parameters and accumulated analysis of deviance of the fitted model see appendix I). For isolate PLY72 symptom development was less pronounced on sweet chestnut and rhododendron compared to isolate BRC01 in October, but considerably more on rhododendron in August. For bilberry it was not possible to draw any conclusions because this species was only tested in the October experiment due to time limitations,

however this species was the most symptomatic in October, with lesions developing not only over the whole leaf (inoculated regions) but covering the entire shoots.

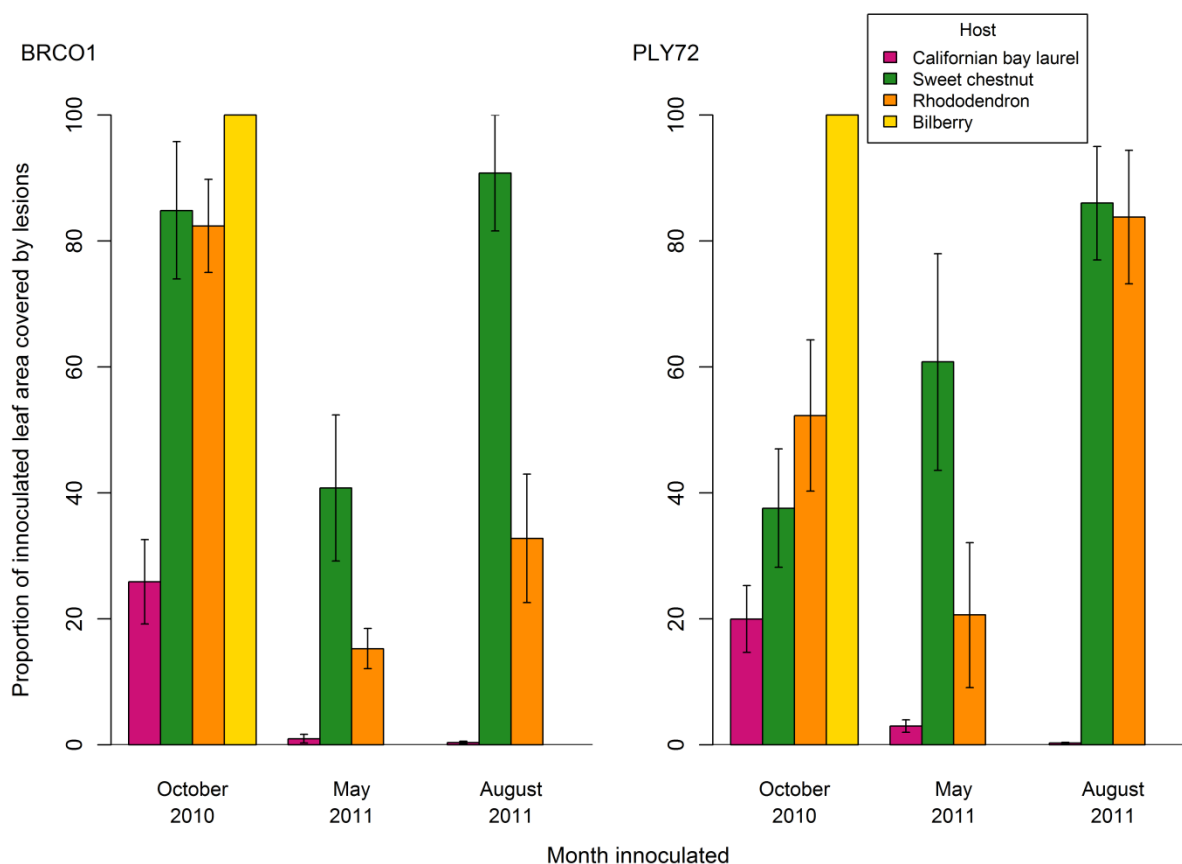


Figure 3.3 - Symptom development from *P. ramorum* infections on non-larch host foliage based on the percentage of inoculated leaf surface area that developed necrosis after seven days incubation, during different months of the year (there are no results for bilberry in May and August as it was not included in these experiments) ($n = 11$ for Bay laurel and sweet chestnut in October 2010, for all other hosts at in each month $n=12$). Error bars represent the standard error of the mean. Different letters indicate differences among treatments determined by Fisher's unprotected least significant difference test at $P < 0.05$.

It was also clear that symptom development did not correlate with sporangial production on Californian bay laurel or rhododendron foliage at any time of year tested. Sporangia production and symptom expression was however positively correlated on sweet chestnut in October 2010 and May 2011 [Figure 3.4].

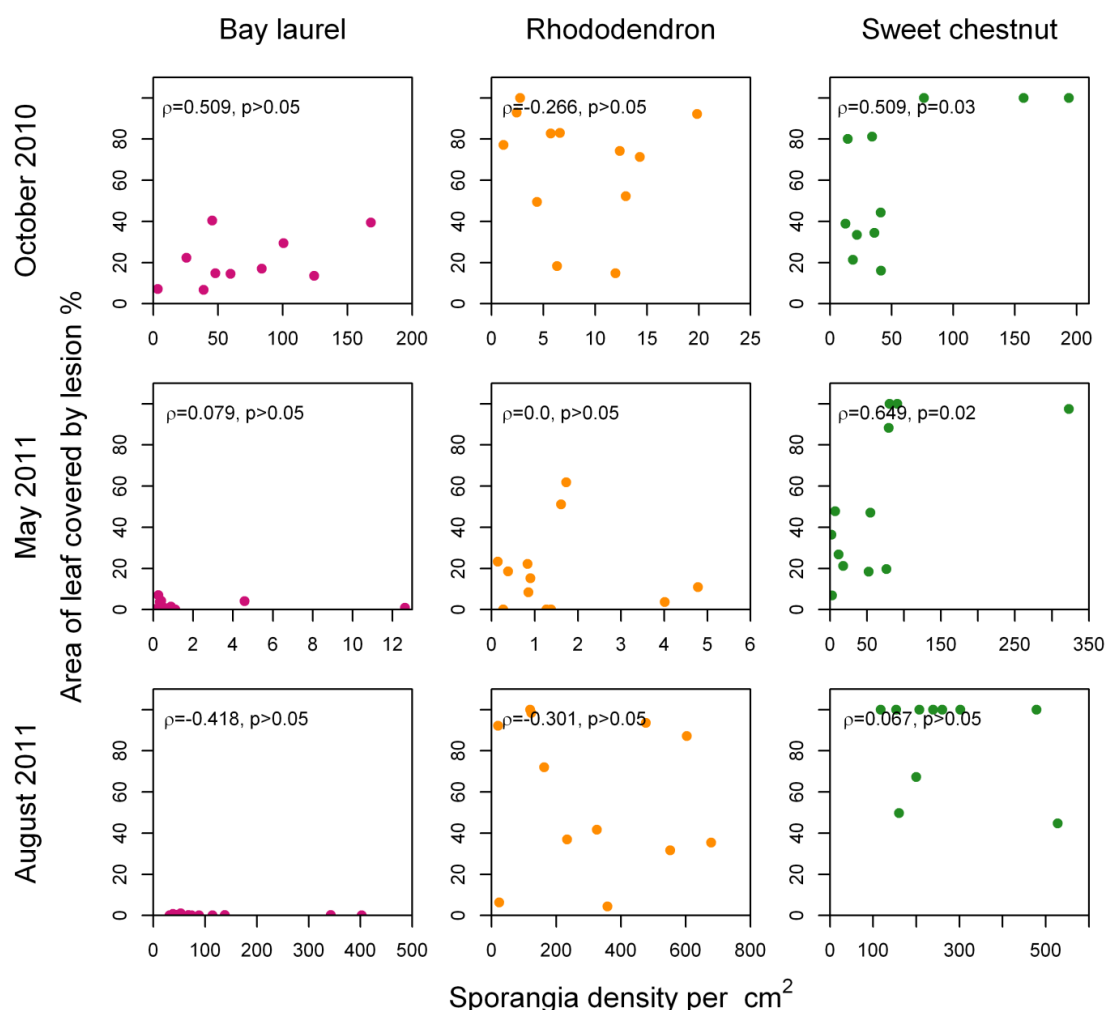


Figure 3.4 - The relationship between the sporangial production of *P. ramorum* on host leaf surface and symptom development (characterised by leaf lesion size), at three different times of year.

3.3.3 Reisolation success of *P. ramorum* from host foliage at different times of the year

Phytophthora ramorum could be reisolated on to SMA plates from all infected host foliage and at all times of year the study was carried out [Figure 3.5]. Reisolation success however, differed significantly according to host and time of year ($F(5, 208) = 6.86$, $p < 0.001$ and $F(2, 208) = 13.36$, $p < 0.001$ respectively), although it was similar for the two isolates ($F(1, 164.4) = 0.49$, $p = 0.487$). Reisolation was nearly always successful from infected bilberry, sweet chestnut and rhododendron. In contrast, reisolation success from larch hosts was significantly lower than non-larch hosts, ranging from 13 to 21% reisolation success for hybrid and European larch respectively, although reisolation from all three larch species was similar. Overall, *P. ramorum* could be reisolated from host foliage most readily in October 2012 and least in May 2011.

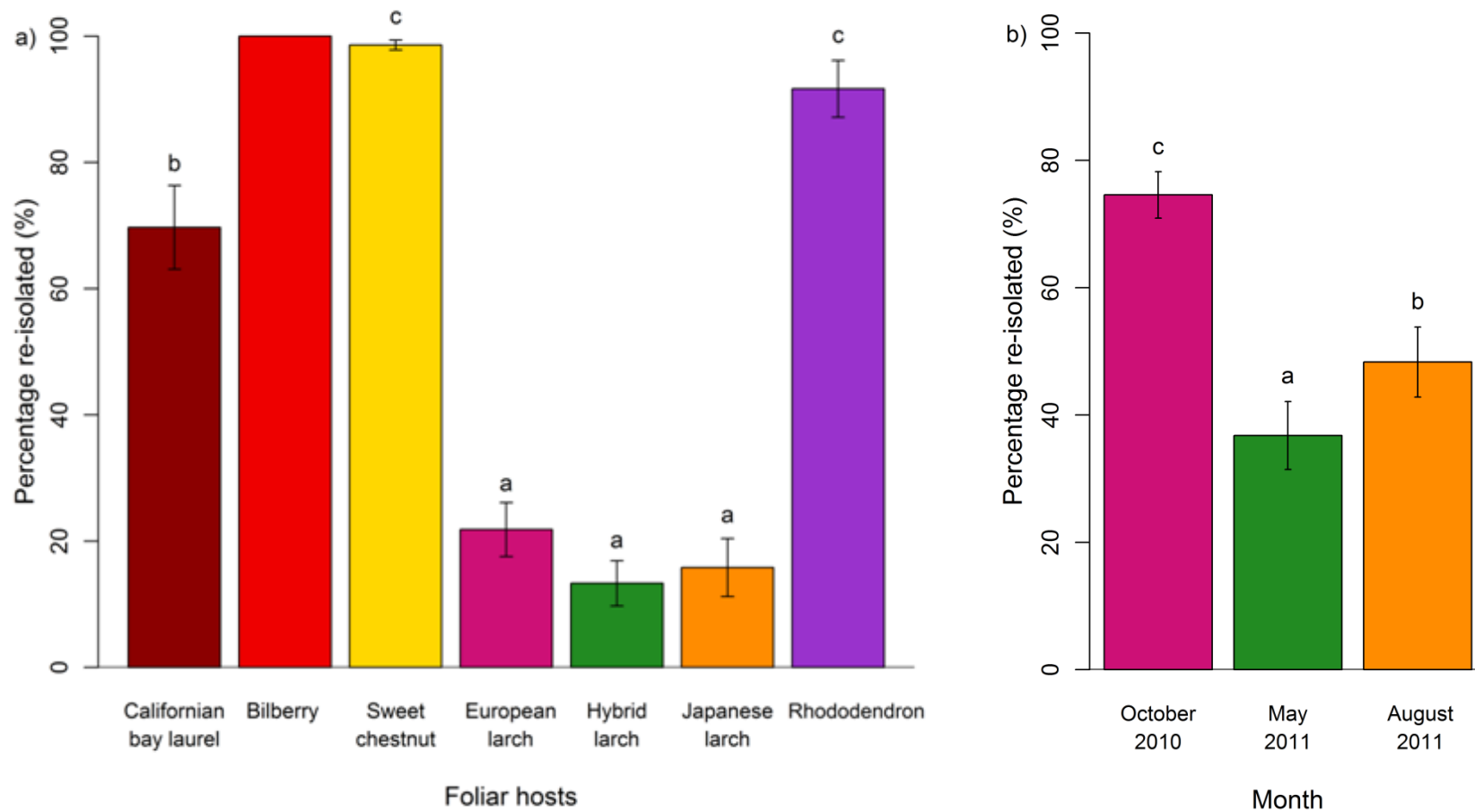


Figure 3.5 - Success of *P. ramorum* re-isolation based on mean percentage re-isolation from; a) different infected host foliage (n = 36 for all hosts except Bilberry where n = 12), b) at three different months of the year (n = 84, 72 and 72 for October, May and August respectively). Error bars represent the standard error of the mean. Different letters indicate significant differences among treatments determined by Fisher's unprotected least significant difference test at $P < 0.05$.

3.3.4 Variation in sporulation potential between isolates of *P. ramorum*

A more detailed study of sporulation potential on larch and rhododendron foliage was undertaken using six isolates of *P. ramorum*. The isolates comprised four EU1 lineage (BOC07, BRC01, P2540 and P1376) and two NA1 lineage (P1349 and P1403) [Table 3.1].

All isolates of *P. ramorum* produced both sporangia and chlamydospores on foliage surfaces of all hosts, but sporangia were produced in significantly higher numbers than chlamydospores [Figure 3.6]. Chlamydospores were observed in abundance on both the surface and within larch needles [Plate 3.3] differing from the previous sporulation study (see section 3.3.1). It was also notable that the number of sporangia produced in this experiment was considerably higher from an isolate (BRC01) also used in the previous study in October 2010 [Table 3.4].

Table 3.4 - Mean sporangia density for isolate BRC01 on inoculated host in October 2010 and 2011.

Host	October 2010 (experiment one)		October 2011 (experiment two)	
	sporangia per cm ²	std	sporangia per cm ²	std
Japanese larch	344.8	± 119.9 (n=30)	2567.4	± 468.7 (n=36)
European larch	61.5	± 16.9 (n=30)	7062.9	± 888.6 (n=36)
Rhododendron	6.0	± 2.8 (n=6)	34.9	± 14.2 (n=6)

Sporangia and chlamydospore production was significantly positively correlated for all isolates on larch except for P2540 and P1403 on European larch; the observed correlation for P1403 was almost zero [Figure 3.7 & Figure 3.8]. This suggests that there is no trade off in sporangia vs chlamydospore production on larch. No significant correlations were observed between sporangia and chlamydospore production on rhododendron, although this may have been influenced by the low numbers of rhododendron samples (n=6) [Figure 3.8].

The different isolates of *P. ramorum* showed considerable variation in their total sporulation potential (summed sporangia and chlamydospores) on hosts. Average densities ranged from 5,289 ± 605 to 13,931 ± 910 (± 1 s.e., n=30) spores per cm² on European larch, 1,124 ± 173 to 15,631 ± 1076 (± 1 s.e., n=30) spores per cm² on Japanese larch and 12 ± 0.6 to 185 ± 19 (± 1 s.e., n=6) spores per cm² on rhododendron.

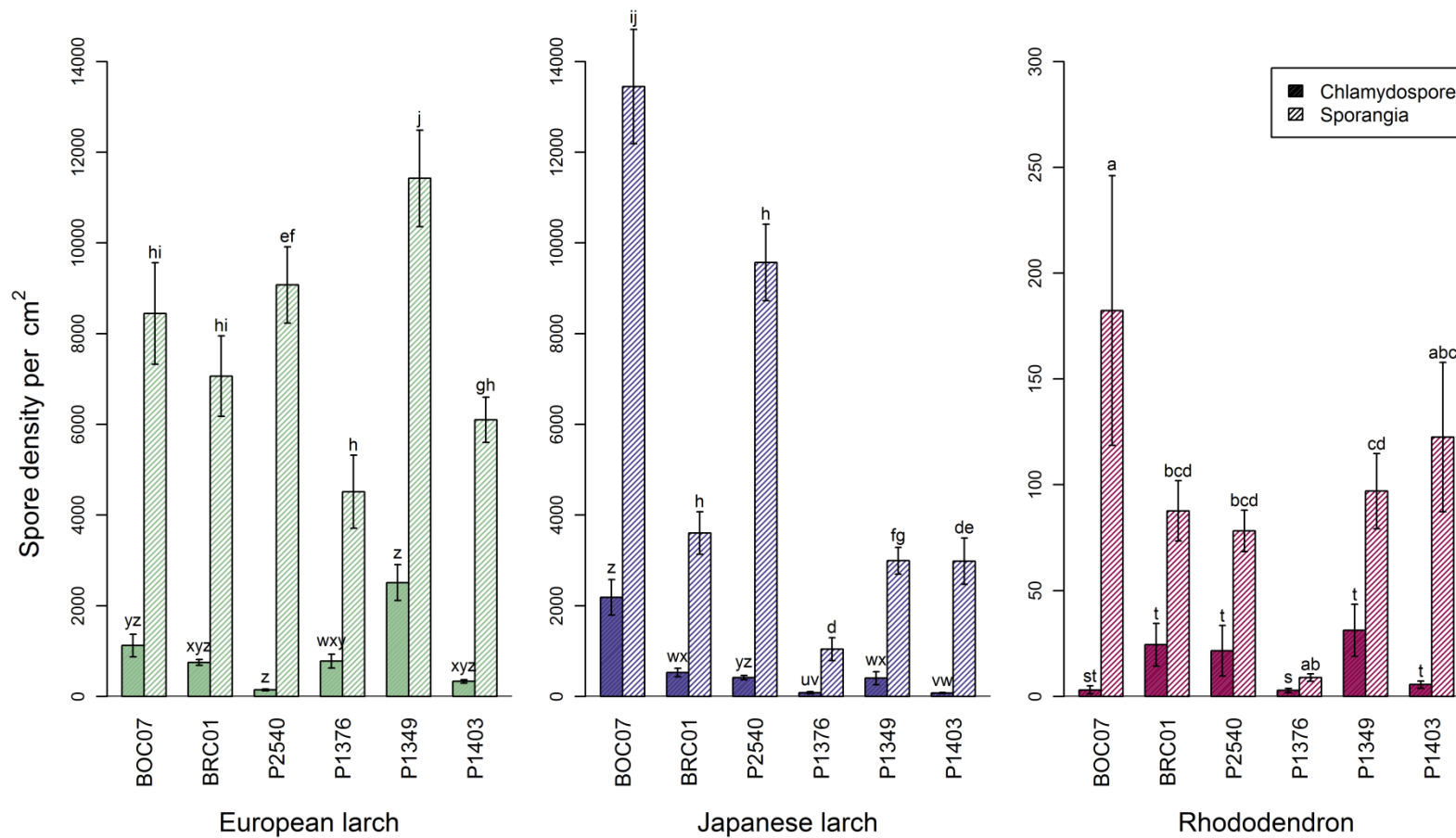


Figure 3.6 – Mean sporulation potential of *P. ramorum* isolates on the leaf surface of hosts. Error bars represent the standard error of the mean ($n = 30$ for counts of sporangia and chlamydo-spores on European and Japanese larch for each isolate and $n = 6$ on rhododendron for each isolate). Different letters indicate differences among isolates determined by Fisher's unprotected least significant difference test at $P < 0.05$, sporangia and chlamydo-spore density was analysed separately.

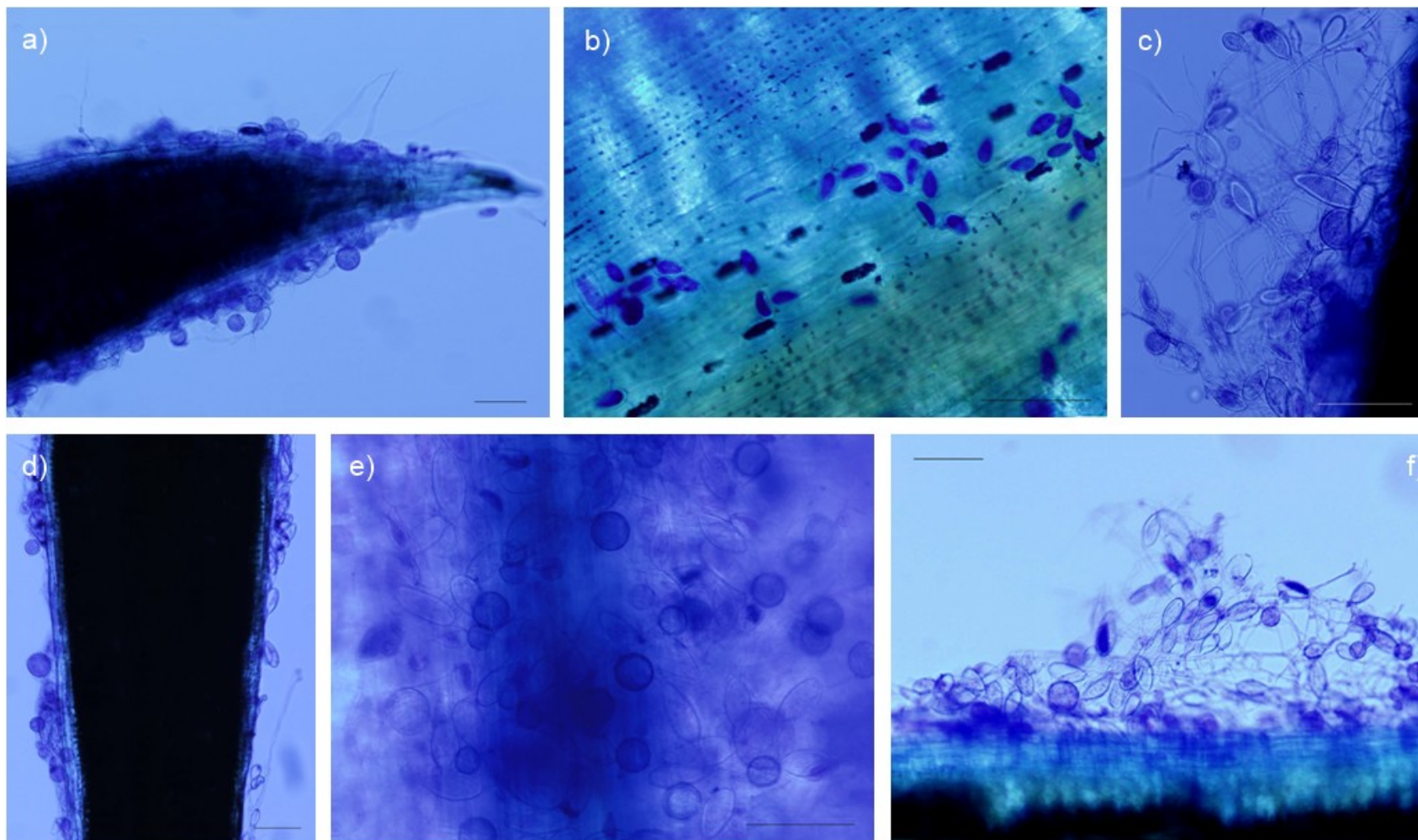
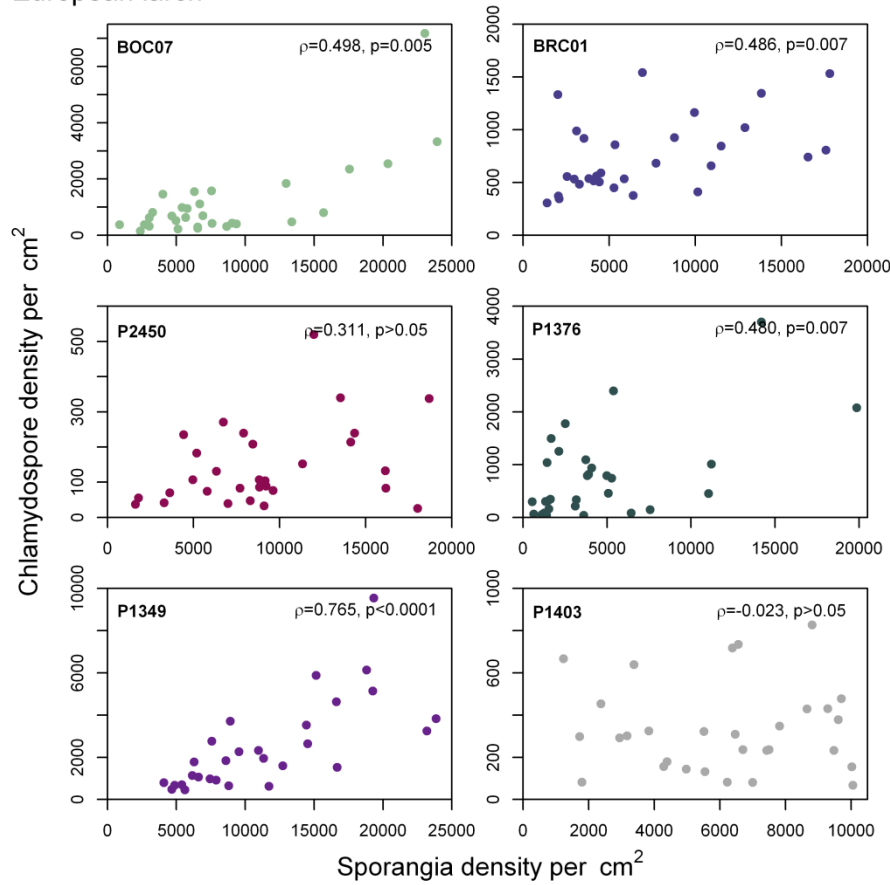


Plate 3.3 – *Phytophthora ramorum* sporangia and chlamydospores growing on the surface of Japanese larch needles after seven days incubation; a) sporangia, chlamydospores & mycelium on needle tip, b) rows of stomata on needle surrounded by sporangia, c & d) sporangia and chlamydospores on the edge of needles, e) large chlamydospores on needles surface, f) sporangia and chlamydospores on the edge of needles (scale bar = 100 μm).

European larch



Japanese larch

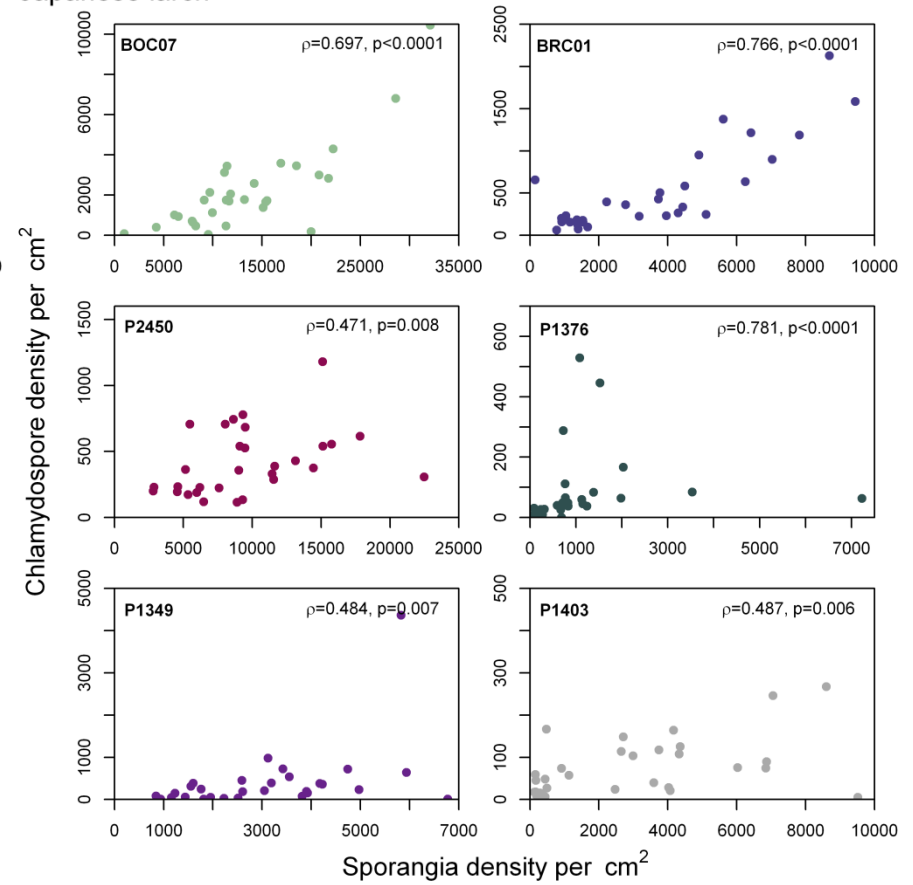


Figure 3.7 - The relationship between the sporangial and chlamydospore production of *P. ramorum* isolates on the surface of European and Japanese larch needles.

Rhododendron

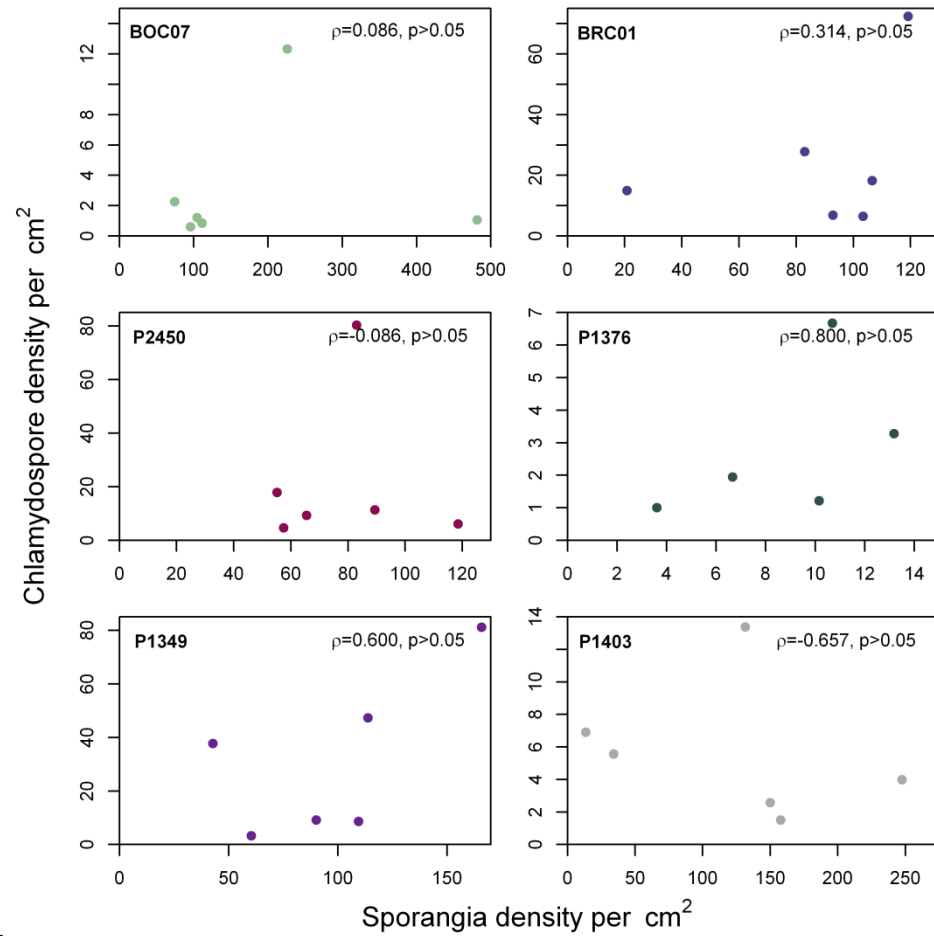


Figure 3.8 - The relationship between the sporangial and chlamydospore production of *P. ramorum* isolates on the surface of rhododendron leaves.

Analysis of the data showed the overall mean sporulation potential varied significantly between isolates ($F(5, 54.8) = 18.01, p < 0.001$) and according to host ($F(2, 5.4) = 78.16, p < 0.001$). Both these factors also interacted significantly ($F(10, 52.2) = 3.79, p < 0.001$). Isolate BOC07 proved the most virulent isolate, producing the highest mean density of propagules on both Japanese larch (15,631 per cm²) and rhododendron (185 per cm²), and the second highest density of propagules on European larch (9,564 per cm²). Isolate P1349, an NA1 isolate, produced the highest mean propagule density on European larch and the second highest propagule density on rhododendron [Figure 3.9].

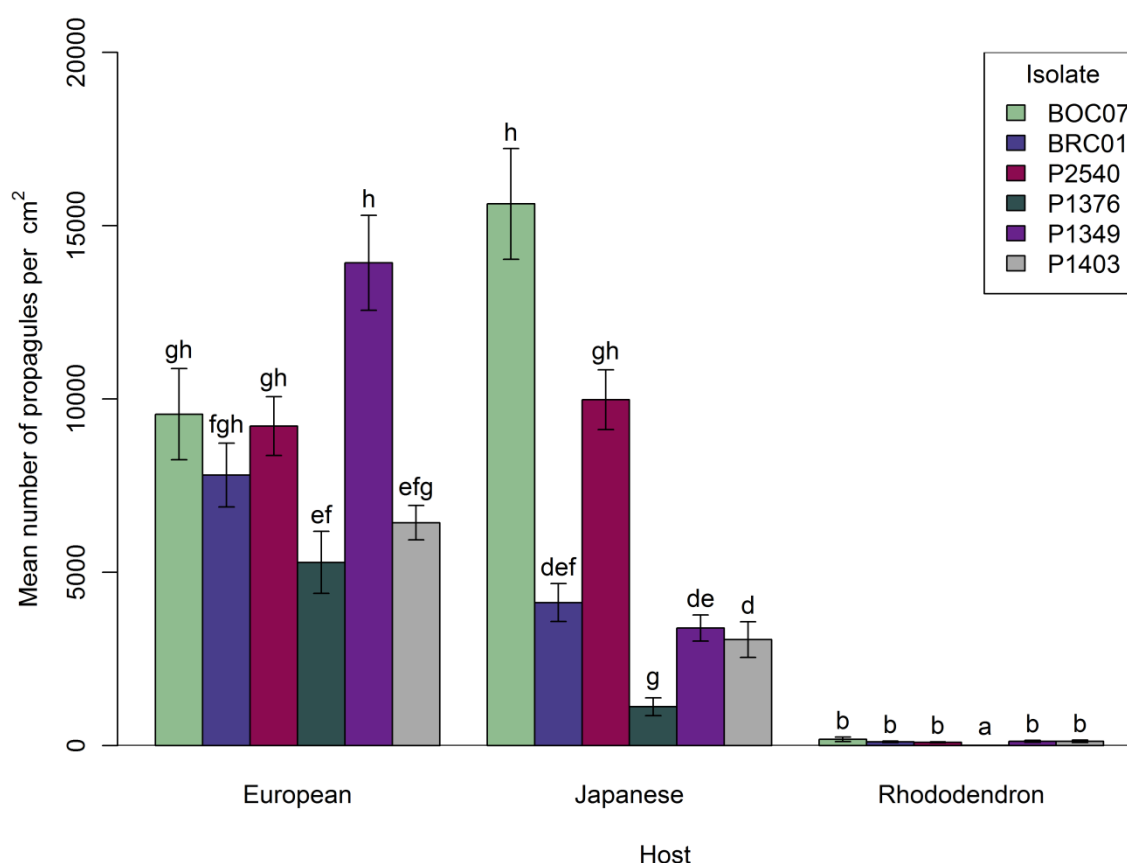


Figure 3.9 - Mean sporulation potential (sporangia and chlamydospores) of *P. ramorum* isolates on the leaf surface of hosts ($n = 30$ for each isolate on Japanese and European larch, $n = 6$ for each isolate on Rhododendron). Error bars represent the standard error of the mean. Different letters indicate differences among treatments determined by Fisher's unprotected least significant difference test at $P < 0.05$.

Isolate P1376 (a UK EU1 standard) was the least virulent isolate, producing the least propagules on all hosts. Sporulation potential of the two NA1 isolates (P1349 and P1403) differed significantly on the larch species, suggesting that the NA1 population of *P. ramorum* may show similar variation in sporulation potential on larch as genotypes of the EU1 lineage. Isolates originating from larch (BOC07, BRC01 and P2540) had the highest levels of sporulation on Japanese larch foliage and the second, third and fourth highest levels on European larch. On rhododendron the second and third highest levels of sporulation came from isolates originating from understory shrubs (P1349 and P1403).

Phytophthora ramorum isolates consistently produced significantly fewer spores on rhododendron foliage compared to Japanese and European larch foliage [Figure 3.9]. Overall, European larch supported higher mean sporulation levels than Japanese larch [Table 3.5]. This contrasted with the previous sporulation potential study (section 3.3.2.1) in which there was no significant difference between Japanese and European larch. Although in the present study there was a significant difference between sporulation levels of *P. ramorum* on European and Japanese larch, this difference was dependent on the isolate, as revealed by the significant interaction between host and isolate.

Table 3.5 – Mean spore density (± 1 s.e.) of *P. ramorum* on host foliage (per cm²).

Host	n	Sporangia	Chlamydospores	Propagules
European larch	179	7749 $\pm$ 394	929 $\pm$ 98.9	8678 $\pm$ 458
Japanese larch	180	5605 $\pm$ 432	614 $\pm$ 89	6219 $\pm$ 500
Rhododendron	35	98 $\pm$ 15	15 $\pm$ 4	113 $\pm$ 16

3.3.5 Variation in symptom development on foliage with different isolate of *P. ramorum*

Symptom development on rhododendron leaves differed significantly according to isolate ($p = 0.001$) [Figure 3.10]. [For estimates of parameters and accumulated analysis of deviance of the fitted model see appendix II]. Isolate P1349, a standard NA1 isolate cultured from rhododendron in 2000, caused the greatest amount of necrosis on rhododendron leaves, followed by P1403, also a NA1 standard from Californian huckleberry in 2002 and isolate BOC07 originating from larch in 2011. The most recently obtained isolate, P2540 from Japanese larch incited the least symptoms.

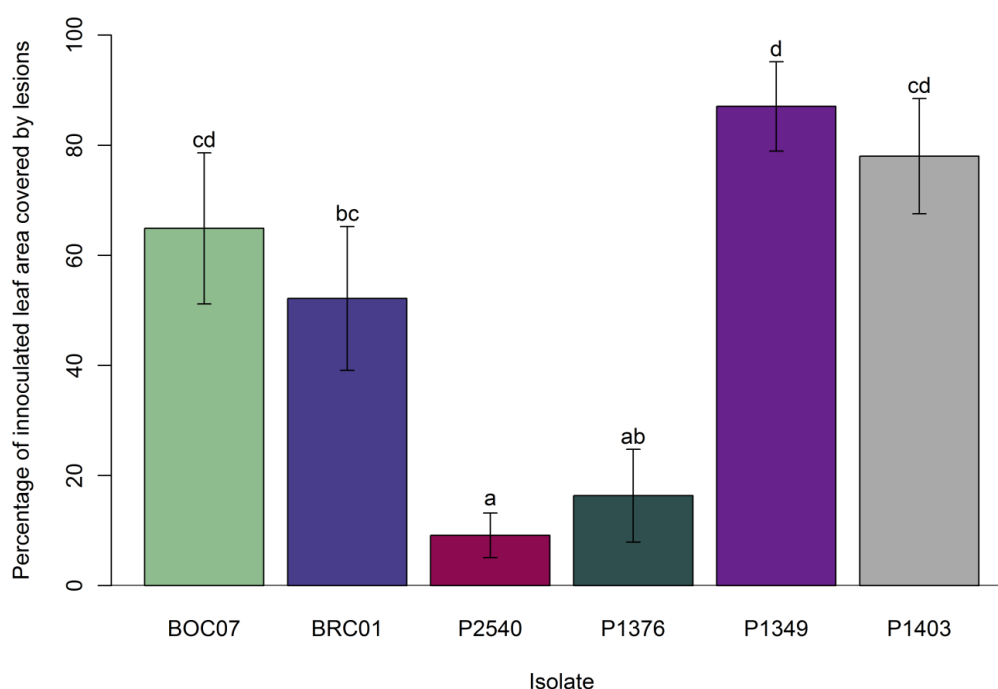


Figure 3.10 - Symptom development on rhododendron leaves caused by different isolates of *P. ramorum* based on the percentage of inoculated leaf surface area that developed necrosis after seven days incubation ($n = 6$). Error bars represent the standard error of the mean. Different letters indicate differences among isolates determined by Fisher's protected least significant difference test at $P < 0.05$

3.3.6 Reisolation success of *P. ramorum* isolates from host foliage

All isolates of *P. ramorum* were reisolated from inoculated hosts, however, reisolation success differed significantly between hosts ($F(2, 8.6) = 33.89$, $p < 0.001$) and unlike previous reisolation experiments (section 3.3.3) between isolates ($F(5, 95.4) = 6.78$, $p < 0.001$) [Plate 3.4 and Figure 3.11]. Reisolation from rhododendron was the most successful

and, as found in the previous study was least successful from Japanese larch. The model predicted a 99% and 13% chance of reisolating the pathogen from rhododendron and Japanese larch respectively. Reisolation success also differed significantly between the larch species, unlike the previous study. *Phytophthora ramorum* was reisolated more readily from European larch needles with the model predicting a 69% chance of reisolating the pathogen compared to 13% in the earlier experiment.

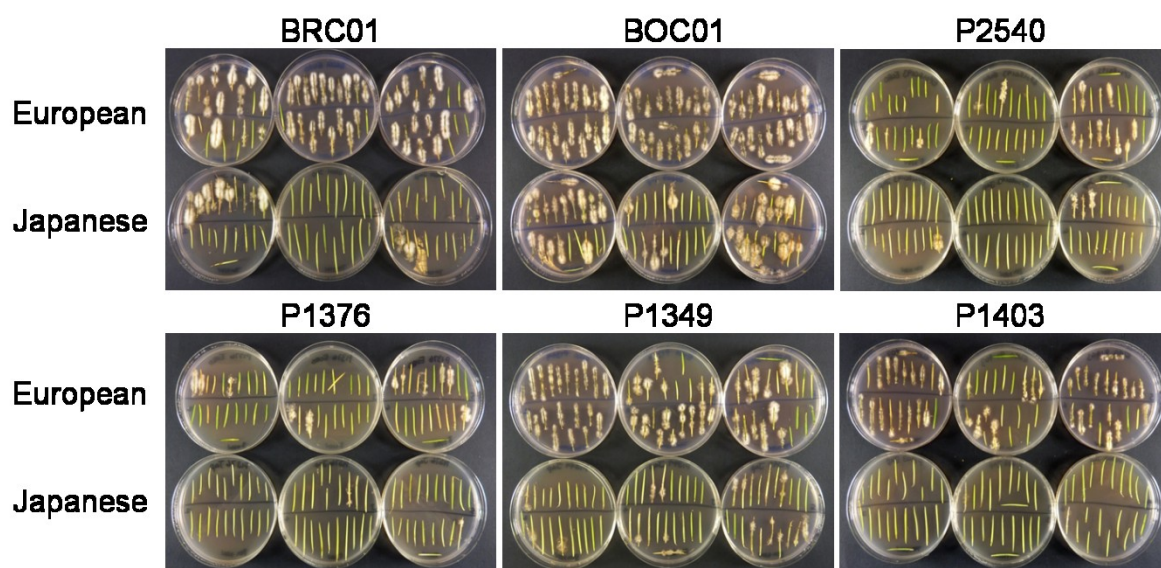


Plate 3.4 – Reisolation of *P. ramorum* isolates from European and Japanese larch on SMA plates.

Reisolation of *P. ramorum* isolates from all hosts varied significantly, ranging from 38–87%. Reisolation success may be correlated with sporulation potential, with isolates exhibiting higher sporulation potential and/or virulence tending to be those that were reisolated more readily. For example, isolate P1376 had the lowest average sporulation levels and reisolation success whereas isolate BOC07 had the highest average sporulation and highest reisolation success [Figure 3.9]. Spore counts, however, could not be undertaken on the needles used to reisolate the pathogen, so direct correlations were not possible. Nevertheless, *P. ramorum* DNA was detected on all larch needles that the pathogen could not be reisolated from, suggesting that they had been infected but that another factor or factors inhibited outgrowth of the pathogen on agar plates.

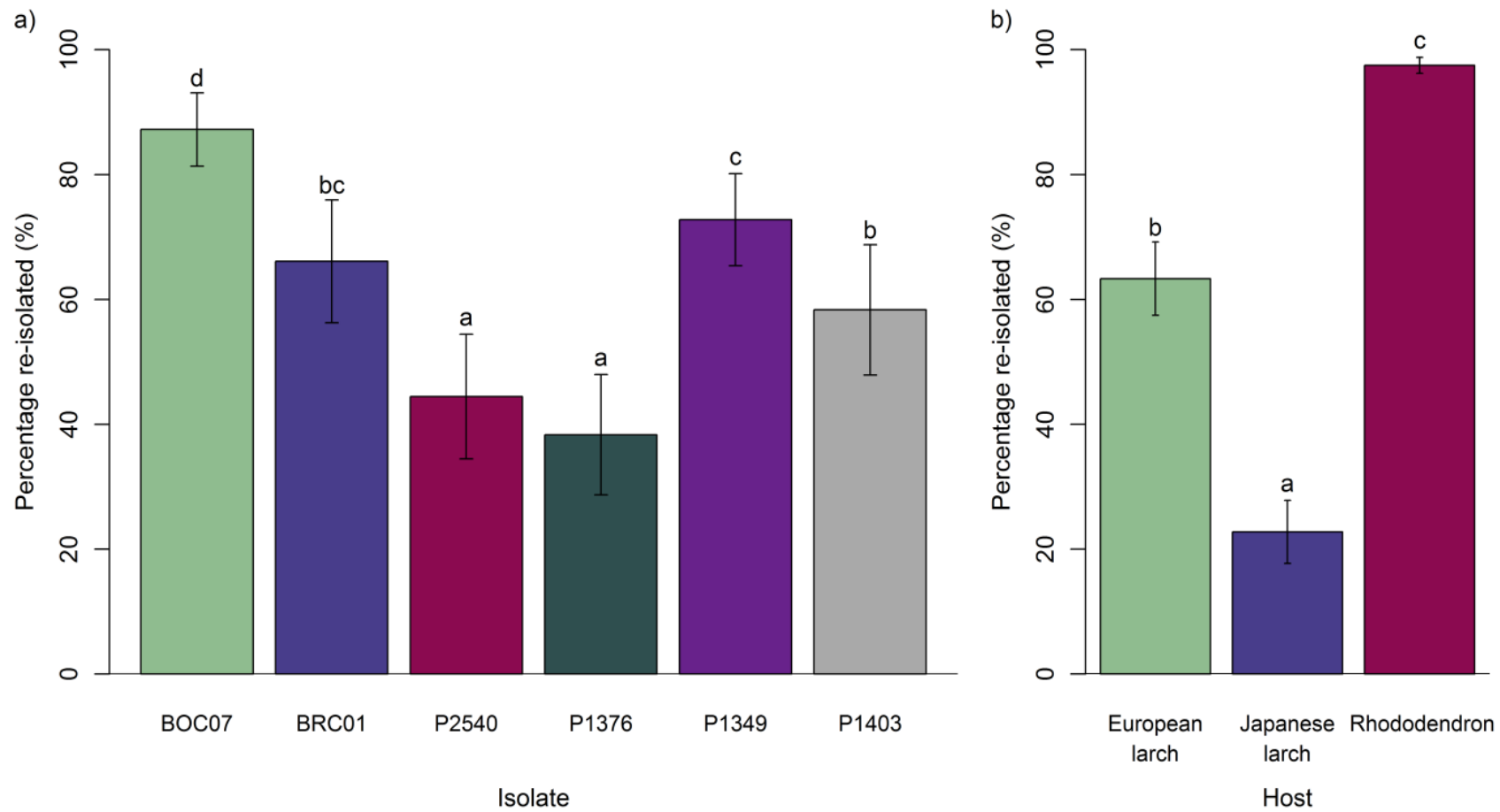


Figure 3.11 - Reisolation success of *P. ramorum* a) isolates from infected foliage (n = 18) and b) different hosts (n = 36), based on mean percentage. Error bars represent the standard error of the mean. Different letters indicate differences among treatments determined by Fisher's unprotected least significant difference test at $P < 0.05$.

3.4 Discussion

Disease development in a plant community depends on the traits of the pathogen, host, and environment. During the year, environments change due to seasonal and climatic influences and this affects both host susceptibility and pathogen vigour. The speed at which plant disease epidemics develop is largely dependent on initial levels of inoculum present (Van der Plank, 1975; Tooley, Browning & Leighity, 2013), so knowledge of pathogen sporulation potential is an important factor for predicting disease spread and management.

3.4.1 Sporulation potential of *P. ramorum*

The findings of this study are the first confirmation that the foliage of European and hybrid larch are both susceptible to *P. ramorum* and able to support its sporulation. Moreover, the sporulation on all larch species exceeded that of all other tested hosts, including Californian bay laurel, which is considered the most epidemiologically important host and the primary source of inoculum driving the Sudden Oak Death epidemics in California (Davidson *et al.*, 2005; Davidson, Patterson & Rizzo, 2008; Meentemeyer *et al.*, 2008).

There are several possible reasons why *P. ramorum* is capable of higher levels of sporulation on larch foliage compared to the broadleaved hosts tested in this study. Firstly, increases in nutrient availability have been shown to increase sporulation (Defra, 2005b), therefore, the nutritional composition of larch needles may be more suitable for sporulation of *P. ramorum* compared with other hosts. Additionally, larch foliage may exhibit less resistance physically and/or chemically towards the pathogen compared to broadleaf hosts. *Phytophthora* species require sterols for sporulation, as they are unable to synthesise their own (Hendrix, 1970) and therefore have to rely on uptake from plant hosts. Differences in the quantitative and/or qualitative plant sterol profiles in host foliage are therefore likely to influence sporulation rates of *P. ramorum* (Strong *et al.*, 2013). Along with plant sterol composition, tannins play an important role in a pathogen's sporulation potential. They form part of the plant's defence mechanism against pathogens and have been shown to affect sporulation through direct toxicity towards the pathogen and inhibiting sterol uptake, possibly by binding with the plant sterols (Manter *et al.*, 2010a; Strong *et al.*, 2013). Variation in tannin content in the foliage of the various hosts may, therefore, affect the sporulation potential of *P. ramorum*. As such, an interesting future study would investigate the sterol content of *P. ramorum* sporulating and non sporulating hosts, with the potential to use such methods as a predictor of plant susceptibility and/or sporulation potential, as proposed by Strong *et al.* (2013). Additionally, needles on the larch shoots may provide a microclimate

with a more optimal environment for sporulation than the broadleaved hosts. As the air moves over the surface of the shoots the needles may slow the movement of air resulting in high humidity (Landsberg & Thom, 1971), generating a more stable and favourable temperature and humidity for sporulation compared to the flatter surface of the broadleaved hosts.

It was notable that sporangial counts varied considerably between larch needles, even those on the same shoot, producing high levels of variance in the data, affecting the likelihood of statistical significance being reached, despite observed differences. Although not significantly different, a higher level of sporulation was observed on Japanese larch compared to European and hybrid larch at all times of year tested. This may be attributable to the anatomy of larch needles. Japanese larch typically have five stomatal bands either side of the midrib on the underside of the needle, and a few obscure lines of stomata on the upper surface of the needle, whilst European larch typically has only one to three stomatal bands on the underside of the needle (Eckenwalder, 2009). This higher density of stomata on Japanese larch may facilitate the production of more sporangia simply by providing a shorter and less energy-demanding route for infection through the leaf epidermis. Sporangia were typically found developing on mycelium emerging from stomata as opposed to mycelium rupturing the leaf cuticle. A recent study exploring how *P. ramorum* infects rhododendron, found it used stomata for both infection and sporulation (Werres & Riedel, 2013) and supports this hypothesis.

Interestingly sweet chestnut foliage supported the highest level of sporulation out of the broadleaved hosts tested. Despite this, sweet chestnut has not played a significant role in the disease epidemic in the UK. It is most likely that whilst the foliage can support *P. ramorum* sporulation, in nature the environment in which sweet chestnut grows may be less conducive to disease development. Sweet chestnut trees have typically been used for landscaping and as urban trees, and are best suited to more continental parts of Britain (i.e. <1500 mm rainfall per year) (Forest Research, 2013), whereas *P. ramorum* is more suited to a maritime climate, hence its distribution along the west coast. The occurrence of sweet chestnut in affected woodlands also tends to be more sporadic, countering the build up of inoculum and formation of disease foci.

The difference observed in the sporulation potential of *P. ramorum* throughout the year is not surprising and supports the findings of previous studies (Davidson *et al.*, 2005; Denman *et al.*, 2006). Seasonal changes in temperature and humidity can impact on the growth and sporulation of *P. ramorum*, with the pathogen favouring temperatures between 16 and 22°C

for sporulation (Englander, Browning & Tooley, 2006) and requiring high levels of humidity (approaching 100%) (Erwin & Ribeiro, 1996). The present study, however, took place under controlled conditions in the lab, with each inoculation experiment being carried out under the same light regime, humidity and temperature. The differences observed are therefore likely to reflect the differences in the host plant physiology throughout the year as opposed to changes in the phenology of the pathogen, which had been stored in a stable controlled environment. The high sporulation levels observed in the lab in August may be attributable to high nutrient availability during the summer months as a result of high photosynthetic rates.

Previous studies, in mixed evergreen forests in California show sporulation of *P. ramorum* to be at its highest in the mid to late rainy season and lowest in the dry hot months (Davidson *et al.*, 2005); the pathogen is also negatively affected by increasing light intensity (Englander, Browning & Tooley, 2006). In addition increases in tannin content in plant foliage associated with increased irradiation as observed in Dahurian larch (*L. gmelinii*) seedlings (Yan, Lu & Yan, 2013) may lead to a reduction in sporulation of *P. ramorum*. The differences associated with the time of year on sporulation levels reflect the importance of climate and environment to the pathogen. Whilst nutrient availability may be at its highest during August, other environmental factors such as light and humidity may limit the sporulation potential of the pathogen in nature, with increased irradiation and lower humidity negatively impacting on sporulation levels. In the lab the pathogen is not exposed to these environmental fluctuations.

An important finding of this study was that sporulation is not linked to needle abscission as has been previously suggested but can occur on larch needles throughout the year. However, field conditions during the summer are unlikely to be climatically suitable for sporulation. Previous lab studies have also shown sporulation levels decreased dramatically in August on sweet chestnut and rhododendron (Denman *et al.*, 2006), whereas this study indicates an increase. Interestingly, sporulation levels the following year in October 2011 using the same *P. ramorum* isolate was markedly higher than in previous similar experiments in 2010 and 2011 [see Table 3.4]. Again the differences observed may be due to changes in nutrition within the foliage between the years, and the needles' physiological state. For example, needle senescence may have started earlier in October 2010 resulting in lower nutrient and higher tannin content compared to October 2011

As with previous studies, spore production varied among *P. ramorum* isolates and on different hosts (Turner, Jennings & Humphries, 2005; Englander, Browning & Tooley, 2006; Englander, Browning & Tooley, 2006; Manter *et al.*, 2010a). Differences in the sporulation

potential of isolates may be due to genotypic variation between these isolates. Results from a previous study looking at two isolates from the NA1 lineage suggest that individuals from this lineage may have a lower sporulation potential when compared to those from the EU1 lineage (Defra, 2005b). Yet another study found that the sporulation potential of EU1, NA1 and NA2 isolates varied significantly, but isolates of the NA1 lineage produced the most sporangia and those of the EU1 lineage the least (McDonald & Grünwald, 2007). In the present study, sporulation potential between the NA1 isolates differed, with some isolates sporulating significantly more than EU1 isolates, others less so. A recent microsatellite analysis of the EU1 nursery and garden population has shown that whilst there is relatively little variation in this population, different genotypes are emerging within the UK (Fera, 2012a). These genotypes show variation in sporulation potential when exposed to different hosts. Whilst this variation may be due to changes in gene sequences it may also be caused by epigenetic changes either in gene expression or cellular phenotype.

There were not only differences in the numbers of sporangia produced on its different hosts but also in the number of chlamydospores. European and Japanese larch foliage, along with rhododendron supported production of both spore types, but sporangia were always produced in higher numbers than chlamydospores, corresponding with previous findings (Defra, 2005b; Turner, Jennings & Humphries, 2005). Although chlamydospores have been observed growing on host leaf surfaces in natural conditions (Davidson *et al.*, 2002), they are more commonly observed growing within host tissues (Tooley, Kyde & Englander, 2004; Pogoda & Werres, 2004; Lewis & Parke, 2006; Fichtner, Lynch & Rizzo, 2007; Parke & Lewis, 2007). A considerable amount is known about the conditions required for sporangia production and germination and chlamydospore germination. However, very little is known about the optimum conditions for chlamydospore production *in planta*. It has, been reported to be suppressed by moist conditions in redwood soils (Fichtner, Lynch & Rizzo, 2009) but increased during dry summer periods in forest soil (Fichtner, Lynch & Rizzo, 2006; Fichtner, Lynch & Rizzo, 2007). Conditions in the lab may be just too moist and cool for higher chlamydospore production but highly suitable for sporangia production. Sporangia production is rapid, whereas chlamydospores are only visible two to three days after inoculation on detached rhododendron leaves in the lab (Smith, 2007) and is connected to increases in environmental stress (Erwin & Ribeiro, 1996). Therefore the optimal conditions for growth and sporulation used in this study are unlikely to have been optimal for chlamydospore production and therefore may not reflect the pathogen's chlamydospore production potential. Importantly, lack of negative correlation between sporangia and chlamydospore numbers suggests there was no apparent trade-off in the production of the different spores. The ratio of sporangia to chlamydospore production may reflect the

pathogen's survival strategy, producing optimal numbers of sporangia for dispersal whereas, chlamydospores which allow long term persistence, may only be produced in abundance when conditions are sub-optimal.

3.4.2 Susceptibility of host foliage

The extent of necrosis, along with infection incidence and reisolation are typically used as indicators of severity in disease epidemics and differ from measures of sporulation potential which is used to assess dissemination potential. Assessment of disease severity can have important implications for disease management strategies.

Whilst all hosts were susceptible at different times of year and the pathogen could be reisolated from them all, results from this study revealed distinct differences in disease incidence, reisolation and host susceptibility to *P. ramorum*. Susceptibility of broadleaved hosts to *P. ramorum* corresponded with the literature. There were relatively minor symptoms on Californian bay laurel (Davidson, Patterson & Rizzo, 2008; Dileo, Bostock & Rizzo, 2009) but large necrotic lesions on bilberry, rhododendron and sweet chestnut (Denman *et al.*, 2005; Sansford *et al.*, 2008). Although symptoms did occur on larch needles they were minor and less pronounced than previously described by (Webber, Mullett & Brasier, 2010; Webber, Turner & Jennings, 2010), but most obvious on European larch compared to Japanese larch.

Symptoms were also most pronounced on the larch in October, corresponding with anecdotal evidence, and least developed in May. Symptom expression on larch has been reported to vary according to larch host and *P. ramorum* isolate by other researchers (Chastagner, Riley & Elliot, 2013). The findings of the present study, however, conflict with a study in North America which found symptoms of *P. ramorum* infection were limited on Autumn tested western (*L. occidentalis*), eastern (*L. laricina*), Japanese and European larch (Chastagner, Riley & Elliot, 2013). Differences between the two studies may be due to plant age, as foliage from mature trees (approximately 30 years old) was used in the present study, whereas Chastagner Riley & Elliot used foliage from young larch seedlings. Host susceptibility to *P. ramorum* is known to change with needle age (Hansen, Parke & Sutton, 2005; De Dobbelaere *et al.*, 2010; Jinek *et al.*, 2011).

Most strikingly symptom expression on Japanese larch was absent in August, but sporulation was at its peak, indicating that *P. ramorum* is able to sporulate at high levels asymptotically on Japanese larch. Asymptomatic sporulation has been reported on naturally infected rhododendron leaves (Denman *et al.*, 2008), and on both detached leaf

and whole plant inoculation studies on balsam fir (*Abies balsamiae*) and eastern larch (*L. laricina*) where asymptomatic sporulation occurred on 90% of the needles (Jinek *et al.*, 2011). Initial inoculum levels were high in this present study and it has been reported that symptom development tends to be linked linearly to inoculum concentration (Tooley, Kyde & Englander, 2004). Despite this, at least over the seven days of the experiment asymptomatic sporulation occurs on larch foliage. This is a significant finding, as it not only suggests sporulation may occur during the summer months on larch, but if it does, infection may go unnoticed and allow the build up of inoculum in the environment.

Symptom expression differed depending on the isolate of *P. ramorum*. This was especially evident from the experiment using six *P. ramorum* isolates (October 2011 study). The highest level of symptom development on rhododendron was caused by isolates BOC07, P1349 and P1403, the latter two belonging to the NA1 population, which is typically slower-growing and less aggressive on host plants compared to EU1 isolates (Brasier, Kirk & Rose, 2006; Elliot *et al.*, 2011). However, the number of isolates used in this study was not sufficient to draw conclusions between the two lineages but instead highlights some of the variability in host susceptibility to different *P. ramorum* genotypes.

Differences in symptom development is also modified by host plant anatomy and chemistry. Plant host resistance to *P. ramorum* is thought to operate at the level of leaf penetration (De Dobbelaere *et al.*, 2010), with leaf anatomy and its effect on water retention being responsible for foliage symptom characteristics, as opposed to susceptibility within the lamina (Moralejo *et al.*, 2006). Changes in leaf physiology throughout the year also explain differences in symptom development on hosts. Susceptibility in rhododendron species has been correlated with the physiological status of the plant, in particular leaf age. However, De Dobbelaere *et al.* (2006) also found that foliage in autumn and winter was less susceptible to *P. ramorum* compared to newly formed leaves in the spring.

3.4.3 Reisolation from infected foliage

Reisolation success of *P. ramorum* from inoculated host foliage was significantly influenced by time of year and host. These results broadly correspond with findings by Denman *et al.* (2008) who reported greater reisolation success in summer compared to winter for conifer species. However, in this study, tests were not carried out in winter as there is no foliage on larch, but the results showed an increase in reisolation from spring to summer and summer to autumn, across all hosts.

Denman *et al.* (2008) also reported lower reisolation success from conifer foliage compared to broadleaved hosts. This is supported by the results of this study, in which reisolation success from larch was considerably lower than broadleaved hosts and nearly always limited to less than 50%. Denman *et al.* (2008) concluded that conifer foliage is considerably less susceptible to infections from *P. ramorum* than broadleaves as a result of low levels of necrosis and less frequent reisolation. This, however, proved not to be the case for the larch in this study as microscopic examination showed needles could be heavily infected with *P. ramorum*. Surprisingly, reisolation assays and pathogen detection were not in agreement with levels of infection or symptom development on larch. Despite positive detection of the pathogen using PCR based detection, symptoms did not always occur and the pathogen was frequently difficult to reisolate, in marked contrast with the broadleaved species. Difficulty in reisolating *P. ramorum* on detached needles has been reported before. Sporangia have been observed growing on the surface of balsam fir (*Abies balsamea*) and eastern larch needles but the pathogen has failed to grow out on to agar plates (Jinek *et al.*, 2011). The authors concluded that this could be due to error in their methods. This current study, however, used alternative methods to Jinek *et al.* (2012) and still found the same result. Instead, low reisolation success from larch needles may be due to leeching of inhibitory compounds, such as phenols from the infected needles into the agar preventing the growth of pathogen. It also suggests that levels of *Phytophthora* infection in conifers may be frequently underestimated, possibly because of limited symptom expression, but more significantly because of the difficulty around isolation. The high degree of infection on the larch needles in this study despite limited symptom development and lack of reisolation indicate that these features cannot be used as indicators of disease levels and infection potential of *P. ramorum* on larch.

3.5 Summary

The main aims of this study were: to test the ability of *P. ramorum* to infect larch foliage, to assess symptom development, to test the pathogen's ability to produce sporangia at different times of year and to investigate the variation in spore production between different isolates of the pathogen.

Assessing the results from the three experiments carried out in October 2010, May 2011 and August 2011 on sporangia production and symptom development as well as the experiment carried out on isolate sporulation variation in October 2011, five findings emerge:

1. Sporulation occurred on all hosts tested in each time period during which the tests were undertaken, but varied between times of year. The foliage of all three larch species consistently supported the highest levels of sporulation, exceeding that of bay laurel which is the most significant sporulating host in California and has been shown to drive the epidemic there (Davidson, Patterson & Rizzo, 2008).
2. Spore production varied amongst isolates of *P. ramorum*. European and Japanese larch foliage supported production of both spore types, however sporangia were always produced in higher numbers than chlamydospores. There was also little evidence of a trade-off between sporangia and chlamydospore production by *P. ramorum* on larch.
3. Symptom development predominantly occurred on older foliage and late in the year for all hosts. For the larch species, host symptom development was most pronounced on European larch and least pronounced on Japanese larch, with hybrid larch intermediate between the two.
4. Reisolation success was also influenced by the time of year, with greater success on older foliage. Reisolation from larch was less successful than from non-larch hosts and nearly always less than 50%. Out of the larch species successful reisolation was always the highest in European larch and lowest in Japanese larch, with hybrid intermediate between the two.
5. Sporulation potential did not correlate with symptom development and reisolation success. These measures are therefore not accurate indicators of the disease level and infection potential of *P. ramorum* on larch.

Chapter four - The susceptibility of *Larix* bark to isolates of *Phytophthora ramorum*

4.1 Introduction

Sporulation studies in the previous chapter gave insight into how *Phytophthora ramorum* proliferates on larch foliage at different times of the year and the factors that influence this. However, it gave little insight into the pathogen's capacity to cause tree mortality. *Phytophthora ramorum* is known to cause lethal bark cankers in some tree hosts. Coastal live oak (*Quercus agrifolia*) and tanoak (*Notholithocarpus densiflorus*) bark in coastal California and Oregon, are particularly susceptible (Rizzo *et al.*, 2002b). Lesions on these species can be over 2 m in length and are typically found 1-2 m above ground but have been reported up to 20 m high (Rizzo *et al.*, 2002a). In the field, infected Japanese larch in the UK has been observed with abundant resin bleeds on trunks, branches and side shoots, with reports of up to 80 separate cankers on the branches and stems of affected individuals (Webber, 2011). Tissue necrosis in the inner bark of these trees typically results in crown dieback followed by tree death as the lesions effectively girdle the whole stem, preventing movement of plant nutrients beyond the lesion (Rizzo *et al.*, 2002a).

Koch's postulates have been satisfied for *P. ramorum* on the foliage of Japanese larch (Webber, Mullett & Brasier, 2010) but have not been fulfilled to date for bark tissue, with tests on Japanese larch logs in the field proving inconclusive (Webber, 2011). In addition, *P. ramorum* has been reported to infect European and hybrid larch in the field, however Koch's postulates have also yet to be satisfied for these species. In Britain over 134,000 hectares of commercial larch plantations are at risk from *P. ramorum* (Forestry Commission, 2012a). It is therefore important to determine the susceptibility of European and hybrid larch bark girdling infections by *P. ramorum* so that the relative risk to the three commercially grown species can be understood. These data could form part of the advice on whether larch species can continue to be grown commercially in Britain.

Prior to the discovery of ramorum dieback on Japanese larch, tree infections in Europe were rare. Only 14 trees in the Netherlands and 82 trees in the UK were reported with infection between 2003-2007 and only 20 of those with stem infections (Sansford & Woodhall, 2007). However, infection of thousands of larch trees has allowed *P. ramorum* a huge turnover in biomass, so the pathogen may have undergone genetic change in these epidemic areas.

Therefore this study aims to establish if the pathogen may be showing evidence of adaptation to its new host by exploring pathogenicity on larch. Material from this study will also be used to evaluate isolation success from different regions of the bark lesion in order to improve disease detection which has proven difficult in the past (Webber, Turner & Jennings, 2010).

The objectives of this study were to:

- 1) Establish the relative susceptibility Japanese, European and hybrid larch bark to *P. ramorum*
- 2) Compare the pathogenicity of different isolates of *P. ramorum* to larch bark
- 3) Determine how host susceptibility to *P. ramorum* changes over the growing season
- 4) Assess relative reisolation success of isolates based on positive or negative reisolation of *P. ramorum* from Japanese, European and hybrid larch bark

4.2 Materials and Methods

The relative susceptibility of Japanese, European and hybrid larch bark to *P. ramorum* and isolate pathogenic variation was tested using log inoculation methods devised by Brasier and Kirk (2001) described in section 2.9. Experiments were carried out in summer (August) 2011 and spring (May) 2012.

4.2.1 Plant material

Logs were cut from mature healthy trees (average stem diameter 30 cm), grown at the Alice Holt Research Station. Two trees of each species were used in each study. Two logs were cut from each tree in summer 2011, three of which were used as experimental logs and the other as lesion development indicator, whilst in spring 2012 five logs were cut from the two trees, two from each tree were used for the main experiment and the remainder as a lesion development indicator log.

4.2.2 Isolates and inoculum production:

Each log was challenged with six isolates of *P. ramorum* in both summer 2011 and spring 2012 [Table 4.1]. Isolates were sourced from recent disease outbreaks on larch along with standard isolates, used in previous studies reported by Sansford *et al.* (2008). The isolates were cultured on CA and incubated at 18-20°C in continuous daylight (60W daylight™ bulb suspended 30 cm above the plates) for 14 days prior to inoculation.

4.2.3 Inoculation, incubation & sampling

Logs were inoculated and incubated as described in section 2.9.2. After five weeks of incubation, lesion development on the indicator logs was checked using sampling methods described in section 2.9.2. Lesion development on the indicator logs in the summer 2011 test was unexpectedly small (approximately 4 cm²) and so the remaining logs were incubated for a further three weeks (August-September) before sampling. In spring 2012, after five weeks incubation (May-June), large lesions had developed on the tester logs and so all logs were sampled at that time.

Table 4.1 - Origins of the *Phytophthora ramorum* isolates used in this study

Isolate	Location	Sampled host	Isolated	Lineage
BOC07	Cornwall, SW England	<i>Larix decidua</i>	Mar 2011	EU1
BRC01	Somerset, SW England	<i>L. kaempferi</i>	Sep 2010	EU1
PLY72	Devon, SW England	<i>Pseudotsuga menziesii</i>	Aug 2010	EU1
P1376*	Sussex, SE England	<i>Viburnum tinus</i>	Apr 2002	EU1
P1403*	Oregon, USA	<i>Vaccinium ovatum</i>	Unknown	NA1
P2470	Devon, SW England	<i>L. kaempferi</i>	Jul 2011	EU1
P2738	Cornwall, SW England	<i>Rhododendron</i>	Aug 2009	EU1

* Standard isolates included for comparisons with host susceptibility tests carried out by Sansford *et al.* (2008).

4.2.4 Re-isolation

Attempts to re-isolate the pathogen from all lesions and logs were made using methods described in section 2.6.1. In addition, isolation was attempted from different coloured phloem zones within the lesions [Plate 4.1] for all isolates and hosts in the summer 2011 experiment and from each lesion on a log of each species in the spring 2011 experiment.

Isolation success was recorded on all re-isolation attempts. Where *P. ramorum* could not be re-isolated from the bark after seven days incubation, bark chips were removed from the SMA plates, transferred to microtubes and stored at -80°C and later prepared for DNA extraction (see section 2.7.1) and pathogen detection via real time PCR (see section 2.8.2)

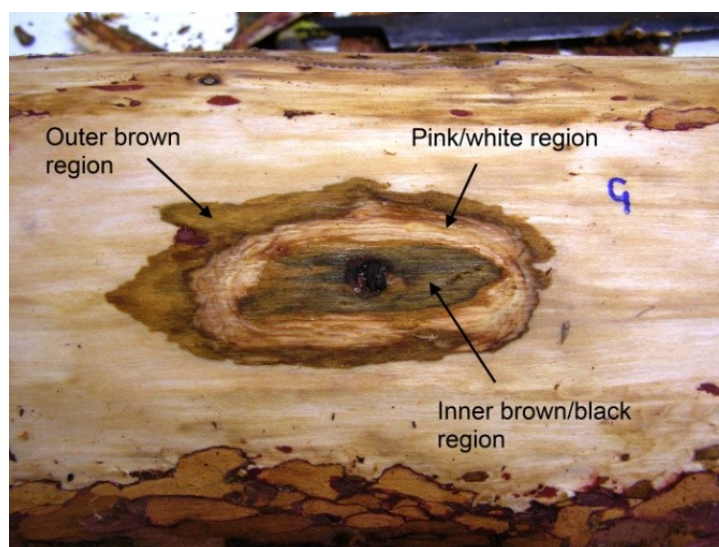


Plate 4.1 - Different colour zones within a *P. ramorum* lesion on larch.

4.2.5 Experimental design and statistical analysis

The relative susceptibility of Japanese, European and hybrid larch bark to *P. ramorum* was tested using seven isolates of *P. ramorum*. Two trees of each species yielded three logs in summer 2011 and four logs in spring 2012 for use in the two experiments. Each isolate was replicated six times in the summer experiment and eight times in the spring experiment with a log representing one block. Each block contained two replicates of each isolate and two carrot agar plugs as controls. Data on the following parameters was recorded:

Parameter 1 – Relative susceptibility of Japanese, European and hybrid larch bark to *P. ramorum*, based on differences in the mean lesion size (cm²).

Parameter 2. – Relative susceptibility of Japanese, European and hybrid larch bark to *P. ramorum* at two different stages in the growing season, based on differences between the mean lesion size (cm²) on bark in summer 2011 and spring 2012.

Parameter 3. - Pathogenic variation between isolates of *P. ramorum* based on differences between the mean lesion size (cm²) generated by each *P. ramorum* isolate.

Parameter 4 – Reisolation success based on positive or negative reisolation of *P. ramorum* from each host and lesion region (determined by zone colour).

Data was analysed using GenStat (16th Edition). For parameters 1 to 3 a restricted likelihood model (REML) variance components analysis was fitted to $\log_{10}$ transformed data on lesion area to test for differences in lesion sizes on larch hosts, between isolates and at two different times of year. Host genotype (tree one or two of each larch species), log (position from either the base or middle of trunk) and band (inoculation points either at the top, middle or bottom of billet) were treated as random effects in the model-building. Fisher's unprotected least significant difference test was carried out on the data to draw out significant differences between individual *P. ramorum* isolates

For parameter 4, reisolation success, the experimental design consisted of 15 to 20 reisolation attempts from each lesion region (where possible) on the three larch species. A generalised linear mixed model with a binomial error structure and logit function was fitted to the data to test for differences in reisolation success between lesion region and larch host. Lesion size was also tested to determine if this influenced reisolation success. Host genotype and log were treated as random effects in the model-building.

4.3 Results

4.3.1 Larch susceptibility and variation in pathogenicity of *P. ramorum* isolates

All seven isolates of *P. ramorum* tested in summer 2011 and spring 2012 caused necrotic lesions in the inner bark of Japanese, European and hybrid larch logs [Plate 4.2]. Bark of the logs remained moist during the five and eight week incubation periods in all three species. In some cases, callus was produced at the cut ends of the logs indicating they were alive and physiologically active. An almond aroma could be detected from the necrotic larch tissues during sampling. Lesion shape and colour varied but was typically dark cinnamon-brown in the innermost section and pink/white on the outermost section. In general larger lesions had an additional ring of cinnamon-brown tissue on the outermost regions of the lesion [Plate 4.1 and Plate 4.2]. Bright pink to maroon-red lesion margins described by (Webber, Mullett & Brasier, 2010) were not observed on any of the lesions. Only on a few occasions did the lesions extend into the xylem, generating black regions in the very outer sapwood layers around the inoculation points. However, the pathogen could not be reisolated from these regions.



Plate 4.2 - Lesions on *Larix* logs caused by *Phytophthora ramorum* isolate P2738 in summer on; a) European larch, b) Hybrid larch and c) Japanese larch. in spring 2012 on; d) European larch, e) Hybrid larch and f) Japanese larch, g) Japanese larch log from spring experiment with multiple lesions, h) lesion caused by isolate P1349 (NA1) on Japanese larch in spring.

4.3.1.1 Host comparisons

When results of both experiments (summer 2011 and spring 2012) were combined, susceptibility of the three larch species did not differ significantly ($F(2, 5.9) = 2.88, p > 0.05$). Average lesion sizes on European, hybrid and Japanese larch were $83.72 \pm 30.7 \text{ cm}^2$, $54.66 \pm 29.53 \text{ cm}^2$ and $62.66 \pm 39.9 \text{ cm}^2$ ($\pm 1 \text{ s.e.}$, $n=4$) respectively. However, the two experiments demonstrated that susceptibility of larch bark of *P. ramorum* declined significantly as the growing season progressed ($F(1, 6) = 26.86, p < 0.01$), decreasing from an average lesion size of $102.17 \pm 28.9 \text{ cm}^2$ (spring experiment) to $23.6 \pm 30.2 \text{ cm}^2$ ($\pm 1 \text{ s.e.}$, $n=6$) (summer experiment) after five and eight weeks of incubation respectively.

When the two experiments were analysed separately, overall European larch was more susceptible to *P. ramorum* in summer, whereas bark of all three species was equally susceptible in the spring [Table 4.2]. There was no significant interaction between season and larch species ($F(2, 6.0) = 1.95, p > 0.05$).

Table 4.2 - Comparisons between the relative susceptibility of larch bark to *P. ramorum* colonisation

Season	Larch species	Mean lesion size (cm^2)	Standard error (n=2)
Spring (May - June)	European	123.74	± 36.17
	Hybrid	104.98	± 39.06
	Japanese	120.90	± 56.09
Summer (August – September)	European	53.48	± 27.35
	Hybrid	16.56	± 8.06
	Japanese	7.02	± 4.59

4.3.1.2 Isolate comparisons

The individual isolates of *P. ramorum* differed significantly in their pathogenic potential, evidenced by mean lesion areas [Table 4.3]. Most variation in lesion size, however, was explained by the interaction between larch species and isolate ($F(12, 202.9) = 2.32, p < 0.01$) [Figure 4.1] and was due to the behaviour of isolate P1403 (of NA1 lineage), which was equally pathogenic on all three larch species. The mean lesion size for all other isolates was greatest on European larch and similar on hybrid and Japanese larch.

Table 4.3 - Comparisons between the relative pathogenicity of isolates of *P. ramorum* on larch bark combined species data (Different letters indicate differences among treatments determined by Fisher's unprotected least significant difference test (LSD) at $P < 0.05$).

Isolate	Mean lesion size (cm ²)	Standard error (n=12)	LSD	Isolated	Lineage
P2470	70.45	± 18.4	a	2001	EU1
BRC01	62.24	± 17.8	ab	2010	EU1
PLY72	79.96	± 21.1	bc	2010	EU1
P1376	81.94	± 20.6	bc	2002	EU1
P2738	76.12	± 19.0	bc	2009	EU1
P1403	74.76	± 21.4	bc	Unknown	NA1
BOC07	84.01	± 21.4	c	2011	EU1

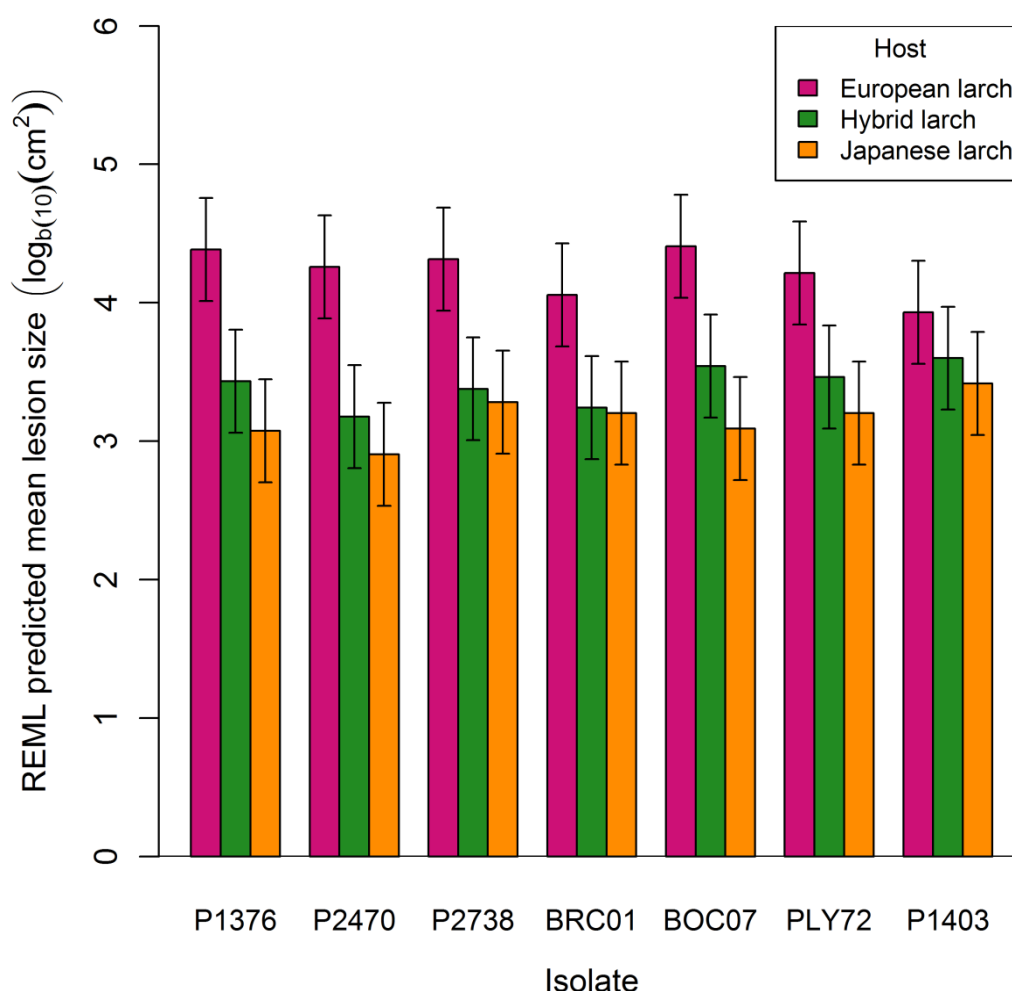


Figure 4.1 – Combined data for the spring and summer experiments showing REML predicted means for log transformed lesion size in the inner bark of larch logs caused by seven isolates of *P. ramorum* colonising European, hybrid and Japanese larch inner bark (error bars represent the standard error of the mean calculated by the REML analysis).

The interaction between season and isolate explained the considerable variation in lesion area ($F(6, 203.2) = 2.55, p < 0.05$). Whilst all isolates were more pathogenic in the spring they were equally pathogenic relative to each within each experiment. The exception to this was isolate BRC01, which was less pathogenic in spring compared to other isolates, but similarly pathogenic in summer [Figure 4.2].

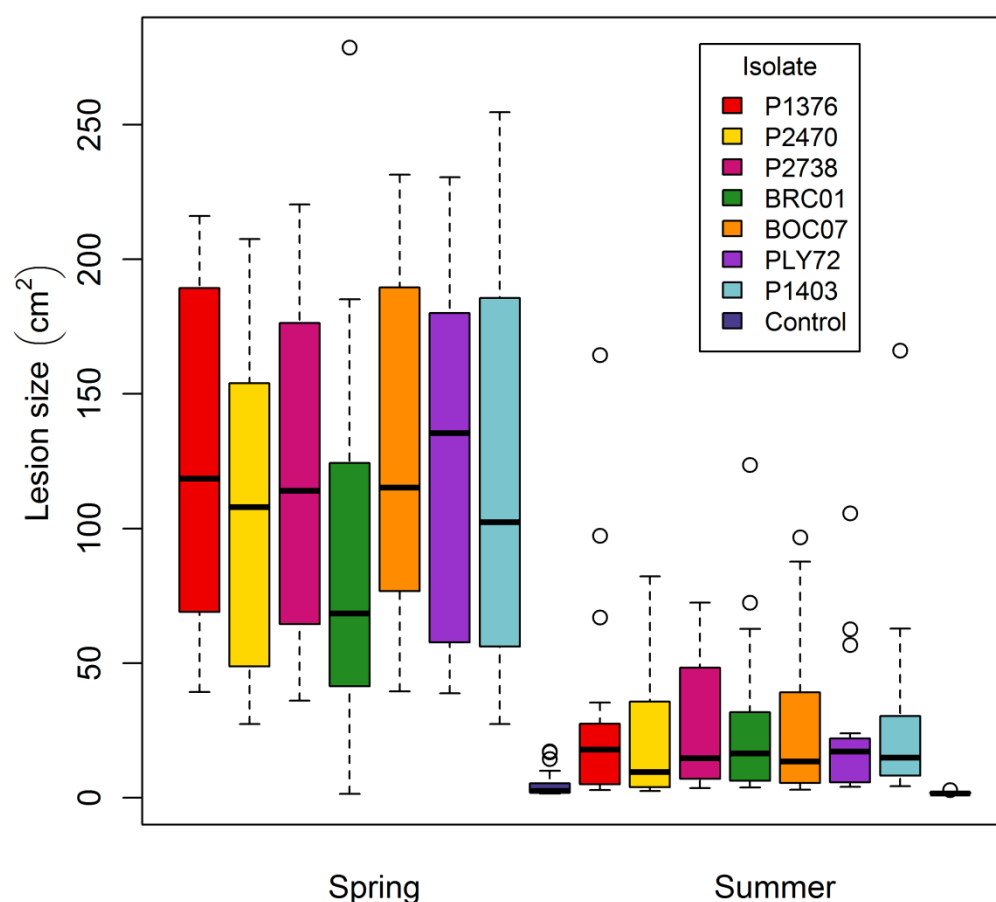


Figure 4.2 – Box and whisker plot of the lesion size of the inner bark of larch logs caused by isolates of *P. ramorum* in spring 2012 and summer 2011.

It was notable that there was considerable variation in the lesion sizes caused by *P. ramorum* amongst all three larch species (see appendix III). Some of the variation within the same tree could be attributable to the tree height from which the logs was taken i.e. the first log taken from the base of the tree or the second log taken from further up the stem. For example, isolate P1403 caused lesions ranging in size from 115.8 – 203.2 cm² on a European larch log cut from the base of the trunk, but lesions ranged from 56.1 - 73.6 cm² on the log originating from further up the stem. Similarly, isolate BRC01 caused lesions ranging in size from 185.1 - 278.7 cm² on a Japanese larch log cut from the base of the tree,

and 142.0 - 159.6 cm² on logs from further up the tree. Overall, lesions on logs from higher up the tree were consistently smaller than those on logs from the tree base.

4.3.2 Success of *P. ramorum* reisolation

Efforts were made to recover *P. ramorum* from all hosts, although not from every lesion. Where it was not possible to reisolate the pathogen, its presence was confirmed by DNA detection. Isolation of cultures from all larch species and each defined region of every lesion was extremely low. The average reisolation success was 10%. Reisolation success, however, was significantly affected by season; it was significantly higher in the summer ($F(1, 202.5) = 6.75, p < 0.01$) with 14.3% isolation success, compared to only 7.6% success rate with spring material.

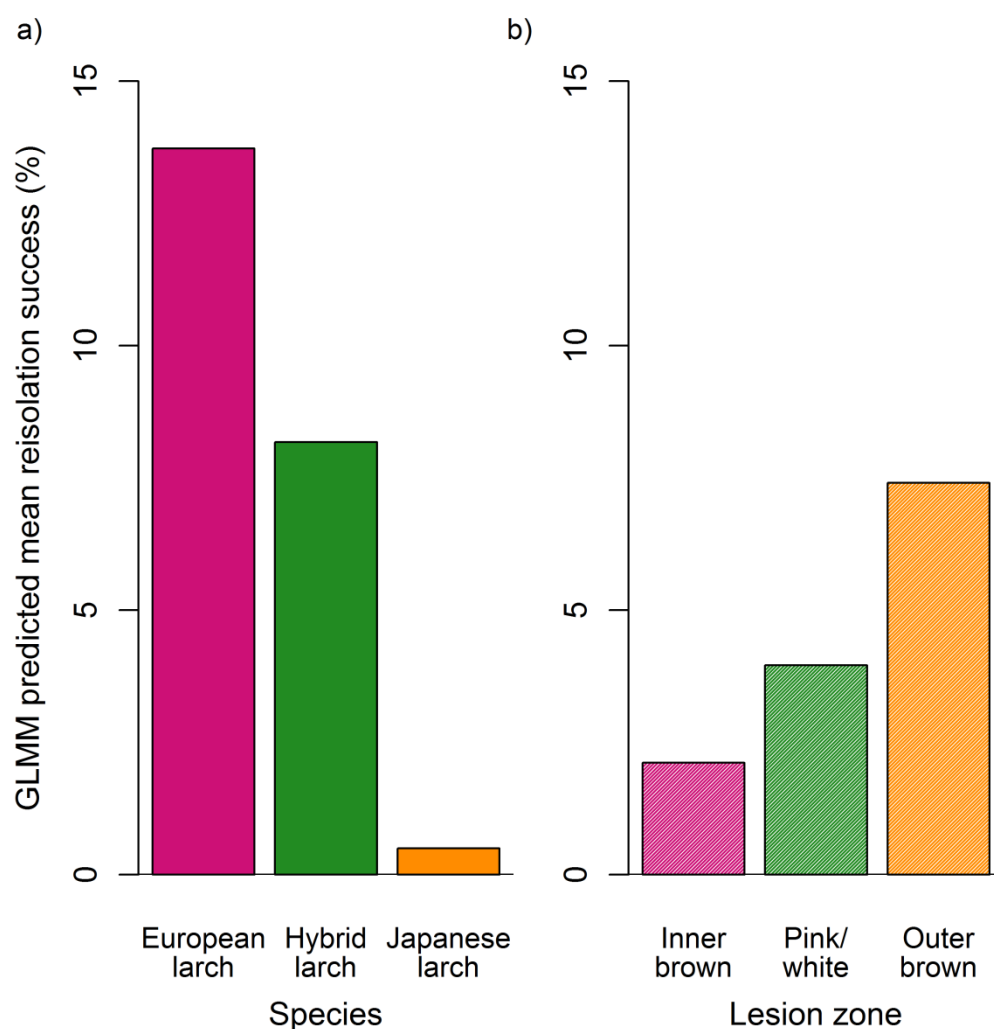


Figure 4.3 – GLMM predicted means for percentage re-isolation success of *P. ramorum* from; a) different species of larch and b) lesion zones, in the inner bark of larch logs

Reisolation success also differed markedly with species ($F(2, 610.3) = 12.11, p < 0.001$) and lesion zone ($F(2, 643.3) = 4.31, p < 0.01$) [Figure 4.3a]. Reisolation success was greatest from European larch, followed by hybrid, and least successful from Japanese larch. When bark pieces taken from the lesions were transferred to SMA plates for reisolation, after several hours of incubation, agar around Japanese and hybrid larch bark pieces turned a brown to red colour [Plate 4.3]. This did not occur with the European larch bark pieces on agar plates.

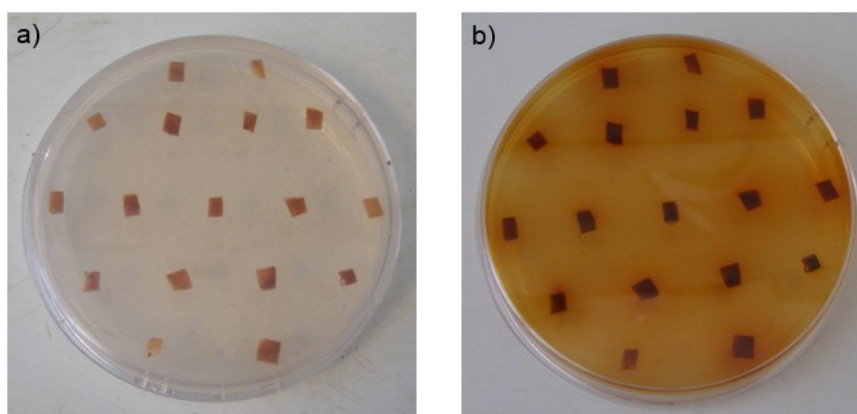


Plate 4.3 – SMA plates with symptomatic tissue from Japanese larch bark after; a) initial placement on the agar, b) after 24 hours of incubation

The pathogen was isolated from all three zones of the lesion, but was more readily isolated from the outer brown region followed by the pink/white region [Figure 4.3b]. Reisolation was least successful from the inner brown region. Lesion size did not have a significant effect on the reisolation success rate ($F(1, 646.5) = 0.72, p > 0.05$), neither did the fixed effects interact with one another ($F(4, 644.3) = 1.59, p > 0.05$).

4.4 Discussion

4.4.1 Larch susceptibility and variation in *P. ramorum* isolate pathogenicity

Prior to this study, although *P. ramorum* had been frequently isolated from girdling cankers on Japanese larch in the field, and very occasionally from similar hybrid and European larch material, Koch's Postulates had not been undertaken for the canker symptoms caused by *P. ramorum* on any larch species. The results demonstrate that the *P. ramorum* is the causal organism of the resinous cankers observed on the bark of all three larch species and therefore satisfied Koch's postulates. In the field *P. ramorum* has been isolated from stem

cankers on at least 30 different tree species, 20 of which are in the UK (Fera, 2012b). In the laboratory, susceptibility testing of bark to *P. ramorum* colonisation has identified 11 tree species as highly susceptible (all from the *Fagaceae*), 14 to be moderately susceptible, 18 to have a low susceptibility, 11 to be resistant and three to be immune to *P. ramorum* (*Phytophthora* database - <http://www.Phytophthoradb.org> [Accessed online, 2014]). However, larch differs from most *P. ramorum* host tree species, as the majority of hosts which display moderate to high levels of bark susceptibility typically show little or no foliar susceptibility (Sansford *et al.*, 2008). *Phytophthora ramorum* is, however, pathogenetic to both larch bark and foliage, with profuse sporulation exhibited in the field and lab on infected larch foliage (see chapter three).

Although data suggest that European larch was more susceptible to *P. ramorum* colonisation in the summer, when compared to Japanese and hybrid larch, analysis revealed that on average the three larch species were similarly susceptible to *P. ramorum* at both times in the growing season. The lack of a significant difference between the species, despite the apparent trend, is most probably due to the low number of replicates used in this study. Two trees of each species were tested for susceptibility in both the spring and summer experiments. In both studies there was a considerable amount of variation in the data that could not be attributed to the fixed test factors but was instead assigned to the natural variation between individual trees and the region of the tree that was inoculated. For these reasons, no significant differences could be drawn out of the comparisons between larch species. These results highlight the need for a larger host genotype sample size when determining the relative differences in the susceptibilities of larch to pathogens such as *P. ramorum*, although such experiments are expensive and labour intensive.

However, it was striking that susceptibility was greater during spring (May-June) than later in the year (August-September). Using the susceptibility scale set out by Sansford *et al.* (2008), all three larch species would be classified as highly susceptible in spring, demonstrating similar levels of susceptibility to many North American tree species belonging to the *Fagaceae*. Later in the year however, European larch would be classified as moderately susceptible, hybrid larch as slightly susceptible, whilst Japanese larch would be classified as resistant. Despite this, statistical analysis did not show the susceptibility of Japanese and hybrid larch to differ significantly in the August-September trial.

The placement of Japanese and hybrid larch in different susceptibility categories, in spite of the lack of statistical difference again reflects the range in intra-species lesion size, suggesting that individual trees within a species can be more or less susceptible to *P.*

ramorum. Variability within a species in susceptibility to *P. ramorum* has also been observed on English oak (*Q. robur*) (Brasier *et al.*, 2006) and other *Quercus* species (Moralejo, Garcia Munoz & Descals, 2009). Such variation may be significant to the larch industry as differences between individuals may indicate the presence of a resistance gene or genes in the population and therefore the potential for breeding resistant varieties. It is also interesting that differences in susceptibility between the individuals of the same species used in this study were so large, particularly as they originated from the same plantation and are therefore likely to be genetically similar, probably having been taken from the same seed stock. This variability however, may not be due to the influence of epigenetic factors controlling host susceptibility as these are typically heritable changes. Instead, abiotic factors may have affected the individual susceptibility of the trees (Schoeneweiss, 1975). For example, environmental stresses such as drought, changes in pH, high or low temperature can all cause changes in plant DNA methylation and chromatin modification (Boyko & Kovalchuk, 2011). Whether this is occurring in the trees in this study is unknown and would require further investigation.

The greater susceptibility of European larch bark to *P. ramorum* colonisation (at least during August-September) has not been observed in field data from the *Phytophthora* survey carried out as part of the disease monitoring and containment programme by Forest Research and Forestry Commission Plant Health teams in England, Scotland and Wales. Out of the 1,258 larch bark samples testing positive for *P. ramorum* infection between 2009 and 2013, seven trees were identified as European larch, 66 as hybrid larch and 943 as Japanese larch (the remaining 242 samples were not identified to species level) (S. Sansici-Frey & J. Webber, unpublished data). The small number of records for European larch tends to suggest it is the least susceptible species. However as Japanese and hybrid larch make up 83% of the larch grown in Britain (Forestry Commission, 2003), the skewed proportion of samples towards Japanese and hybrid larch may simply reflect the greater number of trees available for sampling. Alternatively the difference in these field observations and the results of the current study may suggest that germinating *P. ramorum* zoospores are less effective at penetrating the outer bark of European larch. The method used in this study did not test the pathogen's ability to invade intact host bark, instead it tested the ability of the pathogen to colonise and spread within the inner tissues following wound inoculation. Plants have two classes of defence against invading pathogens. Structural defences which physically prevent the pathogen from gaining entry into the host and chemical defences, produced in the plant cells and tissues which can inhibit pathogen growth (Agrios, 2005). Host resistance in European larch may, possibly, operate at the bark surface limiting the direct penetration of zoospores. Once the pathogen has passed this barrier, however, European larch may be

less effective than Japanese and hybrid larch at defence against the colonising pathogen, especially later on in the growing season. Similar differences have been observed between field studies and experimental data on the pathogenicity of *P. kernoviae* on European sycamore (*Acer pseudoplatanus*). In the field, European sycamore bark is rarely infected but in the lab it demonstrates a moderate to high susceptibility to *P. kernoviae* (Brasier *et al.*, 2006). Further investigation into the ability of zoospores to penetrate outer bark would be needed to test this hypothesis.

A marked seasonal variation in susceptibility of the bark of larch to *P. ramorum* was observed. These findings suggest that the development of lesions on larch bark in the field is likely to be most rapid early in the growing season for all species and moderately restricted as summer progresses into autumn. The physiological basis for these differences is uncertain, but seasonal variation of host bark susceptibility to other *Phytophthora* species has been observed in other studies (Brasier & Kirk, 2001). The authors hypothesise that seasonal differences in susceptibility are likely to be due to changes in bark water content and the mobilization of stored photosynthates and/or defensive metabolites at different times of the year. Other workers have shown the growth of *P. cinnamomi*, on northern red oak (*Q. rubra*) and Jarrah (*Eucalyptus marginata*) bark is reduced by low water potential in the phloem (Tippett, Crombie & Hill, 1987; Marcais, Dupuis & Desprez-Loustau, 1993). Water potential in the inner bark varies throughout the year according to factors such as soil water potential and transpiration rates. Higher relative water content during wetter months of the year may result in an increased susceptibility of hosts to *P. ramorum* colonisation. Rainfall in summer 2011 was lower than the regional yearly average. At Alice Holt (where the trees were growing), rainfall in July 2011 (prior to felling) was extremely low, and trees received only 26.3% of the average rainfall for the month. As such, the inner bark (phloem) is likely to have been relatively dry. By contrast, in spring 2012, rainfall was well above the regional average and resulted in flooding in some areas of southeast England. At Alice Holt rainfall was 284% higher than normal. Approximately 90 mm of extra rain fell in spring 2012 compared to summer 2011 so tree bark in spring 2012 would have a higher water content compared to summer 2011 (weather station data, Alice Holt; Met Office, 2012b). This may well have contributed to increased susceptibility to pathogen colonisation.

Different chemical components in the bark of the three species are also likely to influence the growth of *P. ramorum*. The inner bark of Japanese larch may provide a more hostile environment for *P. ramorum* growth as it has a higher concentration of phenolics compared to European larch (Gierlinger *et al.*, 2004b). Phenolics have been shown to be associated with a reduction in *P. ramorum* lesion size on coastal live oak bark (Nagle, Garbelotto &

Bonello, 2008). Increased pathogenicity of isolates on European larch compared with Japanese and hybrid larch later in the season may also be influenced by differences between concentrations of tannin and phenolic compounds in the bark. Japanese larch has 25% higher tannin content compared to European larch (Aaron, 1982). Comparative Japanese and hybrid larch bark tannin content has not been documented in the literature, however, Japanese and hybrid larch heartwood have a similarly high phenolic content relative to European larch (Gierlinger *et al.*, 2004b), which has been associated with increased resistance to brown rot decay caused by *Coniophora puteana* and *Poria placenta* (Gierlinger *et al.*, 2004a; Gierlinger *et al.*, 2004b). Higher bark tannin contents are associated with reduced pathogenicity of *P. ramorum* (Nagle *et al.*, 2011) and therefore may explain the differing pathogenicity of *P. ramorum* isolates on the three larch species.

Statistical analysis of the data revealed a significant interaction between host species and pathogen isolate. All isolates from the EU1 lineage demonstrated a similar pathogenic behaviour on the three species, being most pathogenic on European larch and equally pathogenic on Japanese and hybrid larch. The significance of the interaction between the two test factors is probably largely due to isolate P1403, the only NA1 lineage isolate. Unlike the EU1 isolates, P1403 was equally pathogenic to all three larch species. This is an interesting find and may indicate variation in adaption to different hosts between the two lineages, although, such conclusions cannot be based on a limited study of one isolate.

Overall, differences in isolate pathogenicity were relatively small. The pathogenicity of most isolates did not differ significantly from each other. Genetic variation in the EU1 population is limited due to the clonal nature of the pathogen (Smith & Gilbert, 2003; Goss, Carbone & Grünwald, 2009; Grünwald *et al.*, 2009), therefore isolates are likely to be genetically similar and therefore have similar behaviour. Nevertheless, isolate BOC07 was significantly more pathogenic than isolates P2470 and BRC01 which were not significantly different from each other, but isolate BRC01 was not significantly less pathogenic than the remaining four isolates. Variation in *P. ramorum* virulence on host tree stems has been linked to the control of epigenetic factors, as opposed to genetic variation (Huberli & Garbelotto, 2012; Kasuga *et al.*, 2012).

4.4.2 Reisolation success of *P. ramorum*

The findings of this study also highlight the difficulty in isolating *P. ramorum* from larch bark tissue. Successful recovery of the pathogen occurred in only 10% of attempts. Differences in recovery success from the three larch species also occurred; the pathogen was more readily isolated from European larch (~14%) compared to Japanese and hybrid larch (~1 and 8%

respectively). These differences may again be due to inhibitory compounds such as phenolics or tannins within the bark tissues. Brown stains were observed around the bark pieces on the SMA plates for Japanese and hybrid larch, suggesting that compounds (possibly tannins) were leaching into the agar. This did not occur with European larch. As previously mentioned, phenolic content is higher in Japanese and hybrid larch compared to European (Gierlinger *et al.*, 2004b). Reduced reisolation success of *P. ramorum* from Japanese and hybrid larch compared to European larch bark may be a result of inhibitory compounds such as tannins leaching into the agar preventing its growth. Seasonal differences in isolation success may also be due to the phenolic compound content being higher in the plant tissues towards the end of the growing season (Wang, Zu & Li, 2007) while the pathogen maybe more active at colonising in the spring because of increased availability of carbohydrates in the phloem tissues (Grainger, 1962).

Phytophthora ramorum was detected in all three regions of a typical lesion in larch bark (inner brown, pink/ white and outer brown). This suggests that the different tissue colours are due to necrosis caused by *P. ramorum*, and not solely a host response in advance tissue invasion. The increased likelihood of isolating the pathogen from the outer brown region of the lesion is not a surprising finding, as the outer region is where the pathogen is actively growing into healthy tissue and is usually easier to isolate from (Davidson *et al.*, 2003). The bright pink to maroon tissue at the edge of lesions on infected trees was not observed in this study. This may not be surprising; although commonly observed on larch bark samples sent to Forest Research for diagnosis these samples invariably come from trees with active defence mechanisms, often manifested as copious resin production from lesion areas. This contrasts with logs, which may have impaired defences, and have also been inoculated at multiple points.

4.5 Summary

The main objectives of this study were to establish the relative susceptibility Japanese, European and hybrid larch bark to *P. ramorum*, determine how host susceptibility to *P. ramorum* changes over the growing season, compare the pathogenicity of different isolates of *P. ramorum* to larch bark and assess the relative success of *P. ramorum* isolates through reisolation from larch bark

Based on results from the two experiments carried out in August 2011 and May 2012 on the susceptibility of larch bark to isolates of *Phytophthora ramorum* five findings emerge;

- 1) The inner bark of European and hybrid larch were susceptible to *P. ramorum* colonisation as well as that of the more commonly affected Japanese larch.
- 2) Overall the bark of all three species of larch was equally susceptible to *P. ramorum*. However, as the year progressed European larch bark was more susceptible relative to Japanese and hybrid larch.
- 3) The susceptibility of larch bark to *P. ramorum* varied over the growing season, and was more susceptible in spring (May-June) but declined in late summer (August-September).
- 4) Variation amongst *P. ramorum* isolates colonising the inner bark of larch was limited. Most isolates were more pathogenic on European larch, but similarly pathogenic to Japanese and hybrid larch, with the exception of a NA1 isolate.
- 5) Reisolation success was also influenced by the growing season, and reisolation from Japanese and hybrid larch was less successful than from European larch.
- 6) *Phytophthora ramorum* diagnostic - DNA could be detected in all regions of the lesion. In contrast, overall reisolation success of the pathogen was limited to just 10%. The pathogen was most successfully reisolated from the outer brown zone of the lesion.

Chapter five - Persistence of *Phytophthora ramorum* in larch forest litter and soil

5.1 Introduction

The previous two chapters have focused on infectivity, sporulation potential and pathogenicity of *P. ramorum* primarily on *Larix* hosts. The ability of a pathogen to persist in the environment is another important epidemiological factor that needs exploring to provide understanding of the ramorum disease epidemic in the UK. This chapter therefore focuses on the persistence of *P. ramorum* on an infected larch plantation over the course of three years. The current strategy to control and contain the spread of *P. ramorum* is to reduce levels of inoculum in the environment (Forestry Commission, 2014b). As such, landowners are required by law to fell all infected plantings of larch and understory rhododendron as these are the main hosts that sustain sporulation. Once sites are cleared of all sporulating hosts, it is uncertain how long the pathogen can persist on site in infected plant litter or soil. Replanting of susceptible host species is not advised for up to three years post-clearing, however this is not always a practical option for restocking of woodlands and plantations (Webber, 2010).

Phytophthora ramorum has been shown to have potential to persist for several years and infect new plantings and re-growth (Davidson *et al.*, 2005; Fichtner, Lynch & Rizzo, 2006; Fichtner, Lynch & Rizzo, 2007). The removal of any infected trees and plants is only the first stage in attempts to control the disease epidemic in the UK (Webber, 2009). Understanding its persistence is key to managing this disease. This study, therefore, aims to determine how long *P. ramorum* persists on cleared larch sites in order to advise landowners when replanting can take place and on the need to monitor sites.

5.2 Materials and Methods

Biosecurity precautions were taken whilst working on infected sites. All sample material was double bagged and sealed. Equipment was disinfected on site by spraying with IMS. Mud and foliage was washed from boots and vehicle wheels with water and sprayed with the disinfectant PropellerTM (which contain IMS) on site.

5.2.1 Research site

Field based experiments and observations were carried out on a site in Wiltshire, Southwest England, where stands of Japanese larch (*Larix kaempferi*) and European larch (*L. decidua*) showed disease symptoms. *Rhododendron ponticum* was the dominant understory shrub across the whole site but showed no disease symptoms. Following the issue of a Statutory Plant Health Notice, the felling of larch trees and rhododendron clearance within the area was initiated in September 2010. Two forest compartments were assessed in this study. The first covered an area of 3.6 hectares comprising of 70% European larch, 20% Douglas fir and 10% mixed broadleaf trees [Plate 5.1]. The second covered an area of 2.8 hectares and consisted of 70% Japanese larch, 10% Douglas fir and 20% mixed broadleaf trees, some of which had symptoms of heavy *P. ramorum* infection [Plate 5.2]. Disease in the European larch compartment was less severe than that of the Japanese larch compartment based on visual symptoms and the number of symptomatic trees. Regardless of the presence/absence of symptoms all potential sporulating hosts (i.e. Japanese larch and rhododendron) were cleared from the Japanese larch compartment in November 2010. Only symptomatic host trees and rhododendron were cleared from the European larch compartment in February 2011.

5.2.2 Sampling

Each stand was surveyed systematically for infected hosts and both needle-litter and soil samples were collected. Sampling quadrats were laid out across both sites. Sampling took place across the length and width of the sites. Five transect lines 5 m apart were run parallel down the length of the site. Sampling took place every five metres along each line. Litter samples were taken from each sampling point within the quadrat, whilst one soil sample was taken from each row. Each sample point was numbered and soil sampling points were randomly selected from each row using a random number generator. A total of 132 samples from the European larch stand (22 soil and 110 litter) [Figure 5.1] and 180 samples from the Japanese larch stand (30 soil and 150 litter) [Figure 5.2] were taken. Litter sampling involved taking a handful (approximately 40 g) of plant litter from the forest floor. Soil samples were collected by firstly removing the litter layer and then taking samples from 10 cm below the soil surface. All samples were placed in sealed plastic bags. Trowels and gloves were sprayed with Propeller™ disinfectant or IMS, which was left to evaporate between each sampling point, to avoid contamination. The sites were mapped using Trimble® GPS and ArcGIS software (Esri). Along with sample points, tree stumps of all species, and symptomatic standing trees were recorded on the maps. Bark samples were taken from



Plate 5.1 – Infected European larch site after felling of infected and symptomatic trees and removal of rhododendron, March 2011

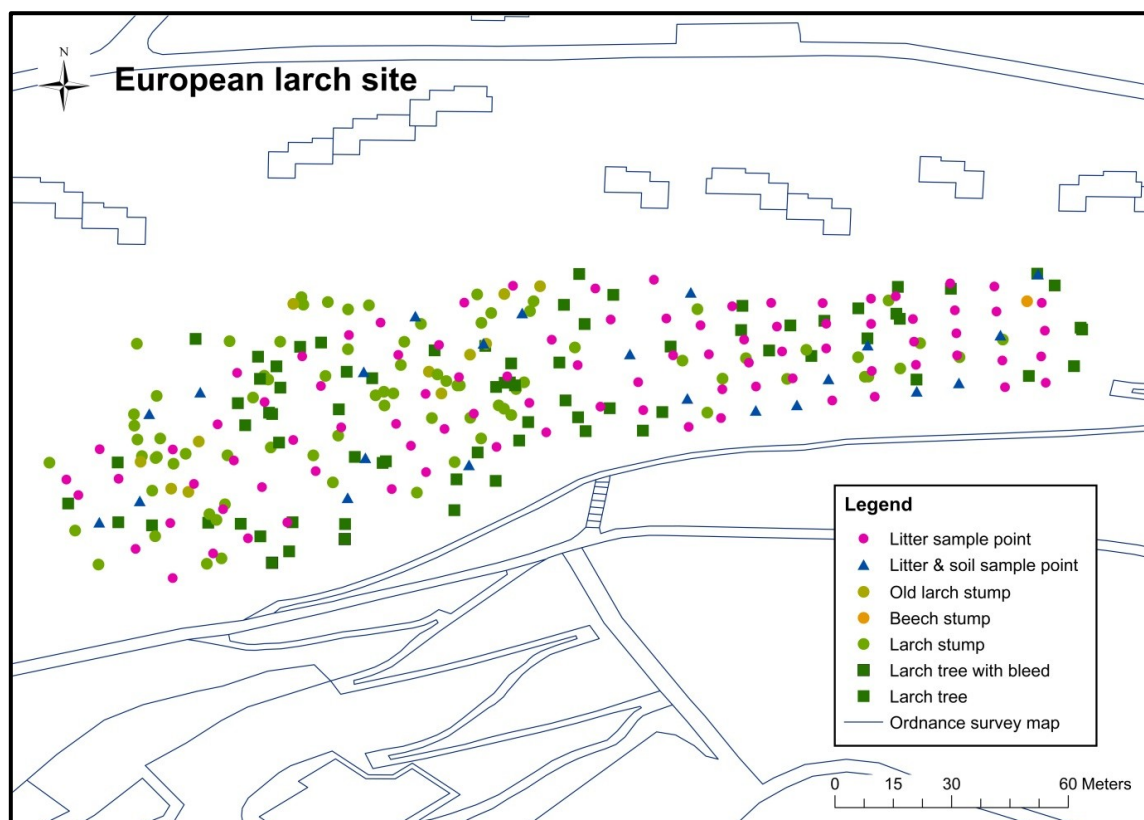


Figure 5.1 - Features and sampling quadrates set-up of infected European larch stand



Plate 5.2 – Infected Japanese larch site after felling of all Japanese larch trees and removal of rhododendron, March 2011. Top and bottom right; symptomatic bleeding cankers on two beech trees (later confirmed infected with *P. ramorum*).

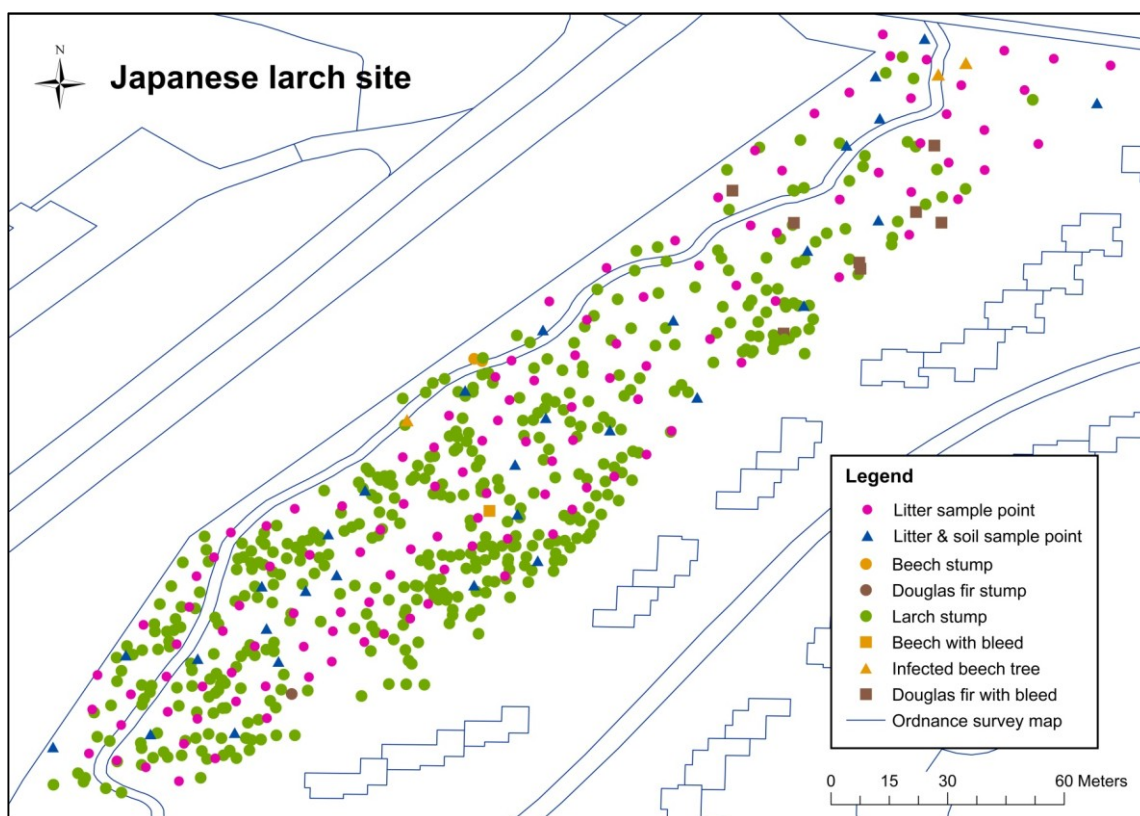


Figure 5.2 - Features and sampling quadrat set-up of infected Japanese larch stand

symptomatic trees on site by extracting a sample of necrotic bark (approximately 4 x 4 cm) from bleeding lesions using a chisel. Small pieces of symptomatic bark were primarily tested for the presence of *Phytophthora* using lateral flow devices (LFDs) on site. Bark samples were then further tested upon return to the lab using methods described in section 2.6.1 to isolate and section 2.8.1.1 to identify the pathogen. A Tinytag[®] Plus 2 data logger (Gemini data loggers) was placed on the litter surface in the Japanese larch stand to record temperature and humidity. Unfortunately, a fault in the data logger occurred generating unreliable records. Therefore, meteorological data over the course of two years were taken from the nearest Met Office weather station located in Larkhill, Wiltshire, 20 miles west of the study sites. The survey was repeated a year later in 2012 and two years later in 2013 on the cleared Japanese larch stand. Additional foliage samples were taken from regenerating rhododendrons on site and mapped. Six leaves were taken from each regenerating plant irrespective of symptoms and were sealed in a plastic bag. The features of both stands can be seen in Figure 5.1, Figure 5.2, Plate 5.1 and Plate 5.2.

5.2.3 Detection of *P. ramorum* from litter, soil and regenerating rhododendron foliage samples

The presence of *P. ramorum* within litter and soil samples was tested by baiting using rhododendron leaf discs (see section 2.6.2). Any fungal growth emerging from the bait was subcultured onto CA for identification (see section 2.8.1.1). In 2013, where the pathogen was not recovered from the baits, the rhododendron discs were removed from the SMA plates, cut into small pieces, placed in MCTs and stored at -20°C. DNA was then extracted (see section 2.7.1) and the presence of *P. ramorum* determined using rtPCR (see section 2.8.2). Rhododendron foliage samples were processed as described in section 2.6.2.

5.2.4 Statistical Analysis

A generalised linear mixed model using SAS[®] 9.4 was fitted to the presence/absence data of *P. ramorum* observed across a non-uniform “rectangular” grid (sampling quadrat) for 2011, 2012 and 2013. In addition to fitting a binomial response model with logit link function, an exponential covariance model was used to model the correlation structure between grid points.

5.3 Results

At the start of the experiment *Phytophthora ramorum* was baited from 101 litter samples (67% of all samples) and from two soil samples (6% of all samples) in the felled Japanese larch site in March 2011 [Figure 5.4]. In contrast, the pathogen could only be recovered from two litter samples (1.8% of samples) and none of the soil samples from the European larch stand [Figure 5.3]. Therefore, no further samples were taken in the following years on this site.

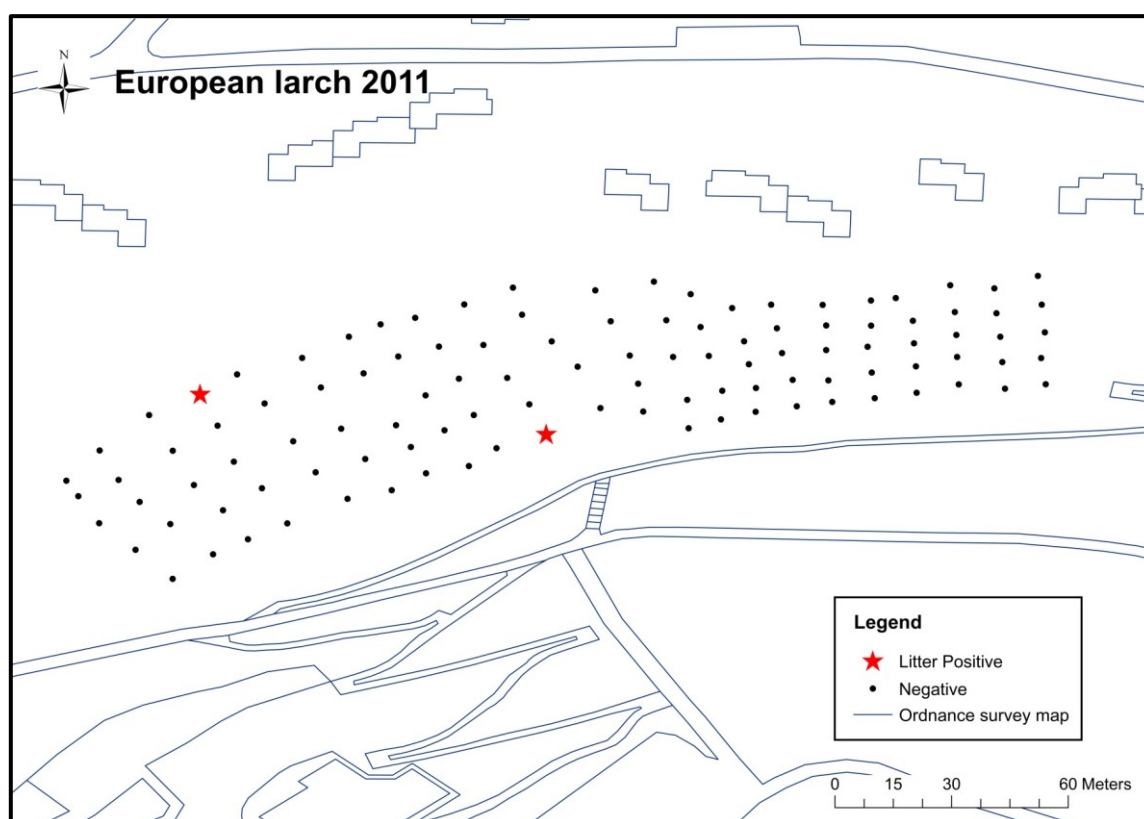


Figure 5.3 – Baiting results for *P. ramorum* in litter and soil from European larch infected site March 2011

A year later, in March 2012, levels of *P. ramorum* within the forest litter of the felled Japanese larch stand declined from the previous year, with the pathogen recovered from 59 litter samples (39% of samples), compared to 101 litter samples (67%) in the previous year. This level of persistence occurred despite three months of low temperatures (December to February) accompanied by snowfall in spring 2012 [Figure 5.5]. *Phytophthora ramorum* could also be isolated from both symptomatic and asymptomatic rhododendron shoots and leaves on two regenerated plants on the site, confirming the pathogen was still active in the area where the Japanese larch had been felled. Of the 59 litter sample points that tested positive for *P. ramorum* in 2012, 18 had not yielded the pathogen in the previous year,

although 58 of the samples points that had tested positive in 2011 were negative in 2012. The pathogen could only be isolated from one soil sample in 2012, which was in close proximity to a rhododendron plant that tested positive for *P. ramorum* infection [Figure 5.4]. *Phytophthora ramorum* was not reisolated from the two soil sample points that were previously positive in 2011. High levels of detection of *P. ramorum* were recorded at both ends of the site where harvested trees and cleared rhododendrons had been stacked awaiting removal in 2011, potentially concentrating high levels of inoculum in these areas. Apart from a small amount of rhododendron regeneration, the site was colonised mainly by grasses, moss and forbs after clearance [Plate 5.3]. The litter was mainly composed of slightly degraded larch needles, cones and dried leaves from broadleaved trees as well as grasses and their roots.



Plate 5.3 – Infected Japanese larch site 2012. Bottom right: symptomatic shoot tip dieback of regenerated rhododendron

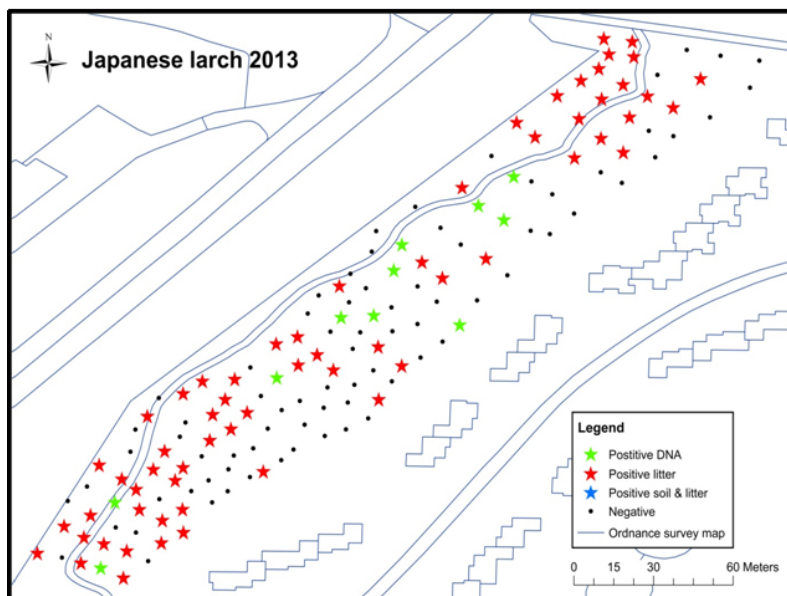
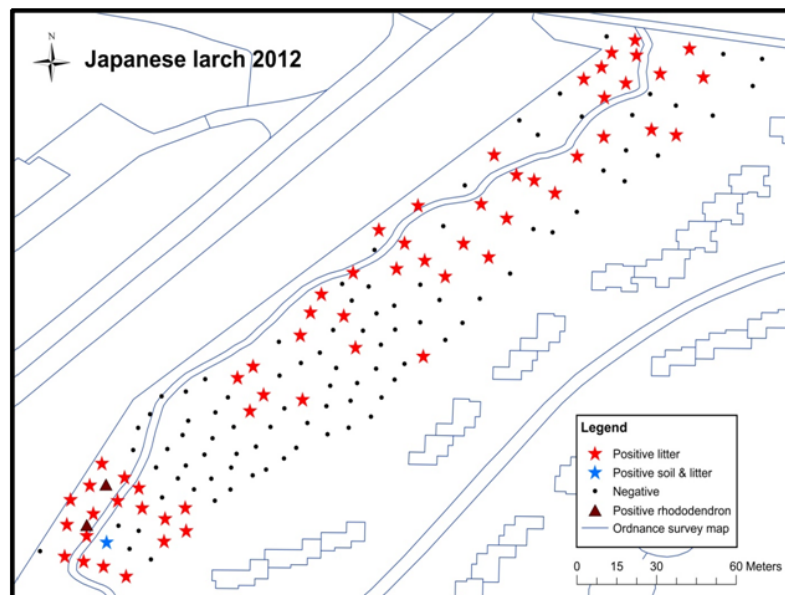
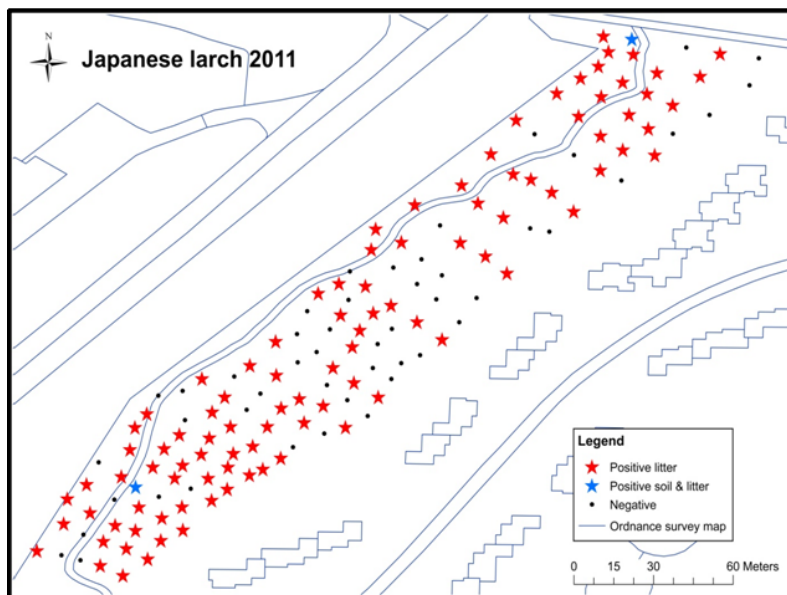


Figure 5.4 – Distribution of *P. ramorum* positives from soil and litter samples taken from an infected Japanese larch site felled in late 2010 and assessed annually from 2011 to 2013

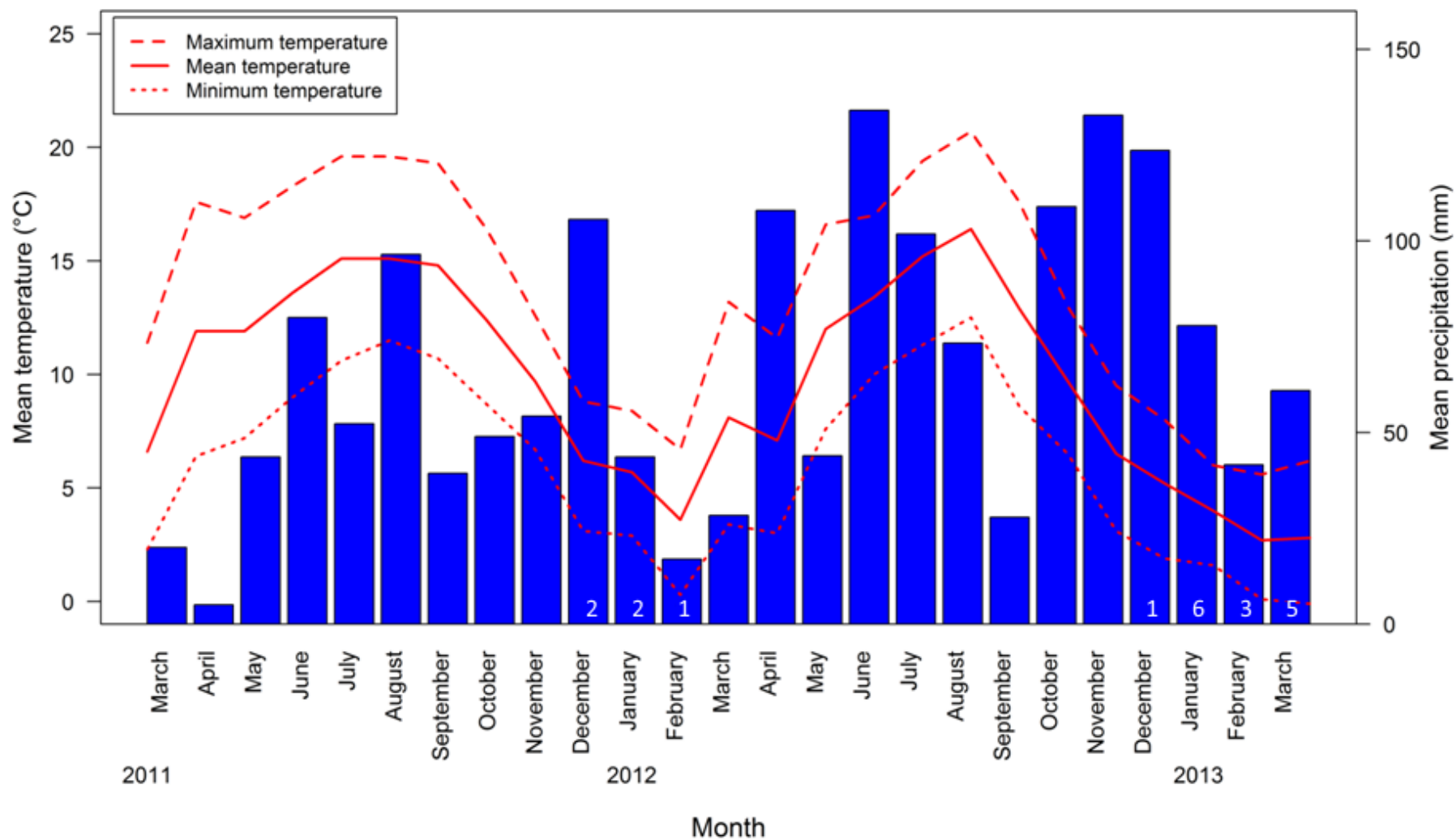


Figure 5.5 – Mean temperature (red lines) and precipitation (blue bars) at Larkhill, Wiltshire meteorological weather station between March 2011-2013. Numbers indicate the number of days that snow was on the ground (Met Office, 2014).



Plate 5.4 – Rhododendron regeneration showing symptoms of *P. ramorum* infection on felled infected Japanese larch site, March 2013: a & b) tip and shoot dieback, c) foliar blight d) extensive rhododendron regeneration, e) chlorotic wilting shoot

By March 2013 extensive rhododendron regeneration had occurred at the site. Most of the plants appeared healthy and symptom-free. However, a few showed signs of possible infection, having black wilted shoots [Plate 5.4] although none of the leaves had necrotic lesions. *Phytophthora ramorum* was isolated from the foliage of five of the 46 rhododendron plants sampled. The pathogen was also isolated from an additional soil and litter sample taken from underneath a strongly symptomatic rhododendron [Figure 5.6]. In addition to extensive rhododendron regeneration, restocking had taken place on this site. Grass and forbs remained dominant [Plate 5.5] but compartment was mainly restocked with broadleaved and pine saplings. As such, much of the soil and litter had been disturbed and moved around. The level of larch needle degradation within the litter as well as moisture content differed across the site, but the litter still consisted largely of larch needles, cones and broken twigs, as well as broadleaf leaves.

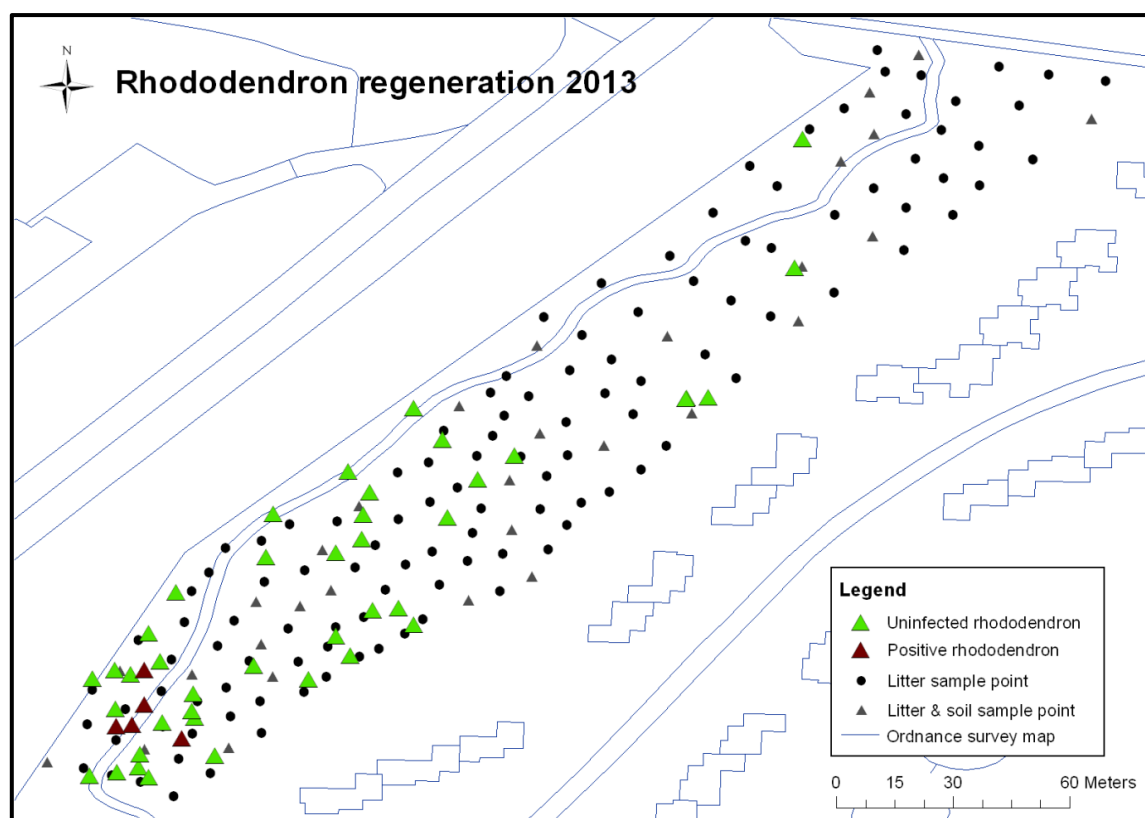


Figure 5.6 - Baiting results for *P. ramorum* from regenerating rhododendron foliage on the felled infected Japanese larch site, March 2013



Plate 5.5 – Infected Japanese larch site, March 2013.

Not surprisingly statistical analysis of the percentage change in reisolation of the pathogen revealed a significant difference ($p < 0.05$) between the three sampling events. The presence of *P. ramorum* in the litter decreased significantly between March 2011 and March 2012. Its presence in the litter between March 2012 and March 2013 increased significantly when taking the PCR positive rhododendron baits into account but not if comparing only samples where an isolate of *P. ramorum* was obtained [Figure 5.7]. There was a significant correlation in the likelihood of a sampling point being positive for the pathogen in both 2011 and 2012 if the adjacent sampling point was positive. There was not an increased risk of a sample point being positive if a neighbouring sample point was positive for the pathogen in 2013.

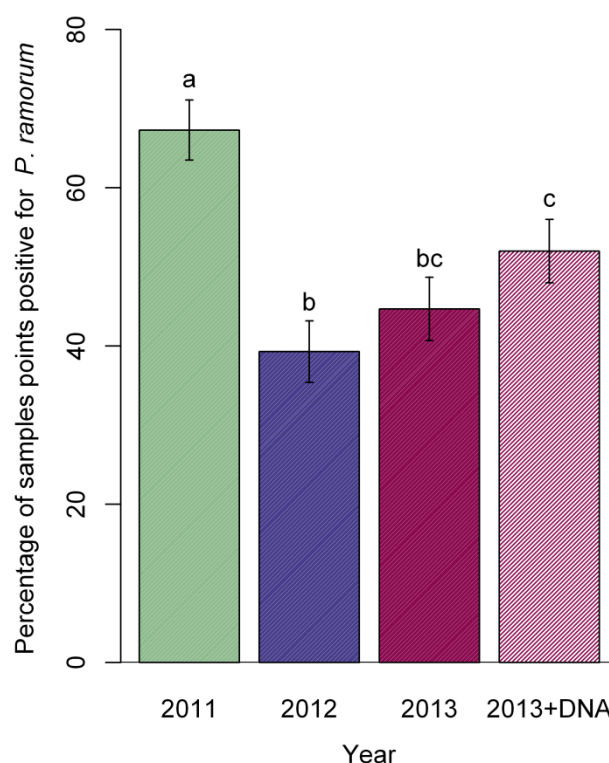


Figure 5.7 – Persistence of *P. ramorum* within the litter in a felled Japanese larch stand. Persistence was determined by percentage of samples yielding *P. ramorum* isolates. 2013+DNA includes the percentage of isolates yielded plus the percentage of rhododendron baits testing positive for *P. ramorum* DNA. Different letters indicate differences among treatments determined by Fisher's least significant difference test at $P < 0.05$.

5.4 Discussion

Whilst the disease potential of a pathogen is largely dependent on its ability to sporulate and disperse, it is also partially dependent on its capacity to survive throughout periods of unfavourable conditions for both growth and sporulation. Under such conditions the ability of a pathogen to persist in either host material or non-host substrates such as organic material and soil can be significant. This study looked at the ability of *P. ramorum* to persist in naturally-infected larch host material in the litter layer and soil of felled larch stands in south west England after statutory requirements of host removal were imposed. The findings demonstrate that *P. ramorum* can remain viable within the Japanese larch litter at least two years after sources of inoculum have been removed but it is largely absent from the forest soil layer. The findings of low level site contamination under European larch support field observations that disease development on European larch is less than on Japanese.

The role of *P. ramorum* in forest soil and litter is still not fully understood. Many *Phytophthora* species are soil-borne and thus adapted for survival and propagation within soil environments, with many initiating infection in host roots e.g. *P. quercina* on European oaks (Jung *et al.*, 1999). *Phytophthora ramorum* is, however, considered an aerial species with host symptoms restricted to above the soil line (Rizzo *et al.*, 2002b; Garbelotto *et al.*, 2003). As such there has been limited research into its natural role in the soil. Studies into survival and detection in mixed evergreen forests and Californian redwood forests in North America have highlighted the pathogen's varying ability to survive in soil and leaf debris-litter throughout the year and in different forest types (Davidson *et al.*, 2005; Fichtner, Lynch & Rizzo, 2006; Fichtner, Lynch & Rizzo, 2007). These studies suggest that in North America *P. ramorum* (NA1 lineage) can persist in plant material for up to 49 weeks incubated at either the litter or soil layer (Fichtner, Lynch & Rizzo, 2009). Furthermore, they emphasize that the persistence of the pathogen in forest environments is limited by high temperature, with recovery decreasing dramatically during the hot dry summer months. It is perhaps not surprising, therefore, to discover that *P. ramorum* is capable of persisting in the litter of a felled larch stand in the UK for at least a few years. *Phytophthora ramorum* is considered a temperate pathogen with an optimal growth temperature of 20°C and minimum and maximum temperatures of 2°C and 30°C respectively. Thus, the UK climate, with its cool summers and mild winters is likely to provide suitable conditions for the pathogen's persistence. In this study, average summer temperatures did not peak above the pathogens optimal temperature for growth, so temperature is unlikely to have been a limiting factor for survival. In addition, average temperatures were seldom low enough to halt the pathogens growth or be lethal to any pathogen within the litter layer. The pathogen has previously been shown to be capable of overwintering in artificially colonised leaf tissue of rhododendron and lilac (*Syringa vulgaris*), both at the soil surface and buried 5 cm below the surface in the UK, with night temperatures reaching a minimum of -9°C (Turner, Jennings & Humphries, 2005).

The high levels of rainfall throughout the study may also have been conducive to the persistence of the pathogen on the site, as drying has been reported to have detrimental effects on the survival of various *Phytophthoras* (Erwin, Bartnicki-Garcia & Tsao, 1983; Davidson *et al.*, 2002; Smith, 2007). Wetter conditions, however, may facilitate the decomposition process of the larch needles providing suitable conditions for antagonistic and/or competitive microorganisms, potentially resulting in a reduction of the pathogen's presence on site. Nevertheless, pathogen survival between sampling efforts was greater between March 2012 and March 2013 compared to March 2011 and March 2012, coinciding with wetter, cooler weather over these periods. Dobrylovska (2001) noted that the

decomposition of larch needles is typically slow and this may protect *P. ramorum* for longer in the context of competing organisms and the outside environment.

The perennating spore of many *Phytophthora* species is typically the chlamydospore, which over time becomes thick-walled and capable of resisting environmental extremes (Erwin, Bartnicki-Garcia & Tsao, 1983). *Phytophthora ramorum* allocates a large proportion of its resources to producing abundant, large, thick-walled chlamydospores and in the absence of oospores it can be argued that chlamydospores are the most important survival structure in the disease cycle. It is likely that the pathogen persisted on the study site as chlamydospores in larch needle tissue. Chlamydospores were often observed growing both on the surface and within the tissues of larch needles in studies in chapter three [see Plate 3.4]. The lack of recovery of the pathogen from the inorganic soil layer, which lacked plant debris, suggests that the pathogen persists in host plant tissues and does not survive outside this protective environment in forest soils. Many reports in the literature refer to ability of *P. ramorum* to survive in plant material buried in the soil as opposed to the inorganic soil alone. However, the pathogen has also been shown to persist in a soil/gravel substrate in an infected garden in south east England for three years despite removal of infected plants and application of disinfectant (Turner, 2007). In addition a study carried out in the Netherlands found that *P. ramorum* could be recovered after at least one year from sandy soil at depths of 20 cm (Aveskamp, van Baal & de Gruyter, 2005). These findings, along with studies in North America which showed *P. ramorum* to be present in soil but absent in litter (Fichtner, Lynch & Rizzo, 2006), suggest that spores of *P. ramorum* are able to percolate down into the soil substrate and survive there in some circumstances.

The almost complete absence of *P. ramorum* in the soil during this study could be due to the differences in forest litter. The deep needle litter layer on site may have prevented the spores from being washed down into the soil, potentially acting as a filter and contrasting with the thinner litter layers of broadleaf leaf litter at other infected sites both in the UK and North America. In addition microsatellite analysis of soil populations of *P. ramorum* suggests that persistence of the pathogen in this substrate is less than one year as often the same genotype cannot be recovered. Apparent persistence of the pathogen may therefore be due to the addition of new inoculum (Eyre, Kozanitas & Garbelotto, 2013). The authors of both studies reporting persistence in the soil in the UK garden and the Netherlands do not rule out the potential for new additions of inoculum. In the study from the Netherlands there was considerable regeneration of infected rhododendrons on site allowing for new inoculum production (Aveskamp, van Baal & de Gruyter, 2005; Turner, 2007).

Survival time of *Phytophthora* species chlamydospores, in free soil and host material can vary greatly according to species but appears to depend on spore size and wall thickness (Erwin, Bartnicki-Garcia & Tsao, 1983). Chlamydospores of *P. ramorum* have the third largest average diameter (52.4 μm) and the fourth average thickest wall (2.3 μm) of all the chlamydospore producing *Phytophthora* species (Smith, 2007). Chlamydospores of *P. lateralis*, the closest related species to *P. ramorum*, also produces large spores (average diameter of 40.1 μm , the fifth largest) but they are relatively thin walled (average 0.8 μm thick) in comparison to *P. ramorum*. Nevertheless, chlamydospores of *P. lateralis* have been known to persist in both soil and root systems for at least six and seven years respectively (Hansen & Hamm, 1996). It has been suggested that the persistence and chlamydospore production of *P. ramorum* in the soil of a Californian Redwood-Tanoak forest was due to the high levels of organic matter within the soil which retained moisture (Fichtner, Lynch & Rizzo, 2007). The soil in the felled Japanese larch stand in this study varied but was mainly composed of clay and low in organic material. On this basis it seems likely that in addition to moisture, *P. ramorum* requires organic material in order to persist in the soil of infected larch sites.

Lack of detection of *P. ramorum* from the majority of the soil samples taken over the three year study does not necessarily confirm the absence of the pathogen. *Phytophthora* chlamydospores are able to enter a dormancy phase which requires an external source of nutrients or water to induce germination (Sussman, 1966). Chlamydospores may have been present in the soil samples but baiting for five days may have been insufficient to break dormancy. Hydration is fundamental in breaking the dormancy of *P. ramorum* chlamydospores (Fichtner, Lynch & Rizzo, 2007). Recovery of the pathogen via baiting from infected rhododendron leaf tissue incubated in the soil and litter layer has been reported to increase by as much as ten percent when hydrated for three weeks as opposed to one (Fichtner, Lynch & Rizzo, 2006). This also has implications for recovery success from the larch litter as well as soil samples and consequently may have resulted in false negatives in the data. The spring of 2011 was exceptionally dry for the UK (Met Office, 2012a) which could have initiated dormancy within the chlamydospores of the pathogen. However, 2012 was one of the wettest years on record in the UK and wet conditions continued into the beginning of 2013, (Met Office, 2012b; Met Office, 2014) and the high availability of water had the potential to break the pathogen's dormancy, facilitating successful recovery of *P. ramorum*.

The method of detection also influenced the proportion of positive findings. DNA based detection increased findings of *P. ramorum*, highlighting the possibility of false negatives in

the data collected in 2011 and 2012. Improved detection of a pathogen through the use of PCR based diagnosis when compared to culturing methods is not a new phenomenon, and has already been reported in relation to *P. kernoviae* (Turner, 2007). However, the ecological significance of the DNA detected should be considered. It does not always indicate that the pathogen is alive, only that it was once present. Using rhododendron baits does indicate that the pathogen is viable as inoculum in the soil or litter must germinate, produce sporangia and release zoospores into the water to actively infect the baits. Therefore in this study baiting based positives were the most ecologically significant. It is however, a conservative measure as, low level infections of *P. ramorum* in rhododendron baits may be outcompeted by other organisms, particularly *Pythium*, when using agar plating for detection (Hendrix, Campbell & Chien, 1971).

The findings of this study demonstrate the importance of host clearance from an infected site. Apart from the residual inoculum in the larch leaf litter the presence of regenerating infected rhododendron on site provided a source of new inoculum and had the potential to increase the presence of the pathogen on site. This was evidenced by the infested soil under a symptomatic infected rhododendron bush in this study. There are several ways in which the regenerating rhododendrons could have been infected, such as (1) regenerating plant tissues from the plant roots coming into contact with infected litter as they grow through the soil (Davidson *et al.*, 2005; Fichtner, Lynch & Rizzo, 2007), (2) the pathogen continues to reside in the roots and remaining stems and then spreads up in to the new shoots and plants (Aveskamp, van Baal & de Gruyter, 2005), (3) newly regenerated foliage may simply become infected due to rain-splash of inoculum from the forest litter (Fichtner, Lynch & Rizzo, 2009). Regardless of how infection occurred the findings highlight the importance of continued site monitoring and disease management. In the future such plants will be the source for an ongoing/increased pathogen presence. As part of the statutory requirements, re-growth of infected sporulating hosts should be prevented (Defra, 2008). This study suggests that site landowners were not complying with regulations, possibly due to limited understanding of pathogen epidemiology and the limited visible symptoms on the regenerating rhododendron. As such, increased surveillance and monitoring of cleared sites to support landowners compliance with regulations could help to limit the spread and persistence of the disease. Complete removal of the infected plants and the leaf litter surrounding infected plants has been shown to be the most effective eradication strategy (Turner *et al.*, 2007). In light of this and the findings of this study, removal of rhododendron including all roots below the soil appears the best way to limit the pathogen on site and reduce the need for continued monitoring of sites for regenerating plants. Additionally, turning over the needle litter may speed up the eradication process as it would move the

underlying litter layers in which persistence is reported to be higher (Fichtner, Lynch & Rizzo, 2009) to the surface exposing the infected material to harsher environmental extremes.

5.5 Summary

This study is the first to report survival of *P. ramorum* in larch litter in the UK. The key findings of this study were;

1. *Phytophthora ramorum* is able to persist in the litter of a felled Japanese larch stand for at least two years in south east England.
2. The pathogen does not appear to persist in the soil layer below the litter layer in a larch forest.
3. Anecdotal low-level site infection of European larch is consistent with limited detection of the pathogen within the litter in a stand of this larch species.
4. Methods of detection relying on reisolation of the pathogen may result in false negatives enabling the pathogen to go undetected on infected sites.
5. Replanting of infected sites with larch or other sporulating plant species within two years of site clearance should be ruled out. Findings also highlight the need for additional surveillance of cleared sites for regenerating infected/infectable hosts such as rhododendron if the pathogen spread with in the UK is to be managed.

Chapter six - Comparative fitness characteristics of the European lineages of *Phytophthora ramorum*

6.1 Introduction

Until recently, *Phytophthora ramorum* has existed across its known geographical distribution in the form of three asexually reproducing clonal lineages, designated NA1, NA2 and EU1 (Ivors *et al.*, 2006; Goss, Carbone & Grünwald, 2009). Since its first detection in Europe in 1993 (Werres *et al.*, 2001) the EU1 lineage has become widespread across most of western and central Europe, occurring in nurseries, gardens, parks and woodlands (see section 1.2.4.1) (Grünwald *et al.*, 2012). In 2011 a new European lineage of the pathogen, designated EU2, was discovered infecting larch in the Antrim plateau, Northern Ireland and the adjacent Argyll/Galloway border region of south west Scotland (approximately 100 km apart) (Van Poucke *et al.*, 2012). The significance of this discovery to forestry and plant health in the UK and Europe is unclear due to the lack of data on the pathogenicity and host range of this new lineage. These characteristics are known to differ significantly between the EU1, NA1 and NA2 lineages (Brasier, Kirk & Rose, 2006; Ivors *et al.*, 2006; Elliot *et al.*, 2011) and define the ecological significance of the pathogen, reflecting its adaptive characteristics and overall strategy for survival (Brasier, 1999). The EU2 lineage has the potential to pose a greater threat to the UK than the EU1 lineage if it has a wider host range, higher sporulation potential and/or greater pathogenic aggressiveness. Assessing such epidemiologically significant properties could help us to understand the recent transfer of *P. ramorum* to larch and determine the likelihood of further damage to ecosystems and industry. If the EU2 lineage poses a greater threat than the EU1 it could require specific quarantine status to restrict its distribution within the UK and prevent its spread to other parts of Europe and beyond.

The aim of this study was to assess the comparative forest and plant health risk posed by the recently discovered EU2 lineage of *P. ramorum* by evaluating continuous variables such as growth rate, pathogenicity and sporulation potential. Such traits have previously been shown to be good indicators of adaptive differences between the EU1, NA1 and NA2 lineages (Brasier, 1999; Brasier *et al.*, 2006; Brasier, Kirk & Rose, 2006; Elliot *et al.*, 2011).

6.2 Materials and methods

Population samples of EU1 and EU2 isolates were compared to determine variation across different defined environments or stress conditions. Variables examined were: *In vitro* temperature-growth response on three agar media, ability to colonise mature bark, sapling stems and rhododendron leaves (i.e. pathogenic aggressiveness) and sporulation potential on Japanese larch needles.

6.2.1 Isolates and inoculum production

Twelve isolates of *P. ramorum*, obtained from infected hosts sampled across the UK were used in this study to represent the populations of both the EU1 and EU2 lineages in the studies described below [Table 6.1]. For growth rate, pathogenicity on rhododendron leaves and sporulation potential on Japanese larch needles, isolates were first taken out of storage under paraffin oil and grown on CA. Mycelial plugs of the chosen isolates were then placed on the abaxial surface of rhododendron leaves and incubated as described in section 2.11.2 until lesions appeared on the leaves. The pathogen was then reisolated from the rhododendron leaves (see section 2.6.1), sub-cultured onto CA and incubated at 18-20°C in continuous daylight (60W daylight™ bulb suspended 30 cm above the plates) for 14 days prior to inoculation/ experimentation. Isolates were 'put through' rhododendron leaves prior to experiments to eliminate possible bias between cultures that had been in storage and freshly isolated cultures, as isolates of *P. ramorum* have been reported to lose vigour in culture (Erwin & Ribeiro, 1996, Kasuga *et al.*, 2012).

Chapter six - Comparative fitness characteristics of the European lineages of *Phytophthora ramorum* in the UK

Table 6.1 - Origins of the *Phytophthora ramorum* isolates used in tests on adaptive behaviour

Isolate	Location	Sampled host	Isolated	Lineage
P1614 ^{2,*}	Cornwall, SW England	<i>Nothofagus</i> bark	2004	EU1
P1898	Cornwall, SW England	<i>Fagus sylvatica</i> bark	2005	EU1
P1959 ²	Cornwall, SW England	<i>Quercus cerris</i> bark	2005	EU1
P2132 ²	Cornwall, SW England	<i>Cinnamomum camphora</i> bark	2007	EU1
P2490 ^{1,2}	Argyll, SW Scotland	<i>Nothofagus</i> bark	2010	EU1
P2590 ^{1,2}	Isle of Mull, Scotland	<i>Larix kaempferi</i> shoot/leaves	2011	EU1
P2600 ^{1,2,*}	Cornwall, SW England	<i>Larix kaempferi</i> bark	2012	EU1
P2609 ^{1,2,*}	Devon, SW England	<i>Castanea sativa</i> shoot/leaves	2012	EU1
P2639 ^{1*}	Devon, SW England	<i>Larix kaempferi</i> bark	2009	EU1
P2720	Cornwall, SW England	<i>Fagus sylvatica</i> bark	2012	EU1
P2732 ²	Devon, SW England	<i>Rhododendron</i> sp. shoot/leaves	2012	EU1
P2733 ¹	Devon, SW England	<i>Fagus sylvatica</i> bark	2012	EU1
P2111 ^{1,2,*}	County Down, Northern Ireland	<i>Quercus rubra</i> bark	2007	EU2
P2460 [*]	County Antrim, Northern Ireland	<i>Larix kaempferi</i> bark	2010	EU2
P2461 ^{1,2,*}	County Antrim, Northern Ireland	<i>Larix kaempferi</i> bark	2010	EU2
P2561	South Ayrshire, SW Scotland	<i>Larix kaempferi</i> bark	2011	EU2
P2565 ¹	County Antrim, Northern Ireland	<i>Vaccinium myrtillus</i> shoot/leaves	2011	EU2
P2566 ²	County Antrim, Northern Ireland	<i>Rhododendron ponticum</i> shoot	2011	EU2
P2583 ^{1,*}	South Ayrshire, SW Scotland	<i>Larix kaempferi</i> foliage	2011	EU2
P2585 ¹	Wigtownshire, SW Scotland	<i>Larix kaempferi</i> foliage	2011	EU2
P2586	South Ayrshire, SW Scotland	<i>Larix kaempferi</i> foliage	2011	EU2
P2587 ²	South Ayrshire, SW Scotland	<i>Larix kaempferi</i> foliage	2011	EU2
P2748 ²	Brigton, Scotland	<i>Larix kaempferi</i> bark	2012	EU2
P2750 ^{1,2}	Brigton, Scotland	<i>Larix kaempferi</i> bark	2012	EU2

¹ Isolates used in pathogenicity tests on rhododendrons in October 2013; ² Isolates used in pathogenicity tests on rhododendrons in March 2014; * Isolates used in sporulation potential test on Japanese larch needles. All isolates were used in pathogenicity testing on mature bark and saplings.

6.2.2 *In vitro* growth response

6.2.2.1 Temperature and media

Radial growth tests were carried out at eight temperatures; 2.5, 5, 10, 15, 20, 25, 27, 29°C on three different agar media; CA, V8 and PDA (see section 2.5). These were selected to put the pathogen under an array of environmental stresses and reveal any genetic divergence between the two European lineages. Temperatures included both known supra- and sub-optimal temperatures for the EU1 lineage to allow comparisons with the unknown EU2 temperature range response. CA and V8 agar were used in this study as they are typically used as suitable media for culturing *Phytophthoras*, with V8 agar considered to provide slightly more nutrient stress than CA (Franceschini *et al.*, 2014). PDA was the most 'stressful' environment due to its high sugar concentration and lack of plant sterols which are required by *Phytophthoras* for growth stimulation and sporulation (Strong *et al.*, 2013).

6.2.2.2 Experimental design and statistical analysis

The two lineages of *P. ramorum* were each represented by twelve isolates with each isolate replicated three times at each temperature and on all three agar types. Four radial growth measurements were taken from each plate (see section 2.12.1). The experiment was blocked by temperature. A REML variance components analysis was fitted to the data on average lineage growth rate and analysed separately for each temperature for each agar. *Phytophthora ramorum* isolate was treated as a random effect in the model-building.

6.2.3 Pathogenicity comparisons on mature tree bark

Pathogenicity comparisons of the two lineages focused on the ability of the pathogen to attack and colonise the phloem (inner bark) of trees, with particular focus on larch due to the current EU1 epidemic in the UK. Host species selected in this study included beech (*Fagus sylvatica*) known to be highly susceptible to *P. ramorum* (Sansford *et al.*, 2008), Japanese and European larch (*Larix kaempferi* and *L. decidua*) and oak (*Quercus robur*) which has bark with low susceptibility to *P. ramorum* (Sansford *et al.*, 2008).

6.2.3.1 Plant material

Logs were cut from mature healthy trees grown at the Alice Holt Research Station in July 2012. Three trees of each species were felled and two logs were cut consecutively starting

one metre up from the base of the tree. An additional log was cut from one tree of each species and was used as an indicator log to guide the best time to sample the trial.

6.2.3.2 Inoculation, incubation sampling and reisolation

The colonising ability of *P. ramorum* isolates was assessed using the wound inoculation methods described by Brasier *et al.* (2001), with logs inoculated and incubated as described in section 2.9.2. Two CA plugs were used in each log as a negative control. After five weeks of incubation lesion development on the indicator logs was assessed using sampling methods described in section 2.9.2. Large lesions had developed on the larch indicator logs and so all experimental logs were then sampled. Attempts to reisolate the pathogen from all lesions was as described in section 2.6.1.

6.2.3.3 Experimental design and statistical analysis

Logs were challenged with twelve isolates of each lineage [Table 6.1]. A log represented a block that was replicated six times for each tree species. Data were analysed using GenStat (13th Edition) and a restricted likelihood model (REML) variance components analysis was fitted to the data. *Phytophthora ramorum* isolate, tree genotype, log and inoculation band were treated as random effects in the model-building. These factors were treated as random effects as lesion development has been shown to vary according to both pathogen and host genotype (Brasier & Kirk, 2001; Sansford *et al.*, 2008; Huberli & Garbelotto, 2012) as well as inoculation position on the stem (Susan Kirk personal communication), with larger lesions tending to develop on logs from the lower part on the tree stem (see chapter 4). Fisher's unprotected least significant difference test was carried out on the data to draw out significant differences between each tree species and lineage.

Re-isolation data were analysed using a generalised linear mixed model with a binomial error structure and logit function fitted to the data to test for differences in reisolation success between lineage and host. Host genotype and *P. ramorum* isolate were treated as random effects in the model-building. Fisher's unprotected least significant difference test was carried out on the data to draw out significant differences between hosts and lineage. The experimental design consisted of twenty reisolation attempts from each isolate on each host, replicated twice.

6.2.4 Pathogenicity comparisons on larch sapling stems

6.2.4.1 Plant material, inoculation, incubation and sampling

Pathogenicity comparisons between the two lineages tested ability to colonise the inner bark of European and Japanese larch saplings (approximately 2 m tall grown in Alice Holt Research Station intensive nursery). Inoculated stems were incubated at 10 and 20°C. The study was carried out in February 2013 on winter host material to determine if the pathogen was active in dormant material. Sapling stems were inoculated, incubated and sampled as described in section 2.10.

6.2.4.2 Experimental design and statistical analysis

Twelve isolates, six from each lineage, were tested against two hosts, Japanese and European larch, incubated at two temperatures, 10 and 20°C. An ANOVA with a quasipoisson error structure was fitted to data. Host genotype and *P. ramorum* isolate were treated as random effects in the model-building. The model tested the effects of lineage and larch species on *P. ramorum* lesion development incubated at two temperatures.

6.2.5 Pathogenicity comparisons on detached rhododendron leaves

6.2.5.1 Plant material

Fully expanded, unblemished, size matched leaves were collected from *Rhododendron ponticum* plants growing on site at Forest Research. Leaves were collected from three plants for the experiments in October 2012 and from ten plants in March 2013. All leaves were rinsed in SDW, air-dried and marked horizontally across the leaf, 5 cm from the tip, using a permanent marker pen, to indicate the inoculation area.

6.2.5.2 Isolate, inoculum production, inoculation, incubation and pathogenicity assessment

Isolates of *P. ramorum* were selected to represent each lineage in this study [Table 6.1] and SDW was used as a control. Inoculum of *P. ramorum* isolates was produced as described (section 2.11.1). Rhododendron leaves were inoculated in October 2012 using two zoospore concentrations, one high consisting of 1×10^5 zoospores mL⁻¹ and a lower concentration of 1×10^4 . The study was later repeated using a zoospore suspension of 1×10^4 zoospores mL⁻¹ and the number of plants tested increased in March 2013, due to variation in the data. Zoospore suspensions were generated using methods described in section 2.11.1, with

leaves inoculated and incubated as described in section 2.11.2 for both trials. The pathogenicity of isolates of *P. ramorum* was assessed as described in section 2.11.4.

6.2.5.3 Experimental design and statistical analysis

The two lineages of *P. ramorum* were each represented by six isolates. Testing of each isolate was replicated six times on three different plants in October 2012 and 20 times on ten plants in March 2013. A generalised mixed linear model with a binomial error structure and logit function was fitted to data to test for differences in the lesion size produced by the two European lineages of *P. ramorum* separately at each spore concentration. Host genotype and *P. ramorum* isolate were treated as random effects in the model-building

6.2.6 Comparative sporulation potential on Japanese larch needles

6.2.6.1 Plant material

In October 2012 and 2013, the current year's long shoots, approximately 15 cm long and bearing individual needles were selected from mature healthy trees, grown in the trial grounds at Alice Holt Research Station. Shoots were marked 4 cm from the tip using a permanent marker pen to indicate the inoculation area. Each isolate was tested on six shoots which came from three different trees (i.e. two shoots from each individual plant).

6.2.6.2 Inoculum production, inoculation and incubation

Four isolates of *P. ramorum* were used to represent each lineage in this study [Table 6.1] and SDW was used as a control. Inoculum of *P. ramorum* isolates was produced as described in section 2.11.1. Larch shoots were inoculated in October 2012 using two separate concentrations of zoospore suspensions; a high concentration 1×10^5 zoospores mL^{-1} and a lower concentration of 1×10^4 . Different zoospore concentrations were used as levels of disease development have been found to be directly related to inoculum concentration and resistance can be overcome by exposures to high levels of inoculum (Tooley, Browning & Leighity, 2013). In previous sporulation studies a zoospore concentration of 3×10^5 zoospores per ml were used based on methods devised by Denman *et al.* (2005) and Fichtner *et al.* (2012). This concentration however is relatively high compared to likely field exposure levels. Therefore, a lower concentration of 1×10^5 zoospores per ml was chosen along with an even lower concentration of 1×10^4 , as these have been used in other studies of virulence in *P. ramorum* isolates on rhododendron and

bay laurel (Huberli & Garbelotto, 2012; Jinek *et al.*, 2011; De Dobbelaere *et al.*, 2010). With previous assessments sporangia were produced at such high a density on the larch foliage that accurate counts could not be made so the study was repeated in October 2013 using an even lower zoospore concentration of 1×10^3 zoospores mL^{-1} . Inoculated shoots were incubated for seven days at 18 - 20°C and the number of sporangia on the larch needles was counted as described in section 2.11.2 and 2.11.3.

6.2.6.3 Experimental design and statistical analysis

In the experimental design each lineage was represented by four isolates of *P. ramorum* (n=4). Six replicate larch shoots were taken from three Japanese larch trees, with a subset of six needle replicates on each shoot (n=36) for each isolate. The data were analysed using GenStat (16th Edition). A restricted likelihood model (REML) variance components analysis was fitted to the square root transformed data. For comparison of lineages the factors isolate, host genotype, shoot and needle were treated as random effects in the model-building. For comparison of isolates the factors host genotype, shoot and needle were treated as random effects in the model-building. Fisher's unprotected least significant difference test was carried out on isolate data to draw out significant differences.

6.3 Results

6.3.1 *In vitro* growth response

All twelve isolates of *P. ramorum* from each lineage were grown on agar media. Differences between the two lineages in colony morphology were observed on all three agars at 25°C and 28°C, but were most distinct on CA and V8 agar [Plate 6.1].

At 25°C EU1 isolates formed rosette colonies and were slower growing than EU2 isolates on both CA and V8 agar. On CA EU2 isolates formed stellate colonies with uniform outer edges and fluffy white inner regions. On V8 agar EU2 isolates formed stellate to petaloid shaped colonies. Colonies from both lineages on PDA grew irregularly from dense submerged petaloid shapes. Colonies of the EU1 lineage were more dense and slightly more uniform than the EU2.

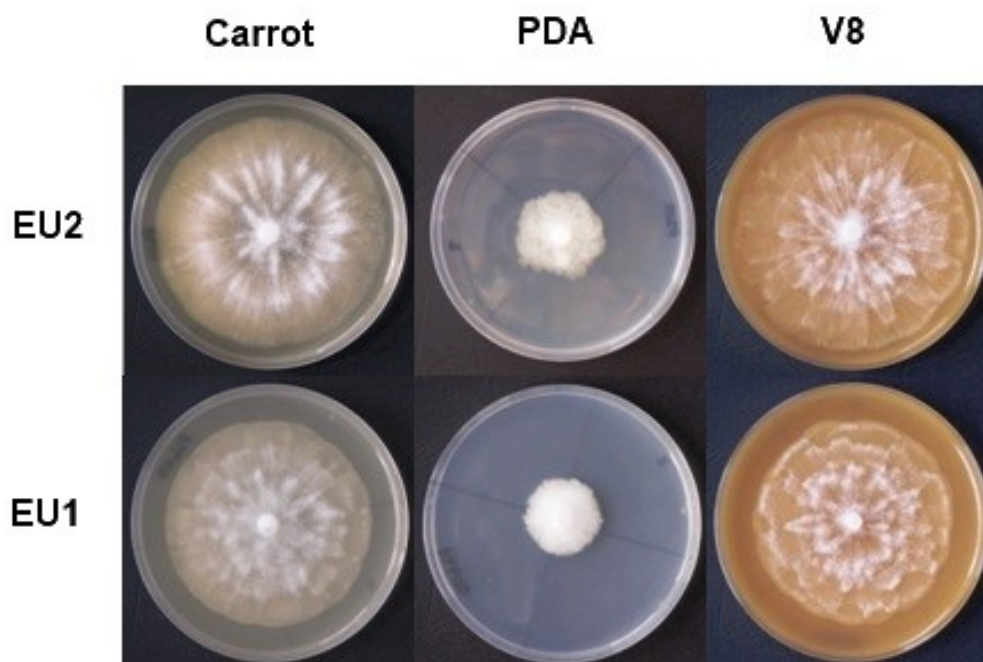


Plate 6.1 - Colony morphologies of isolates from the two European lineages of *P. ramorum* on carrot agar, potato dextrose and V8 agar incubated at 25°C.

On CA both European lineages shared a similar shaped growth curve, with average optimum growth rate at 20°C [Figure 6.1a]. The maximum temperature for growth of EU1 isolates was 27°C whilst isolates from the EU2 lineage were able to grow at 29°C. Growth occurred at the lowest temperature of 2.5°C for both lineages. Whilst the two lineages shared similar growth curves the mean daily growth rates of the lineages differed significantly at temperatures 2.5, 5, 10, 25 and 29°C [Table 6.2], with the EU2 on average having a faster linear growth rate.

The biggest difference in growth rate between the lineages could be seen on V8 agar. In contrast to CA the two lineages did not share a similar growth curve on V8 agar [Figure 6.1b]. Daily mean growth rate peaked again at 20°C for EU1 isolates but optimum growth for EU2 isolates was 25°C. Growth occurred at the lowest temperature tested of 2.5°C for isolates of both lineages. The maximum temperature for growth of EU1 isolates was again 27°C and 29°C for all isolates of EU2, but EU2 isolates were significantly faster growing than the EU1 lineage at all temperatures [Table 6.2].

The linear growth of *P. ramorum* was considerably slower on PDA compared to CA and V8 agar. The average growth curve of both lineages was unusual in that it produced two peaks, one at 20°C and another at 27.5°C [Figure 6.1c]. Similar growth occurred for both lineages

at 2.5°C but at 5°C and 10°C EU2 isolates grew significantly faster than EU1 isolates. At 15°C and 20°C mean growth rates of both lineages were the same. At 25°C the growth rate of both lineages declined, but more so for EU2 isolates, so that the EU1 isolates had a significantly faster growth rate than the EU2 [Table 6.2]. This difference between the lineages continued at 27.5°C, but at 29°C no growth was observed for any EU1 isolates, although EU2 isolates could grow at this temperature.

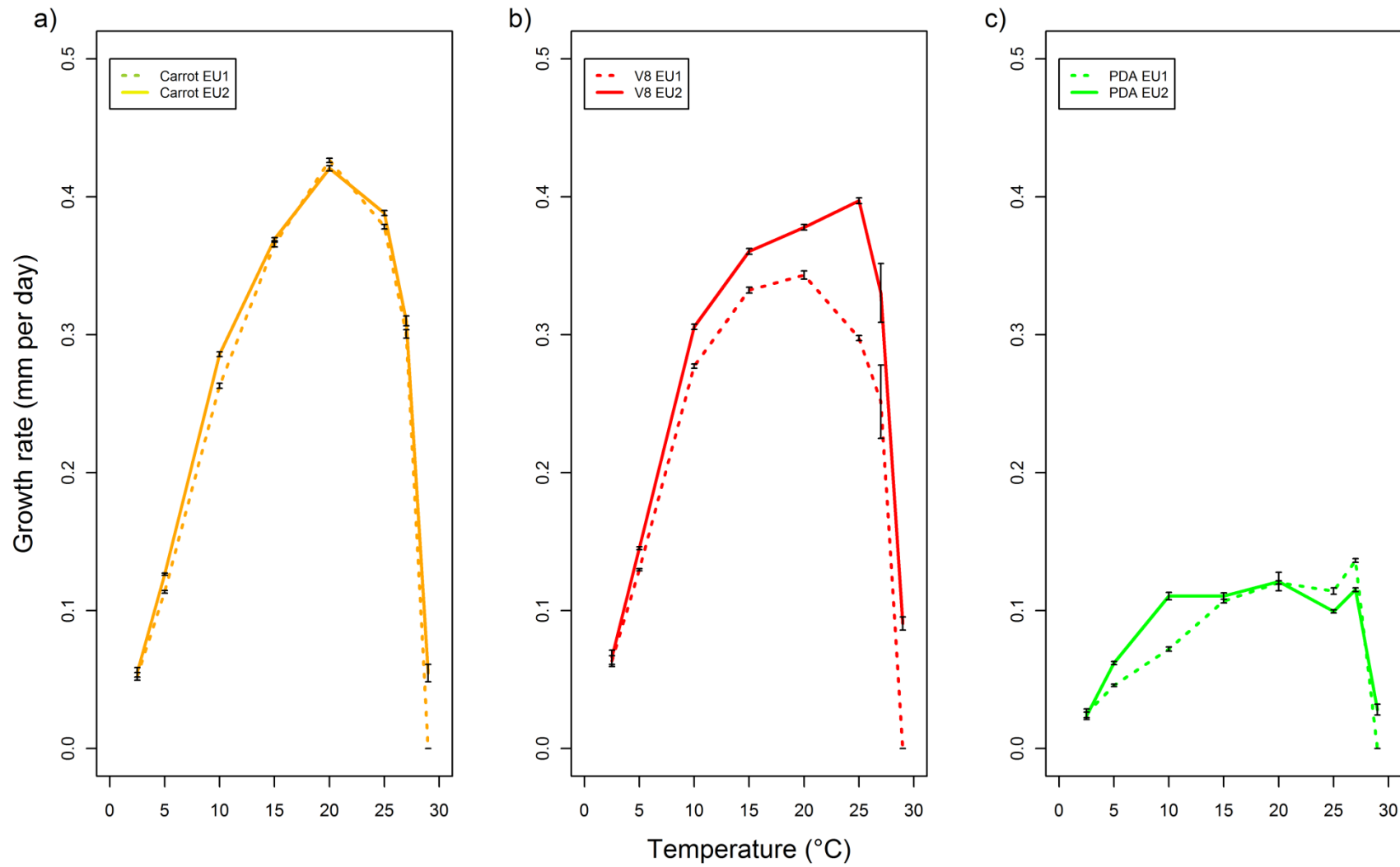


Figure 6.1 – Mean radial growth rate curves of the two European lineages of *Phytophthora ramorum* on a) carrot agar, b) V8 agar and c) potato dextrose agar between 2.5°C and 29°C.

Table 6.2 – Growth rate comparisons between the European lineage of *P. ramorum* (Different letters indicate differences between growth rate at specific temperatures determined by Fisher's unprotected least significant difference test (LSD) at $P < 0.05$).

Temperature (°C)	Lineage	Carrot agar LSD	V8 agar LSD	PDA agar LSD
2.5	EU1	a	a	a
	EU2	b	b	a
5	EU1	a	a	a
	EU2	b	b	b
10	EU1	a	a	a
	EU2	b	b	b
15	EU1	a	a	a
	EU2	a	b	a
20	EU1	a	a	a
	EU2	a	b	a
25	EU1	a	a	a
	EU2	b	b	b
27.5	EU1	a	a	a
	EU2	a	b	a
29	EU1	a	a	a
	EU2	b	b	b

6.3.2 Pathogenicity on mature tree bark

All 12 *P. ramorum* isolates from each lineage caused necrotic lesions on the inner bark of the four hosts, whereas little necrosis was associated with control inoculations and appeared to be entirely due to wound response. Lesion shape and colour varied according to host, with oval shaped lesions which were beige to light brown in colour typically occurring on beech, long narrow, dark brown lesions on oak and cinnamon brown, diamond-shaped lesions on larch [Plate 6.2].



Plate 6.2 – Lesion on the inner bark of logs caused by *P. ramorum* after five weeks incubation.

Overall the pathogenicity of *P. ramorum* based on lesion area in inner bark differed significantly between hosts ($F(3, 8.0) = 5.05, p < 0.05$). Averaged over both lineages, *P. ramorum* was least pathogenic to oak, but similarly pathogenic to European larch, Japanese larch and beech [Table 6.3].

Table 6.3 – Relative pathogenicity based on combined averages of the two lineages colonising inner bark (different letters indicate differences among treatments determined by Fisher's unprotected least significant difference test (LSD) at $P < 0.05$).

Host	Model predicted mean	LSD at the 95% level
Oak	2.316	A
European larch	4.021	B
Beech	4.090	B
Japanese larch	4.201	B

However, when averaged over the four hosts, there was a significant difference in the pathogenicity of the two lineages of *P. ramorum* ($F(1, 486.7) = 412.56, p < 0.001$). EU2 lineage isolates produced a mean lesion area 122% larger than isolates from the EU1 lineage. Most variation in lesion size was explained by the interaction between lineage and host ($F(3, 487.1) = 103.94, p < 0.001$) [Figure 6.2]. Lesion areas generated by the two lineages differed significantly on Japanese larch, European larch and oak but not on beech.

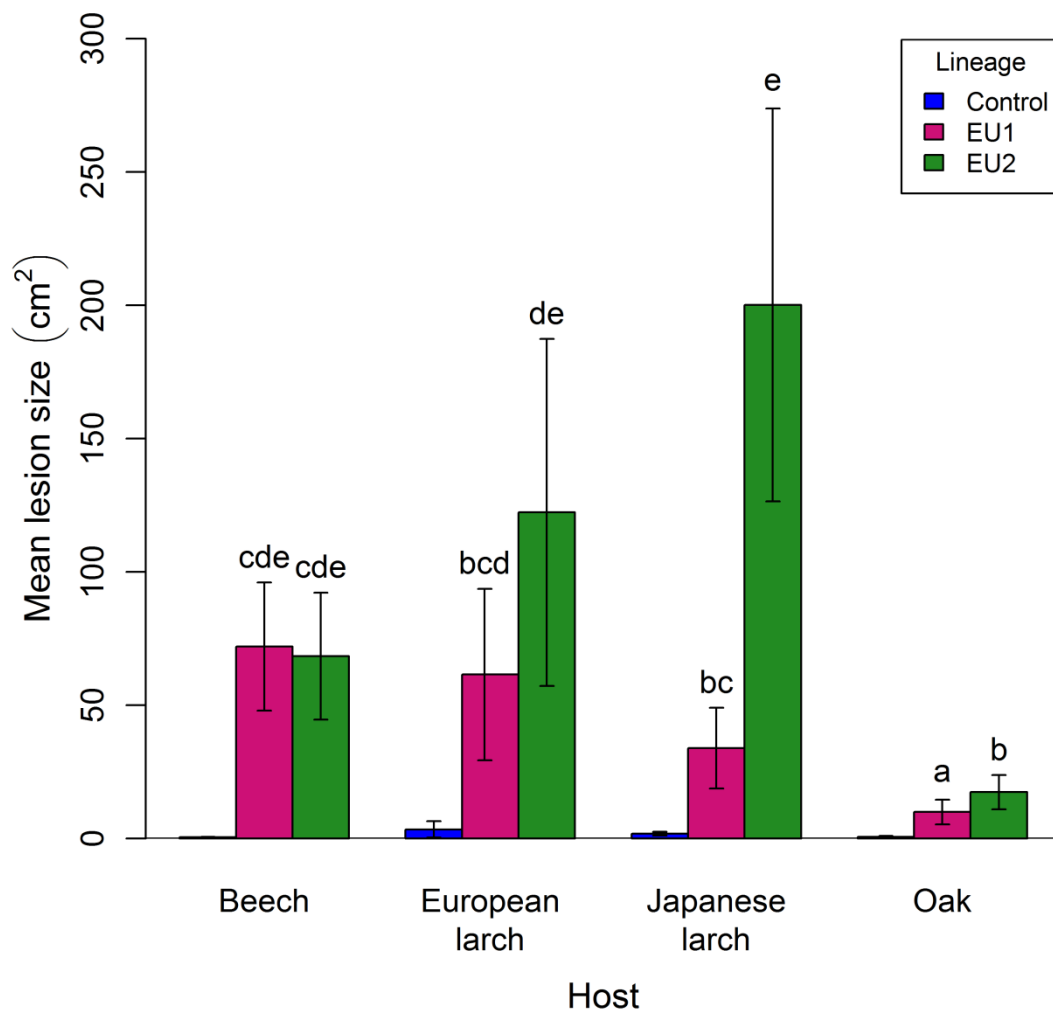


Figure 6.2 – Mean lesion area produced by *P. ramorum* EU1 and EU2 lineages when inoculated into inner bark of tree hosts (Error bars represent the standard error of the mean (n=3). Different letters indicate significant differences among treatments determined by Fisher's unprotected least significant difference test at $P < 0.05$).

6.3.2.1 Reisolation success from host bark

It was possible to reisolate *Phytophthora ramorum* from symptomatic bark tissue produced by EU1 and EU2 on all four hosts tested [Figure 6.3]. However, reisolation success differed significantly according to lineage and host ($F(1, 195.7) = 7.86, p < 0.01$ and $F(3, 2.6) = 18.31, p < 0.05$ respectively). Overall, reisolation was more successful when culturing from lesions incited by the EU1 lineage compared with the EU2. Reisolation was nearly always successful from symptomatic beech and oak bark tissue (92% success rate for both) regardless of lineage.

Reisolation success was significantly affected by an interaction between lineage and host ($F(3, 195.3) = 3.25, p < 0.05$). It did not differ significantly between hosts except for European larch, where isolates of the EU1 lineage were more readily reisolated from bark tissue compared to those of EU2. Reisolation of both European lineages of *P. ramorum* inoculated into Japanese larch and the EU2 lineage inoculated into European larch was significantly lower than reisolation success of both lineages from oak and beech and the EU1 lineage from European larch [Figure 6.3].

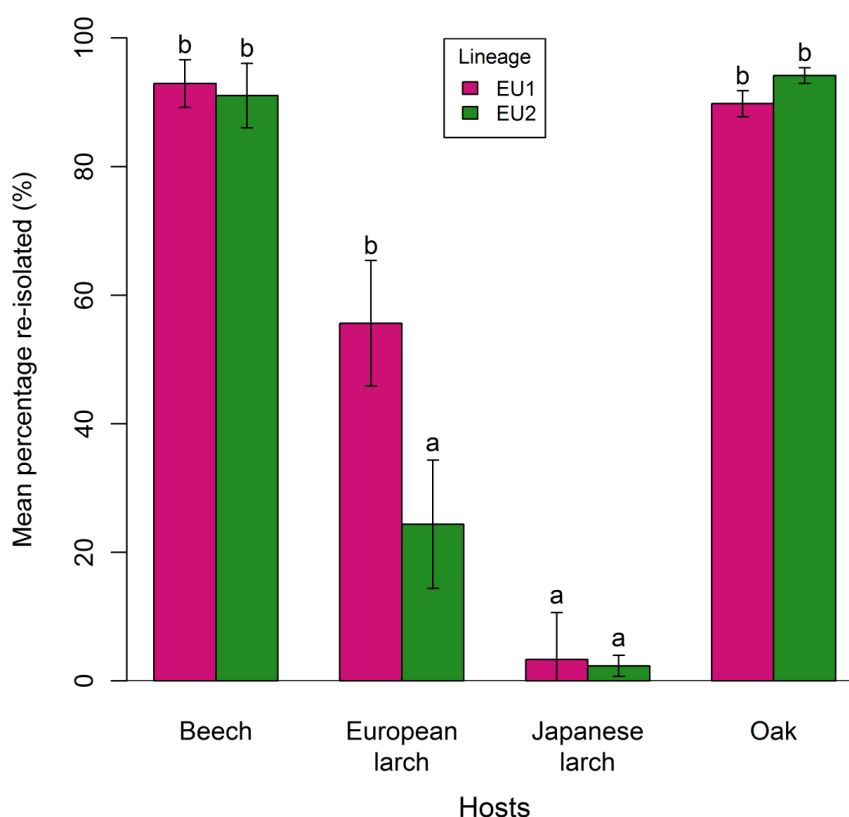


Figure 6.3 - Success of *P. ramorum* reisolation from symptomatic bark based on mean percentage reisolation of isolates of each lineage from different hosts. (Error bars represent the standard error of the mean. Different letters indicate significant differences among treatments determined by Fisher's unprotected least significant difference test at $P < 0.05$).

6.3.3 Pathogenicity on sapling stems in winter

Isolates of *P. ramorum* from both European lineages caused necrotic lesions on the inner bark of European and Japanese larch saplings after incubation at 10 or 20°C [Plate 6.3]. Pathogenicity of the European lineages of *P. ramorum* was significantly affected by temperature ($p < 0.001$) and larch species ($p = 0.026$). When incubated at 10°C, the comparative pathogenicity of the two lineages was similar although the EU1 produced slightly smaller lesions. At 20°C however, based on mean lesion size the EU2 lineage was significantly more pathogenic than the EU1. Overall, EU2 lesions were approximately double the size of those caused by the EU1. The EU1 appeared slightly less pathogenic on sapling bark incubated at 20°C compared to 10°C but not significantly so, whilst EU2 isolates were significantly more pathogenic at 20°C compared to 10°C [Figure 6.4 a]. Pathogenicity of the EU2 was similar on European and Japanese larch. EU1 was similarly pathogenic on European larch. The EU1 lineage was significantly less pathogenic on Japanese larch compared to the EU2 isolates with approximately 50% smaller lesions on Japanese compared to European larch [Figure 6.4 b].

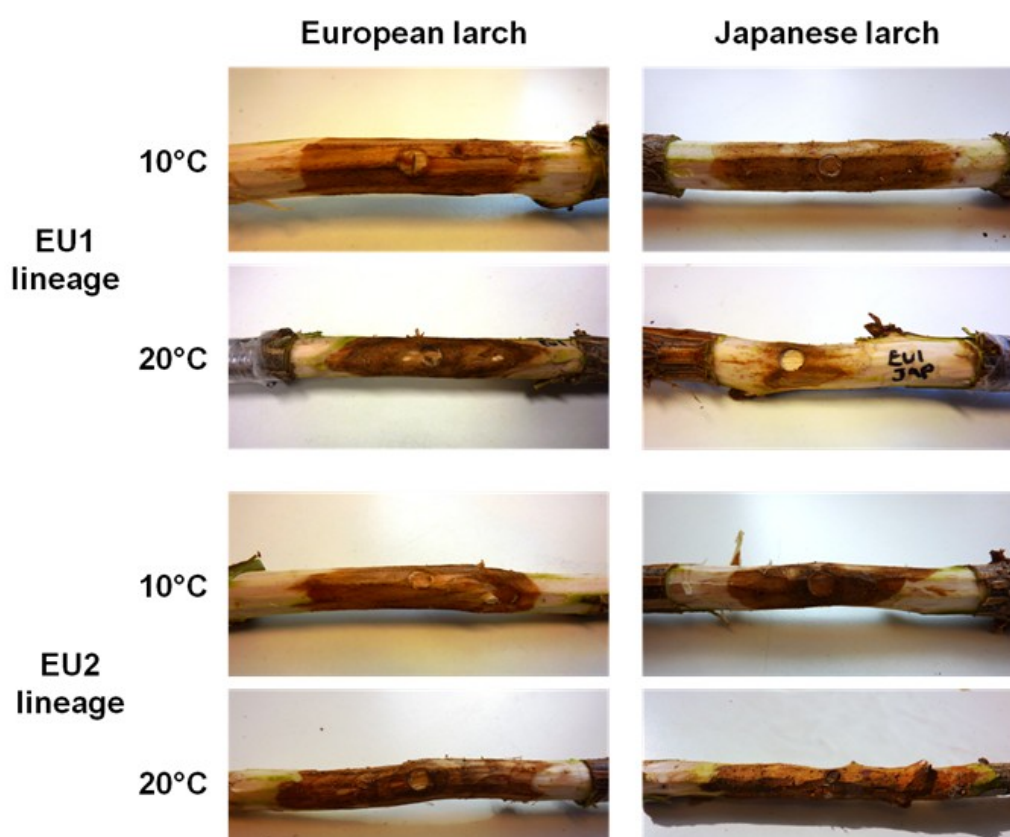


Plate 6.3 – Lesion on the inner bark of larch sapling stems caused by *P. ramorum* after two weeks incubation at 10 and 20°C.

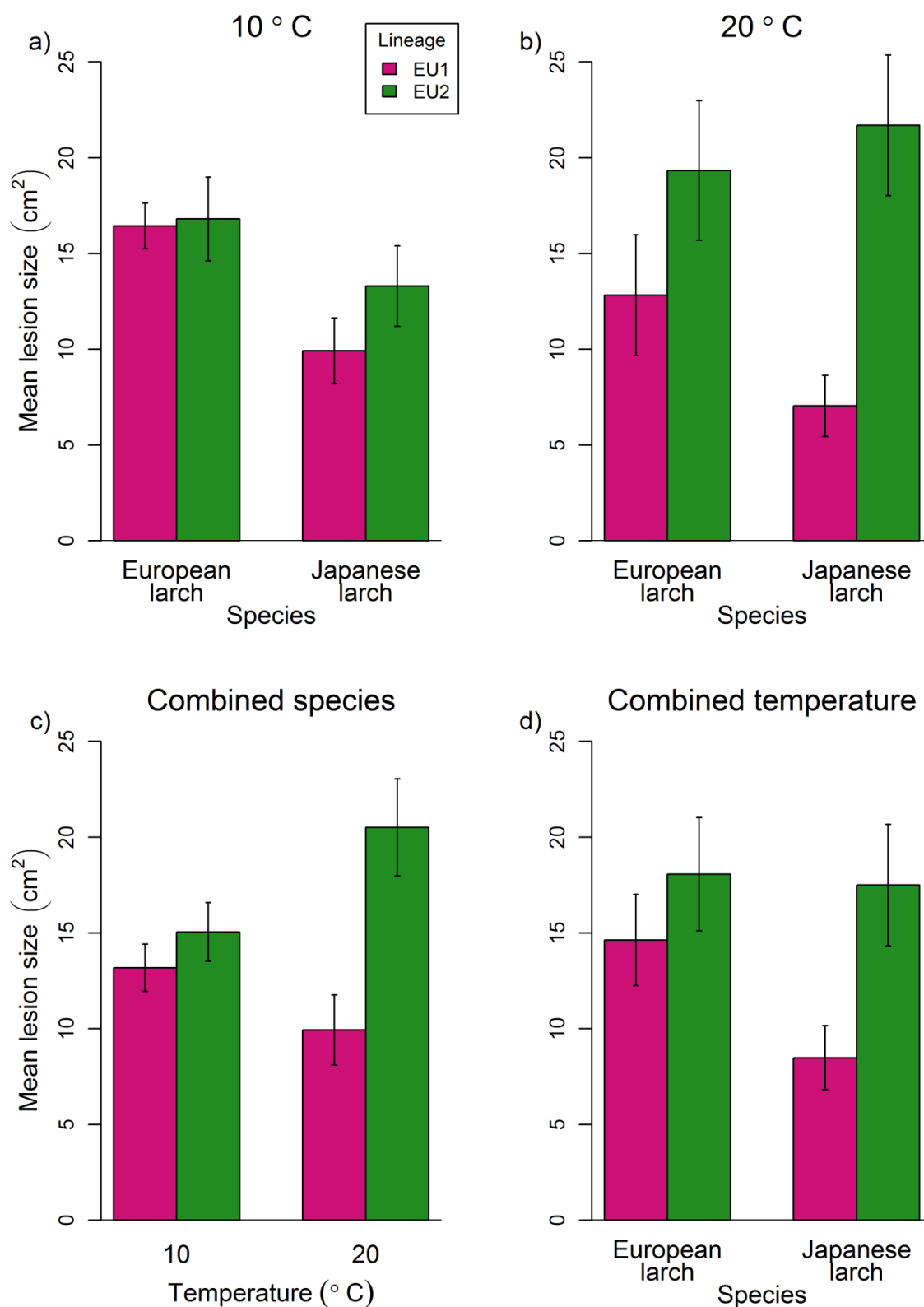


Figure 6.4 – Mean lesion size of isolates of *P. ramorum* belonging to the EU1 and EU2 lineage on the bark of; a) European and Japanese larch sapling stems incubated at 10°C (n=12); b) European and Japanese larch sapling stems incubated at 20°C (n=12); c) larch stems incubated at 10°C and 20°C (n=24); d) European and Japanese larch stems (combined temperature data (n=24)). (Error bars represent the standard error of the mean).

6.3.4 Pathogenicity on detached rhododendron leaves

In test one (October 2013) with the higher inoculum concentration (1×10^5 zoospores mL^{-1}) lesion development on rhododendron leaves was greater with the EU1 than the EU2 lineage [Figure 6.5a], but with the lower concentration (1×10^4 zoospores mL^{-1}) the opposite occurred and the mean lesion area for the EU2 was greater. When the study was repeated (March 2014) using increased number of isolates and replication with spore concentration of 1×10^4 zoospores mL^{-1} , lesion size was on average, larger for the EU1 compared with the EU2 [Figure 6.5b&c]. However, statistical analysis found no significant difference in average lesion size between the two lineages on rhododendron leaves when exposed to the lower inoculum concentrations in October 2013 and March 2014 ($F(1, 125.7) = 1.5, p = 0.22$). Therefore, data from both tests using the lower spore concentrations from October 2013 and March 2014 were combined and compared to the results from tests using the higher concentration of spores in October 2013. This new analysis indicated that spore concentration did not have a significant effect on the pathogenicity of the lineages on rhododendron leaves ($F(1, 152) = 2.93, p = 0.09$). So all data, from both years were further combined for analysis. Overall, although isolates from the EU1 produced larger lesions on rhododendron leaves compared to EU2 isolates [Figure 6.5d], the difference was not significant despite the apparent trend ($F(1, 10.8) = 0.99, p = 0.34$).

It is worth noting that lesion development varied considerably amongst plants and leaves [Plate 6.4], resulting in high levels of variance within the data. Increasing the number of isolates representing each lineage and the number of plants and leaves tested in March 2014 helped to reduce this variance [Figure 6.5c], but the analysis still suggested that the lineages were similarly pathogenic to rhododendron leaves ($F(1, 9.3) = 0.45, p = 0.52$).

The smallest mean lesion size on rhododendron resulted with EU1 isolate P2639 with the average lesion area accounting for 1.9 % of the inoculated leaf area. The largest and therefore most pathogenic isolate, was also an EU1 isolate, P2609 which formed lesions covering 50.7% of the inoculated leaf area. The least pathogenic EU2 isolate tested was P2748 and the most pathogenic P2565 with mean lesion areas of 2.6% and 30.2% respectively. However, despite these apparent differences, statistical analysis showed that pathogenicity did not differ significantly between any of the individual isolates ($F(17, 112.2) = 0.81, p = 0.68$).

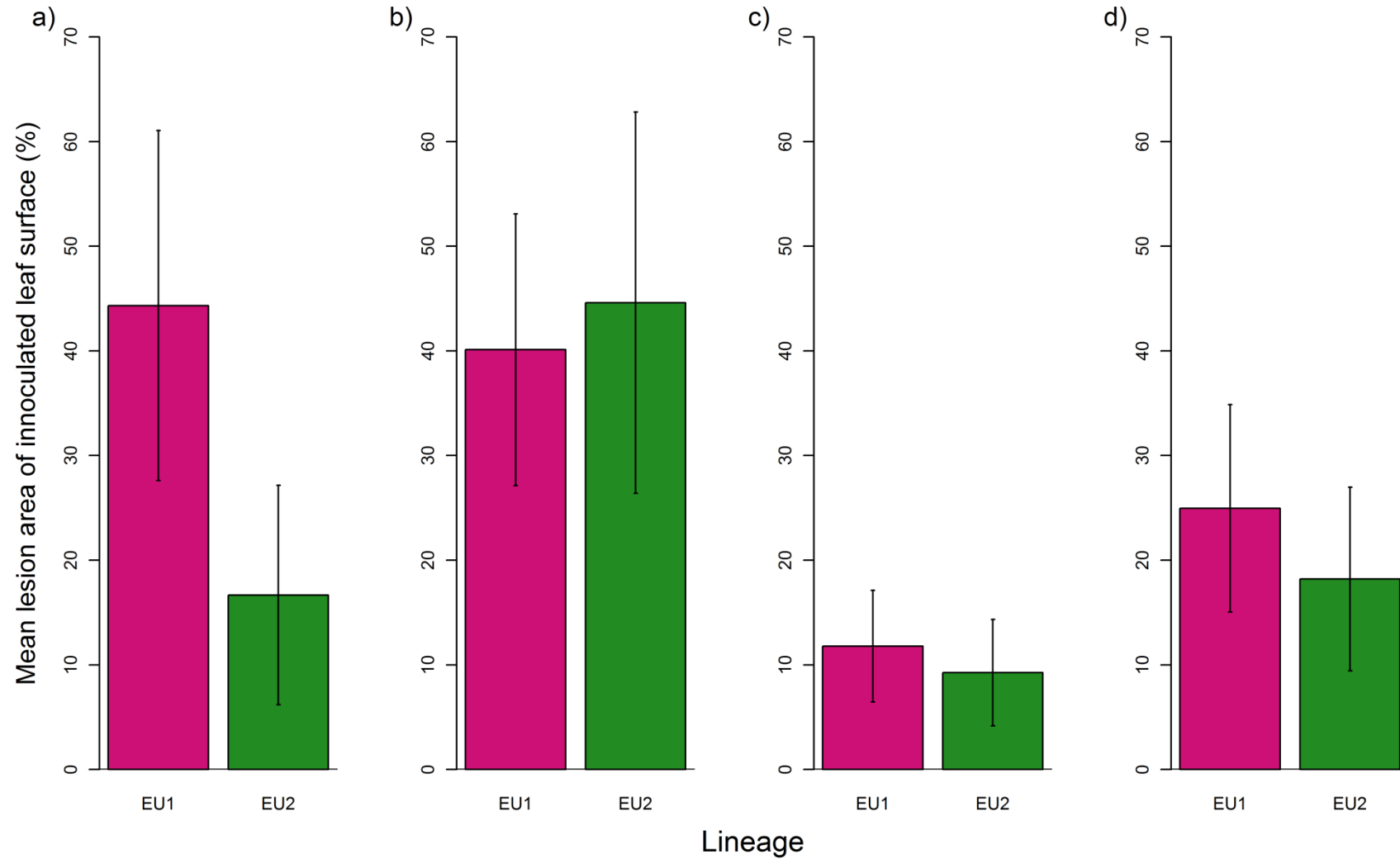


Figure 6.5 - Mean area of leaf surface of rhododendron covered with lesions after exposure to *P. ramorum* zoospore suspension of a) 1×10^5 zoospores mL⁻¹ (n=3), b) 1×10^4 zoospores mL⁻¹ in October 2012 (n=3), c) repeat 1×10^4 zoospores mL⁻¹ in March 2013 (n=10) and d) combined data for October and March experiments. (Error bars represent the standard error of the mean).

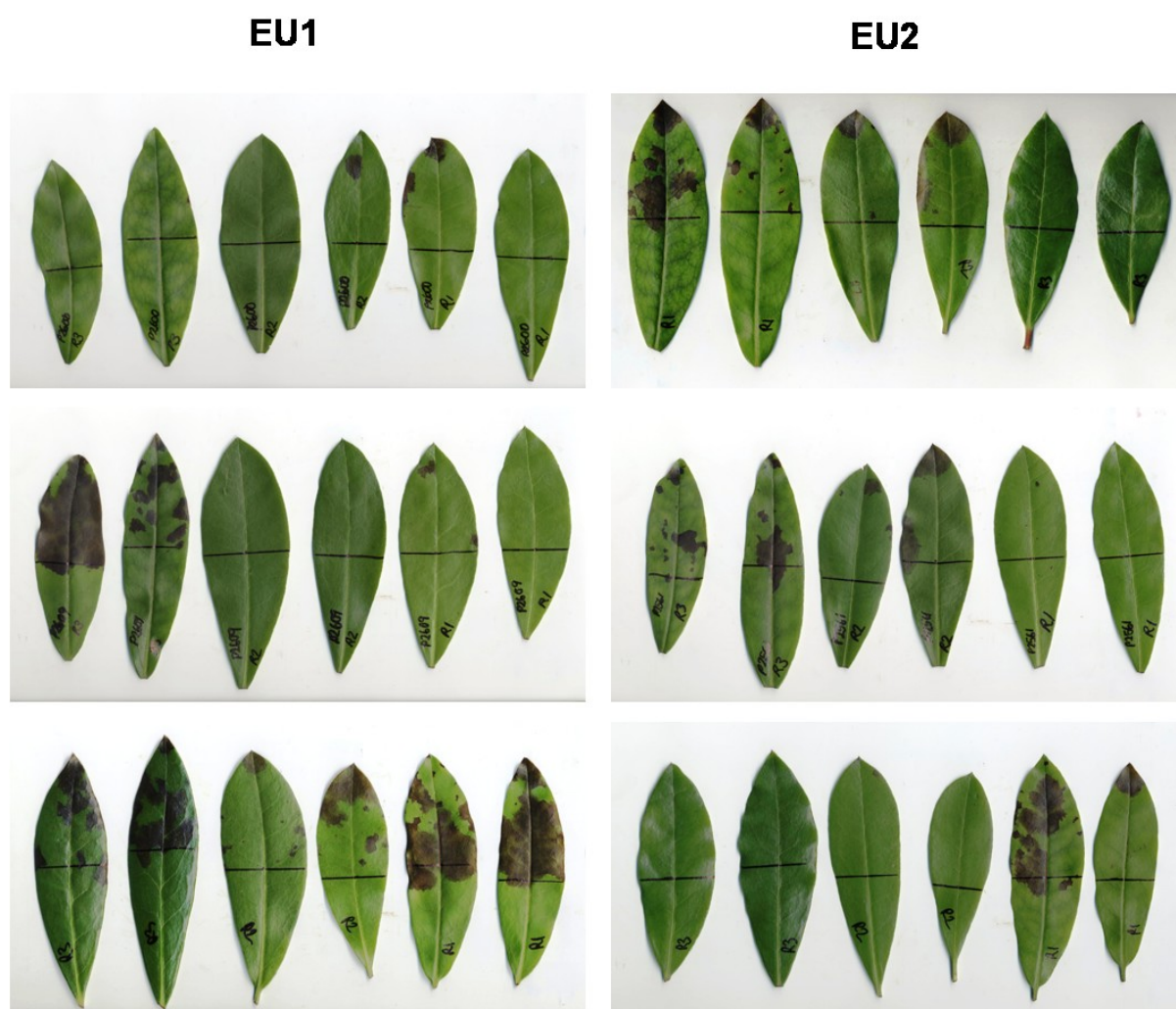


Plate 6.4 – Lesion development on rhododendron leaves exposed to *P. ramorum* zoospore suspensions at 1×10^4 zoospores mL^{-1} concentration and incubated for seven days in October 2013 (EU1 isolates P2600, P2609 & P2590 and EU2 isolates P2750, P2561 & P2583 from top to bottom).

6.3.5 Comparative sporulation potential on Japanese larch needles

Inoculation of Japanese larch needles with inoculum concentration of 1×10^5 zoospores mL^{-1} and 1×10^4 zoospores mL^{-1} resulted in infection and profuse sporulation with thousands of sporangia per needle regardless of lineage. At both concentrations, Japanese larch needles were highly susceptible to infection and produced both sporangia and chlamydospores with both lineages. Differences between the sporulation potential of isolates and lineages could not be assessed as spore number could not be counted accurately.

In the second trial using an inoculum concentration of 1×10^3 zoospores mL^{-1} sporangia were produced on all needles inoculated with EU2 isolates and all but three needles inoculated with EU1 isolates. Overall, the EU2 lineage produced fewer sporangia per cm^2 compared with the EU1 lineage, producing 1113.9 ± 833.3 (± 1 s.e., $n=4$) and 1967.6 ± 1318.2 (± 1 s.e., $n=4$) sporangia cm^{-2} respectively [Figure 6.6a]. Despite the trend for higher levels of sporulation with EU1 on Japanese larch, average sporulation values for EU1 and EU2 did not differ significantly ($F(1, 6) = 4.88$, $p = 0.07$). Chlamydospores were only infrequently observed on the needles' surfaces. Out of the total of 144 needles inoculated for both lineages chlamydospores developed on 29 and 34 EU1 and EU2 needles respectively. Between one and five chlamydospores developed on 20 and 27 needles inoculated with the EU1 and EU2 isolates respectively, whilst fewer than 10 chlamydospores were observed on a total of eight and six needles respectively. On average EU1 isolates produced chlamydospores at a density of $11.9 \text{ cm}^{-2} \pm 22.9$ (± 1 s.e., $n=4$) and EU2 produced them at $8.2 \text{ cm}^{-2} \pm 13.7$ (± 1 s.e., $n=4$).

Whilst there was no significant difference between mean sporulation levels of the two European lineages, there was a significant difference between mean sporangia production with individual isolates ($F(7, 245) = 2.9$, $p < 0.01$). With the exception of isolate P1614, EU1 isolates sporulated more profusely than EU2 isolates [Figure 6.6b]. EU2 isolates P2460 and P2583 produced significantly fewer sporangia per cm^2 (109.9 ± 75.5 (± 1 s.e., $n=6$)) than EU1 isolates P2609 and P2600 (338.6 ± 199.5 (± 1 s.e., $n=6$)).

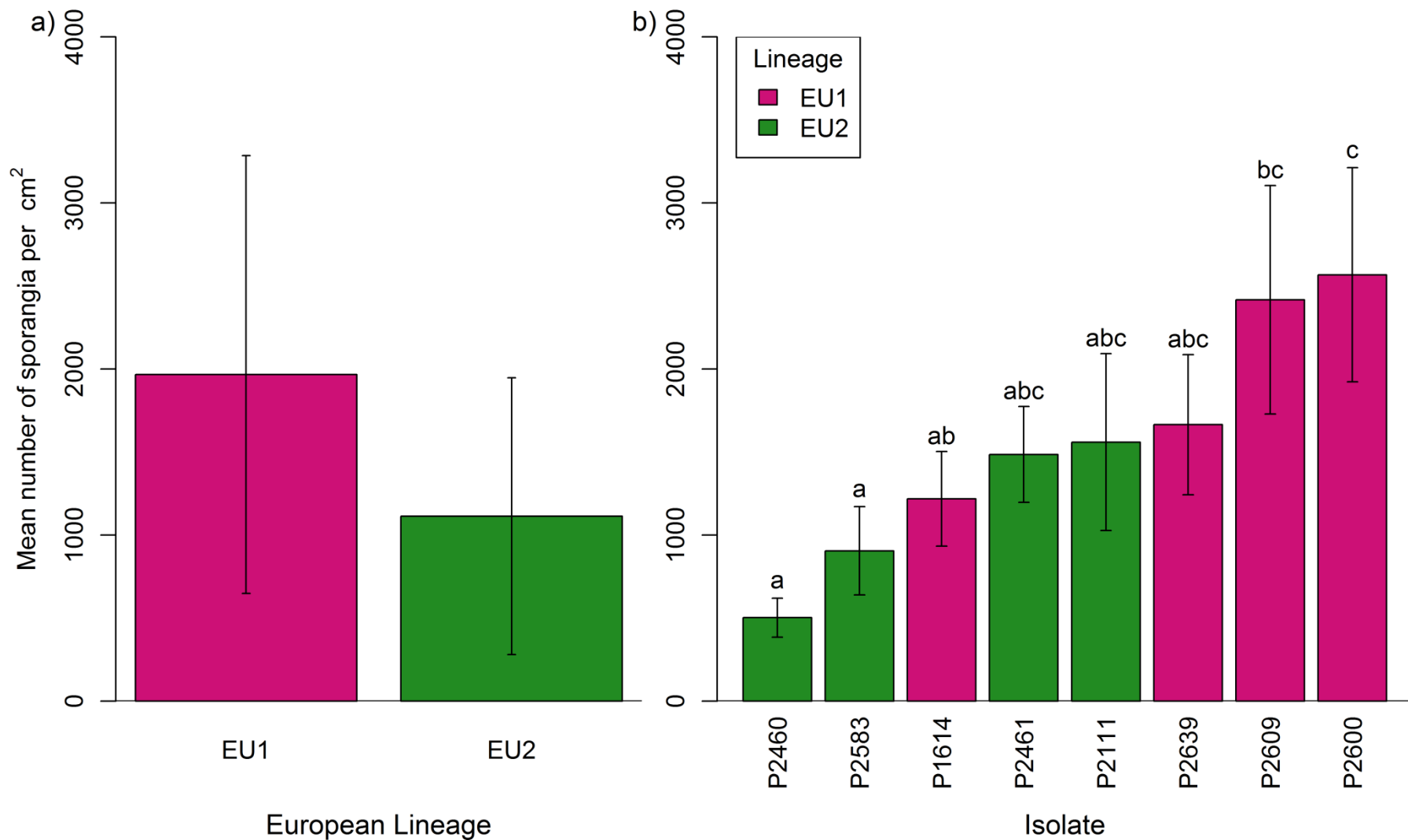


Figure 6.6 - Mean sporulation on Japanese larch of; a) European lineages of *P. ramorum* (n=4); b) isolates of *P. ramorum* (n=6). Error bars represent the standard error of the mean. Different letters indicate differences among treatments determined by Fisher's unprotected least significant difference test at $P < 0.05$.

6.4 Discussion

Virulence, inoculum production and host range are the fundamental traits of a pathogen that influence development of plant disease epidemics. Rate of infection, colonisation and spread are all essential factors in disease development. Virulent pathogens are faster growing, colonise host tissues more readily and produce larger amounts of inoculum, thus improving the chances of spread to other hosts and establishing a disease epidemic (Agrios, 2005). The findings of this study indicate that the EU2 lineage of *P. ramorum* has distinctive characteristics which distinguish it from the EU1 lineage.

Growth rates tests suggest that the EU2 lineage tolerates a wider range of temperatures and is generally faster growing and better adapted to nutritional stress than EU1. Such characteristics could allow it to colonise hosts rapidly, early in the season and to persist until later in the season. The tolerance of temperature extremes may also enable the EU2 lineage to be active earlier in the spring, continue developing throughout the hot summers and remain active for longer towards the end of the growing season compared to EU1, thereby having a deleterious impact on hosts over a longer period. In addition to faster *in vitro* growth, the EU2 lineage was also faster growing *in planta* compared to the EU1 in comparisons of colonisation of the inner bark of mature European larch and Japanese larch. The same outcome also occurred on Japanese and European larch sapling stems at 20°C. This finding adds support to the anecdotal reports from Plant Health surveyors of increased rates of tree mortality through girdling caused by colonisation by *P. ramorum* in the EU2 dominated area of south west Scotland (P. Robertson, personal communication).

Interestingly, mean pathogenicity of EU2 isolates on the inner bark of mature English oak (*Quercus robur*) was also significantly greater than for EU1 isolates. At present there are no records of natural infections caused by the EU2 on oak in Britain and the EU1 lineage of *P. ramorum* has posed little threat to native oak species, English oak and sessile oak (*Q. petraea*). These species have only low susceptibility to *P. ramorum* with only one and four documented infections on sessile oak and English oak respectively in Britain since 2003 (Forest Research, unpublished data). This contrasts with the impact of the NA1 lineage of *P. ramorum* on American oak species such as coastal live oak (*Q. agrifolia*) and California black oak (*Q. kelloggii*). It is estimated to have killed over 5 million trees in coastal California and Oregon, including true oaks and tanoaks (*Notholithocarpus densiflorus*) (Frankel & Palmieri, 2014). Potentially, EU2 infection of native oak in the UK could have serious implications for woodlands and biodiversity if it is more pathogenic. However, the results also

indicate that overall levels of English oak susceptibility were still lower even if larger lesions were incited by EU2 isolates than EU1.

Although EU2 is a more effective coloniser of larch bark, there is no evidence to suggest it is a more abundant sporulator. Indeed, EU1 isolates typically produced nearly 50% more sporangia on Japanese larch needles than EU2 isolates, although the difference was not found to be statistically significant between the lineages. However, individual isolates of the EU1 produced significantly higher levels of sporulation than most EU2 isolates.

The fact that the EU2 lineage does not display both increased pathogenicity and sporulation potential on Japanese larch compared with the EU1 lineage, suggests there may be a trade-off between these two factors, preventing the EU2 lineage from maximising both fitness traits. A similar trade-off between virulence and transmission has been reported to occur in *P. ramorum* by Moralejo, Garcia Munoz & Descals (2006) and was apparent in the response of *P. ramorum* to rhododendron reported in chapter three. By exhibiting high pathogenicity, the EU2 lineage will have the capacity to infect hosts more effectively/extensively but may suffer a fitness cost exhibited as a reduced sporulation rate on a given host.

Recent molecular analysis of the UK *P. ramorum* population has established that the EU1 is currently the more widespread of the two lineages and that the EU2 lineage has not spread rapidly from southwest Scotland where it was first detected in 2007 (date of the oldest known isolate) (Van Poucke *et al.*, 2012; King, Harris & Webber, 2014). Predictions on rate of spread and disease levels are difficult to make as they are controlled by multiple factors concerning the pathogen, host phenology and climatic conditions. The rate of spread and thus scale of epidemic caused by the EU2 lineage could be expected to be reduced in comparison to the EU1 lineage, because lower sporulation levels would lesson capacity to spread and infect. Additionally, although more aggressive colonisation of phloem tissue may kill trees more quickly, this could also limit available live host material for sporulation and consequently limit pathogen spread.

Undoubtedly, ramorum disease on larch has continued to spread in the UK since its discovery in 2009, despite containment strategies that have been in place since 2003. During 2013, an additional 10,000 hectares of infected larch was identified mainly in Wales and south west Scotland (Forest Research, unpublished data). In Wales, the disease epidemic is solely due to the EU1 lineage, whereas in south west Scotland the EU2 predominates (King, Harris & Webber, 2014). The widespread mortality of the larch in south west Scotland has been attributed to the pathogenicity of EU2, but the larch is also grown in large continuous blocks and especially favourable climatic conditions in 2012 (extended and

above average rainfall) are also likely to have enhanced sporulation and dispersal (Garbelotto *et al.*, 2003; Davidson *et al.*, 2005; Davidson *et al.*, 2005; Hansen *et al.*, 2008). For Wales, where the EU2 is absent, the heightened epidemic in 2013 can only be due to the favourable climatic conditions and the large areas of larch planting which are similar for south west Scotland. This emphasises the importance of environmental factors in driving the ramorum epidemic. In addition, as more plantations have become infected over recent years, forest managers have struggled to remove infected, sporulating hosts to contain the disease. Thus, the increasing number of sources and the levels of inoculum in the environment have also contributed to the likelihood of trees at new sites becoming affected. It is difficult to determine the influence of lineage on disease intensity in the wider environment with the many variables that operate and this requires further study in the field. It is clear that 2012 was an unusually wet year following periods of below average rainfall (Met Office, 2012b) and it is likely that this has been a major factor in heightening the ramorum epidemic in some parts of the country.

The presence of the EU2 lineage in the UK poses an additional risk to larch. It may not, however, substantially increase the threat of *P. ramorum* to most non-larch forest trees as significant differences in the bark-colonising ability of the EU1 and EU2 lineages have not been found on beech, Douglas fir, noble fir, Sitka spruce, Norway spruce, Scots pine or birch (Webber *et al.*, 2014). Both lineages were also similarly pathogenic on rhododendron leaves, even when tested at different times of year and when exposed to different concentrations of inoculum. It may be that rhododendron is so highly susceptible to *P. ramorum* and most other Phytophthoras (Erwin & Ribeiro, 1996) that all lineages of genotypes are similarly pathogenic to this shrub.

It is unclear whether greater pathogenicity of the EU2 lineage on Japanese larch will have an increased detrimental effect on the larch industry. Once infected by *P. ramorum*, regardless of lineage, there is a limited chance of tree recovery and the statutory legislation currently requires felling (Commission Decision, (2002/757/EC)). Had the EU2 lineage produced significantly more inoculum than the EU1 lineage on larch foliage, the chances of spread would have been significantly increased, adding to the difficulties of disease containment.

6.5 Summary

The aim of this study was to assess the comparative forest and plant health risk posed by the newly discovered EU2 lineage of *P. ramorum* based on the results of experiments carried out on growth rate, pathogenicity and sporulation potential.

The key findings of this study were;

1. Isolates from the EU2 lineage of *P. ramorum* are both faster growing and able to grow at a wider range of temperatures than EU1 isolates.
2. Pathogenicity of the EU2 lineage is significantly greater than the EU1 lineage on the inner bark of Japanese larch, European larch and English oak trees but equally pathogenic on rhododendron leaves.
3. The EU2 lineage of *P. ramorum* produces lower inoculum loads than the EU1 on larch.

Chapter seven – General discussion

7.1 Overview

Sudden larch death has had a serious impact on the UK larch industry since its discovery in the West Country in 2009. At that time larch forests covered 134,000 hectares of Great Britain, with Japanese and hybrid larch making up 4.5% of total woodland cover and European larch 0.9% (Forestry Commission, 2009). Since then over 1,400 forest sites have been issued with statutory plant health notices (SPHN), an estimated 10,000 hectares of larch forest with SPHN have been felled and an additional 6,500 hectares of larch is likely to be infected but not yet confirmed (Forestry Commission, unpublished data). The overall economic cost of the disease to the industry is currently unknown, but landowners have suffered significant losses through the destruction of immature crops, implementation of biosecurity measures and decreased timber values as the market has been flooded with surplus supplies of felled larch. Since 2010 an estimated volume of 1.6 million m³ over bark of larch round wood has become available and has had to find a market (Forestry Commission, unpublished data).

The severe *P. ramorum* epidemic in Scotland and Wales has left management teams struggling to meet regulated clearance targets due to the thousands of trees that require felling. In Wales and Scotland respectively, 5,500 hectares and 4,000-6,000 hectares of infected larch required felling by the end of 2013. Such a large operation was unfeasible so an alternative strategy was sought. To this end, both countries have adopted a zoning approach. In Scotland the Plant Health (Forestry) (*Phytophthora ramorum* Management Zone) Order 2014 became law on 5th June 2014. This enables free movement of infected material within a management zone (the most severely affected region of south west Scotland) to licensed facilities. In Wales, two zones; the Core Disease Zone (CDZ) where infection is high and the Disease Limiting Zone (DLZ) where infection is limited or not present, have been established to manage the disease and minimise further spread. The aim is to focus all efforts on new infections in the DLZ where SPHN continue to require the immediate felling of infected trees. Eradication is no longer considered an option in the CDZ, instead efforts feed into wider land management (Welsh Government, 2014). Both countries have accepted losses in the management zones and now focus resources on slowing the spread beyond the core zones in lightly affected forests by providing flexibility in the timing of felling in the severely affected regions and so reducing resource requirements.

7.2 Understanding Ramorum Disease on Larch

The aim of this study was to enhance understanding of ‘ramorum’ disease on larch in the UK, in order to facilitate effective management of the pathogen in forest plantations. To achieve this, the study focused on assessing the epidemiologically important factors of inoculum production, pathogenicity, persistence, pathogen adaptability and host susceptibility.

The findings demonstrate that *P. ramorum* has the potential for infectivity and polycyclic sporulation on larch foliage throughout the growing season, resulting in the potential for high levels of inoculum to build up, leading to heightened epidemics on larch, collateral damage to other susceptible species and spread into new regions. These factors, along with the aggressive ability of *P. ramorum* to colonise larch bark in both the growing season and winter account for the rapid mortality of larch and spread of the disease in the field despite management efforts, over the last four years [Figure 7.1] (King, Harris & Webber, 2014; Webber, 2014).

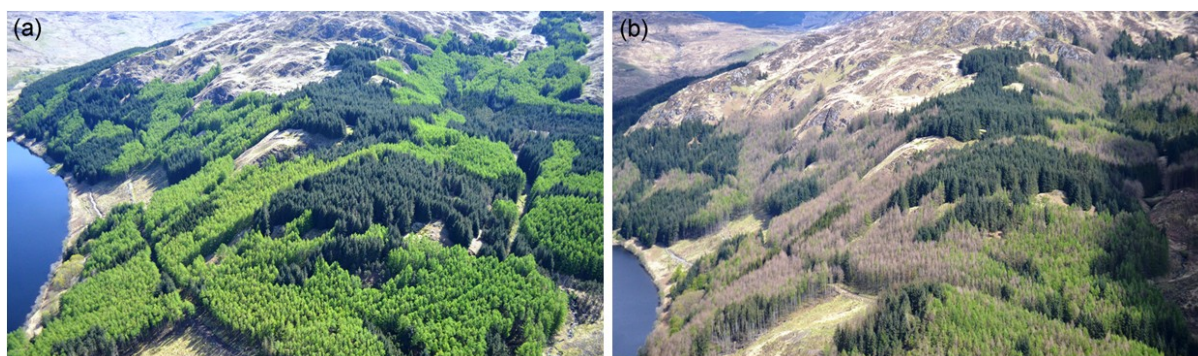


Figure 7.1 – Rapid disease development on larch in south west Scotland; a) apparently healthy trees in May 2012 and b) extensive tree dieback in May 2013 (King, Harris & Webber, 2014).

7.2.1 Influence of environmental factors on sudden larch disease

Plant disease epidemics result from cumulative effects and the interaction between host, pathogen and environment [Figure 7.2]. The nature of the host is determined by its susceptibility, distribution and the presence of new or associated hosts, while the pathogen is defined by its pathogenic potential, inoculum production, viability, ability to disperse, adaptability and survival potential. Environmental factors such as temperature, rainfall, humidity, wind, light and shade also influence the expression of host and pathogen traits (Colhoun, 1973). The clustering of *P. ramorum* outbreaks along the western edge of Britain emphasise the influence of climate on the development of the epidemic on larch and other

susceptible hosts (Forestry Commission, 2014c). Such complexity within a system makes the study of epidemiology challenging and at times can lead to ambiguous results. It is a reminder that host, pathogen and environment are all dynamic entities and require a combination of interacting conditions to come together to cause disease epidemics. Inevitably, experiments are carried out in controlled environments in the lab and can only be undertaken a limited number of times so offer only a 'snapshot' of pathogen behaviour at a particular time.

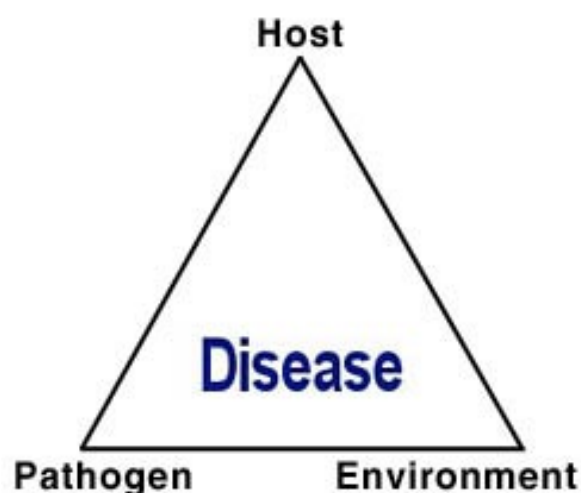


Figure 7.2 – The plant disease triangle incorporating the three necessary causal factors of disease; the interaction of a susceptible host, a virulent pathogen, and an environment favourable for disease development (Francl, 2001).

Seasonal effects on sporulation, susceptibility and pathogenicity were repeatedly observed throughout the current studies when assessing the interaction between larch and *P. ramorum*. Susceptibility of larch bark varied significantly at different times of year, with all three larch species proving more susceptible in spring compared to late summer, although the species differed slightly. Seasonal differences in lesion development in the phloem tissue of bark have been reported for a number of *Phytophthora* species and tree hosts (Gates & Millikan, 1972; Matheron & Mircetich, 1985; Matheron & Matejka, 1989; Robin, Dupuis & Desprez-Loustau, 1994) and the phenomenon linked to relative water content, growth rhythm and active shoot growth. Other research suggests that colonisation is also linked to carbohydrate content in host tissues (Grainger, 1962). In this study *P. ramorum* was most aggressive as a coloniser of larch bark in the spring, following bud burst and active shoot growth, when carbohydrate levels would have been high within the cortical tissues. Similarly, *P. cinnamomi* shows greatest growth in the bark of northern red oak (*Quercus rubra*) in the spring, decreasing towards leaf senescence and growth cessation (Robin, Dupuis &

Desprez-Loustau, 1994). As well as seasonal fluctuation in carbohydrate content, fluctuations in phenolic compound concentrations also occur and influence the colonising ability of pathogens. The concentration of tannins, which inhibit the growth of *P. ramorum* (Strong *et al.*, 2013), for example, increase in autumn within larch bark (Wang, Zu & Li, 2007). Thus, reduced pathogenicity in late summer compared to spring may be caused by accumulating levels of tannins within the bark tissues which limit the spread of the pathogen. Foliage susceptibility to *P. ramorum* infection also varied with time of year. However, the pattern in foliage susceptibility and symptom development contrasted with bark susceptibility. Typically foliar susceptibility increased as the year progressed from spring to summer and into autumn. These differences in susceptibility of different parts of plant hosts at different times of year again draw attention to the complexity of plant disease and highlight the importance of repeated studies throughout the year as tissues age and undergo physiological change.

Epidemiological factors of host and pathogen can also vary not just because of season and climatic conditions but due to host ontogeny. Different experiments assessing sporulation of *P. ramorum* on larch needles were carried out in October for three successive years. Sporulation levels, however, fluctuated considerably from year to year and even from needle to needle. For example the average density of sporangia produced on Japanese larch needles by EU1 isolates of *P. ramorum* was around 200 sporangia per cm² in October 2010 but over 5,500 sporangia per cm² of needle in October 2011. In 2012, the sheer abundance of sporangia on individual needles prevented accurate counts whilst in October 2013 the average was *ca.* 2000 sporangia per cm². Differences in sporulation levels between October 2010 and 2011 could be due to host source and genotype. Foliage was taken from mature trees in 2010 and saplings in 2011, which could affect host susceptibility and pathogen sporulation (Denman *et al.*, 2005; De Dobbelaere *et al.*, 2010). However, studies carried out in October 2010, 2012 and 2013 used foliage taken from the same three mature Japanese larch trees. The considerable difference in levels of sporulation on larch foliage from the same trees, tested under similar controlled environments suggests other factors must affect sporulation potential when environment, host and pathogen genotype remain constant. In this instance, changes in tree physiology conditioned by year to year changes in weather may have affected the quality of foliage (carbohydrate, water and sterol content) and host predisposition; this appears to have a major impact on sporulation potential. Such factors could even influence the amplitude of an epidemic from year to year but are often overlooked.

The effect of changes in environmental conditions on host susceptibility and pathogen infectivity, sporulation and pathogenicity are also likely to be increasingly important as climate change occurs. Predicted changes in humidity, temperature, rainfall and rain events (Met Office, 2011) mean that relatively innocuous *Phytophthora* species may become more prevalent and damaging as higher levels of humidity, temperature and rain events increase sporulation levels, pathogenicity and the potential for dispersal. Trees may also become more susceptible in the UK as projected increases in heavy rainfall increase the risk of flooding, particularly in Scotland, western Wales and the north-west of England (Met Office, 2011). This could also lead to higher plant water content in plant tissues which has been associated with increased susceptibility in some trees to *Phytophthora* infections (Tippett, Crombie & Hill, 1987; Brasier & Kirk, 2001). In addition, increased predicted incidences of drought following flooding can lead to higher susceptibility of trees to root attacking *Phytophthoras* (Marcais, Dupuis & Desprez-Loustau, 1993).

In general, hosts that support sporulation by *P. ramorum* but also have susceptible bark are unusual. Most sporulating hosts tend to be more tolerant of infection (e.g. rhododendron and Californian bay laurel). However this study has shown that all three species of larch grown in the UK are not only able to support prolific sporulation but are vulnerable to bark infection, resulting in the extensive dieback and mortality seen in the field, especially in Japanese larch. In North America *P. ramorum* causes relatively minor injuries to California bay laurel, its major sporulating host, so this species maintains pathogen populations enabling disease to persist in forests despite continued management efforts (Dileo, Bostock & Rizzo, 2009). The abundance of California bay laurel is even predicted to increase in natural forests in North America as other forest plants and trees succumb to infection (Cobb, Meentemeyer & Rizzo, 2010). The *P. ramorum* epidemic in the UK has, on the other hand, the potential to cause the elimination of larch. For this to take place all major larch plantations and forests would have to be affected, not too farfetched a scenario considering the current rate of spore spread and distribution of rhododendron, another sporulating host of *P. ramorum* which could provide a bridge between forested areas (Ireland, Hardy & Kriticos, 2013).

7.2.2 Genetic variation

Phytophthora ramorum has an extremely broad host range and hosts are typically categorised as sporulating hosts (foliar hosts which support pathogen sporulation) or dead-end hosts (hosts that are susceptible to colonisation but not able to support sporulation). As a pathogen *P. ramorum* exists as four near-clonal lineages and although it is a heterothallic species there is little indication that sexual reproduction is occurring in populations

(Vercauteren *et al.*, 2010; Vercauteren *et al.*, 2011). In part this is because of low functionality of the breeding system (Brasier, Kirk & Webber, 2007; Boutet *et al.*, 2010) but also because of the near complete separation of A1 and A2 mating types (Werres & Kaminski, 2005; Vercauteren *et al.*, 2010).

At present, apart from larch, no other conifer species or plantation trees has proven to be a sporulating host or is affected by the disease to the same extent; usually they will succumb only when exposed to the exceptionally high inoculum levels generated by nearby infected larch (Webber, Turner & Jennings, 2010). Nevertheless, disease affecting millions of larch trees has allowed the pathogen a huge turnover in biomass, the numerous generations increasing the chance of mutations occurring within the population. Not surprisingly therefore, there is evidence of some genetic change and the emergence of dominant genotypes has been detected using microsatellite analysis (Fera, 2012a). However, there is no indication so far to suggest that adaptation to larch is occurring (Webber *et al.*, 2014).

However, whilst *P. ramorum* has been found to cause disease on more than 150 plant species, results from this study suggest that pathogenicity can vary between isolates and isolates obtained from epidemiologically important hosts tend to be more pathogenic than those isolates from dead end hosts. All isolates tested in this current study came from epidemiologically important sporulating hosts with the exception of PLY72 from western hemlock. As such, it is not unexpected to find limited variation in pathogenicity, but significant differences were detected between some isolates (BOC07, P2470 and BRC01) and are of interest. Isolate BOC07 originated from European larch and was the most pathogenic, whilst isolates P2470 and BRC01 originated from Japanese larch and were least pathogenic. Recent work has led to the hypothesis that variation in pathogenicity on dead-end hosts in genetically identical isolates results from physiological stress, which disrupts epigenetic silencing of transposable elements and causes changes in gene expression (Kasuga *et al.*, 2012). Therefore, some of the differences observed between *P. ramorum* isolates may reflect a form of 'programming' depending on the history of the individual isolate and the type of host infected. In contrast, differences between lineages in their ability to attack larch bark tissue showed much more disparity, although the striking difference between the bark colonising ability of EU1 and EU2 in larch did not extend to other tree species, with the exception of English oak (*Quercus robur*). Both lineages of *P. ramorum* cause similar amounts of damage to the bark of beech (*Fagus sylvatica*), birch (*Betula pendula*), sessile oak (*Q. petraea*), Douglas fir (*Pseudotsuga menziesii*), noble fir (*Abies procera*), Sitka spruce (*Picea sitchensis*), Norway spruce (*P. abies*) and Scots pine (*Pinus*

sylvestris) when inoculated into bark and all are much less susceptible to colonisation than larch, with the exception of beech (Webber *et al.*, 2014).

7.3 Host Pathogen Interaction and Detection

Accurate diagnosis of ramorum disease has proven difficult due to the limited isolation success from larch bark, which is often less than 25% (Webber, Turner & Jennings, 2010). This has meant that diagnosis often relies on visual symptoms on trees, field tests applied to bark and foliage using lateral flow devices, finding infection on nearby *Rhododendron* in the forest or detecting *P. ramorum* in larch litter from the forest floor. Isolation success rates from larch foliage and bark in this study averaged 17% and 10% respectively, compared to 91% from rhododendron leaves and 92% from beech bark. Typically, symptoms of bark infection include lesions with pink-maroon margins [Figure 7.3], and the pathogen is rarely isolated from this region (Webber, Turner & Jennings, 2010).

Even in inoculated larch logs, this study revealed that distinct zones develop within a lesion, and isolation success varies between the zones. Some of the changes in infected phloem tissues are thought to come about as a result of host response to invasion. In particular, high levels of the resin acids, dehydroabietic acid and methyl abietate have been detected in the pink-maroon lesion margins along with increased levels of monoterpenes, α -pinene and 3-carene in both pink-maroon and brown tissue deeper within the lesion (Kalantarzadeh, 2013). Such resins have been found to inhibit the growth of fungi (and insects) (Phillips & Croteau, 1999) and are thought to be a host defence response inhibiting the growth of *P. ramorum* (Kalantarzadeh, 2013). These resin acids could also leach into the media preventing the pathogen growing into the agar when infected larch tissue is plated out.



Figure 7.3 - Typical pink-maroon symptoms of bark *P. ramorum* infection on larch in the field (Picture provided by Forestry Commission).

During the study it was also apparent that isolation rates varied according to species, tissue type (needles/ bark) and time of year. Isolation success from larch foliage and bark was at its lowest in spring (1% and 7.6% respectively) followed by summer (7% and 14% respectively) but highest in autumn (42% from foliage, no bark isolation studies in autumn). However, this overall trend also hid differences between larch species. Isolate success was greater from Japanese larch in spring (11% success) than summer (1% success), whereas with European larch isolation was 5% and 27% in spring and summer respectively. Concentrations of α -pinene vary over the year in larch tissues, decreasing throughout the growing season and in parallel with nutrient availability (Powell & Raffa, 1999). The presence of these defensive compounds in the bark tissues is likely to influence isolation rates. Lower rates of reisolation of EU2 isolates compared to EU1, from both Japanese and European larch bark could be due to the increased pathogenicity of the EU2 lineage which might induce a greater host defence response than the EU1. This response could result in high concentrations of dehydroabietic acid, methyl abietate and the monoterpenes α -pinene and 3-carene all of which can inhibit pathogen growth on agar (Kalantarzadeh, 2013). Further work would, however, be needed to test this hypothesis.

7.4 Lessons for Disease Management

High levels of sporulation and bark pathogenicity of *P. ramorum* on European and hybrid larch provides clear evidence that we cannot rely on these species to replace Japanese larch in forestry. Such high levels of sporulation of *P. ramorum* highlight the vulnerability of larch and have wider implications for ramorum disease management and containment in the UK. Before this finding rhododendron, which proved to have a significantly lower sporulation potential than larch, was considered the most important sporulating host. Prior to the host 'jump' of *P. ramorum* disease to larch, the disease impact in the UK was much less significant compared with North America, particularly in relation to trees. However, with the current spread of the disease in the UK and the findings of this study, it can be predicted that continuing epidemics in the UK are likely to be severe because all three larch species have a high sporulation potential when infected by *P. ramorum*. Larch is grown in dense monocultures (stocked at 2,500-3,000 stems per hectare) covering areas of many hectares, which will facilitate spread. The mild and often humid conditions of the south west British Isles are also ideal for sporulation, so removal of infected larch is essential to restrict disease spread and prevent inoculum build up.

Disease spread in North America has been linked to contaminated soils and understory vegetation releasing inoculum upwards. Inoculum from these reservoirs goes on to infect the boles (trunks) of native oaks, causing sudden oak death. In the UK the disease dynamic is different; infection takes place high in the larch canopy, infecting foliage and girdling stems and branches without the need for understory sporulating hosts (Webber, Turner & Jennings, 2010). The height of the trees and heavy inoculum loads are likely to increase the spread of the disease with wind-driven rains picking up spores high in the canopy and dispersing them further afield than inoculum produced on understory shrubs that are sheltered under the forest canopy (Gomez, 2007). In Oregon, disease dynamics share some similarities with the UK; sporulation primarily occurs high in the canopy on tanoak, and spreads downwards on to the trees stems and understory hosts (Hansen *et al.*, 2008; Peterson, 2013). Studies in North America have also highlighted the importance of factors such as forest composition, inoculum production and climate (Davidson *et al.*, 2005; Davidson, Patterson & Rizzo, 2008; Hansen *et al.*, 2008; Davidson *et al.*, 2011; Tooley, Browning & Leighity, 2013) on the disease gradient of *P. ramorum*. Knowledge of these factors is essential in understanding the disease epidemiology of this pathogen in the UK. Whilst the findings of this study add to the knowledge of inoculum production potential of major hosts, further understanding of disease gradients on larch is required for improved management.

7.4.1 Interpretation

The findings of this study also draw attention to the fundamental difficulty in studying factors that influence disease epidemics. As already mentioned, predicting disease outbreaks relies on understanding how pathogens interact with their environment and hosts. These experiments were carried out in controlled environments in the lab due to quarantine restrictions. Whilst findings of high levels of *P. ramorum* sporulation on larch foliage appear consistent with infection levels in the field, and the acute pathogenicity of *P. ramorum* on larch bark in the lab corresponds with rapid tree death via branch and stem girdling in the field, some findings were inconsistent with those observed in the natural environment. For example disease incidence and sporulation on larch is at its peak in forests in autumn (Webber, Turner & Jennings, 2010), however, sporulation levels in this study were highest on all foliage in summer (August) and lowest in autumn (October). These contradictory observations are probably due the influence of environment on pathogen. Whilst larch foliage consistently supports high levels of sporulation under controlled conditions in the lab, in the field fluctuations in temperature, humidity and UV radiation will affect both the pathogen's ability to sporulate and host defences. The most critical factor in the development

of disease epidemics caused by oomycetes is moisture (Agrios, 2005) affecting rates of infection, sporulation (Garbelotto *et al.*, 2003), dispersal (Hansen *et al.*, 2008) and survival (Browning *et al.*, 2008). High levels of humidity allow these processes to take place at faster rates. In the UK humidity is typically higher in autumn compared to summer (Jenkins, Perry & Prior, 2008). Consequently sporulation is likely to be lower in the field in summer months than observed in the lab due to reduced humidity. Factors important for pathogen spread such as the number of rain events and wind speed (Davidson *et al.*, 2005; Hansen *et al.*, 2008) are also more frequent in the autumn compared to summer (Jenkins, Perry & Prior, 2008). Consequently disease spread is also likely to be reduced in summer due to fewer rain events and reduced wind speed. These differences in field and lab studies emphasise that caution is required when trying to transfer results directly from laboratory-based studies into the field and other influential factors should be considered. The study's results, however, are still valuable as they give an indication of the highest levels of sporulation of *P. ramorum* under optimal conditions and so predict the worst case scenario. This information, when used alongside knowledge of environmental influences on *P. ramorum* in North America and behaviour of other *Phytophthora* species, can provide an indication of the expected behaviour of *P. ramorum* in the UK.

The variation in isolation success rates from the different host species, tissues and at different times of year make disease diagnosis uncertain if isolation alone is used, as it relies on expression of symptoms on particular host tissues at a certain time of year. Consequently the sole use of cultural isolation of the pathogen for disease diagnosis on larch would allow the pathogen to go undetected approximately 90% of the time. Delayed diagnoses of infected trees can allow high levels of infection and inoculum to build up, increasing potential for further spread and threatening vulnerable habitats. *Phytophthora ramorum* DNA however, was readily extracted from all infected symptomatic host foliage and bark tested, and in some cases even from asymptomatic larch foliage. The high detection rates of *P. ramorum* DNA from infected samples, when pathogen reisolation failed provides an alternative and more efficient method for diagnosis, which does not rely on characteristic visual symptoms in larch or nearby infected non-larch hosts as indicators. High through-put molecular detection, such as real time PCR methods used in this study, provide a quick and efficient method for diagnosis of suspect *P. ramorum* infections and therefore aid plant health officers in the identification of infected trees and forests that require management intervention.

Understanding the aftermath of disease episodes on larch is also critical to the future use of the land and replanting choices. Ideally the study of persistence of *P. ramorum* in the litter of

a felled larch stand would not have been limited to three years. Nevertheless it provides valuable lessons for management of *P. ramorum*. Management strategies stress the need to reduce inoculum levels to epidemiologically insignificant quantities by removing sporulating hosts. The study site used was under a SPHN and should have been complying with regulation by removing regenerating 'sporulating hosts'. This however was not the case. Rhododendron regeneration was abundant on the site and *P. ramorum* was isolated from asymptomatic leaves and in the soil and litter under infected rhododendron for two consecutive years after initial site clearance. It is likely that the land manager was unaware of the continued presence of the pathogen due to the limited symptoms. This finding therefore highlights the need for increased surveillance and monitoring of cleared sites and further education of landowners regarding asymptomatic sporulation and regulation compliance if the disease is to be contained successfully.

7.4.2 Lessons

1. European and hybrid larch should not be considered as an alternative forestry species to Japanese larch as in laboratory testing the bark of all three species is equally susceptible to *P. ramorum* colonization and their foliage supports similarly high levels of sporulation.
2. *Phytophthora ramorum* sporulation on the foliage of larch exceeds that of bay laurel (which is the most significant sporulating host in California) and rhododendron (previously considered the major sporulating host in the UK). Consequently, management efforts should be focused on removing infected trees quickly in order to reduce inoculum levels to try and limit disease spread.
3. Due to persistence of the pathogen in the litter of infested sites, replanting of cleared sites with larch or other sporulating plant species within two years of site clearance should be prevented. Additional surveillance of cleared sites for regenerating infected/infectable hosts such as rhododendron should also be carried out to prevent a resurgence of the pathogen on-site.
4. A lack of symptoms cannot be used as an indicator of disease absence as symptom development is influenced by seasonal conditions and sporulation can occur asymptotically. The disease front is likely to be more advanced than the detectable disease indicated by the presence of symptomatic and dying trees.

Therefore biosecurity precautions should be taken in all larch forests in the vicinity of infection.

5. Success of pathogen isolation from symptomatic material is low, increasing the possibility for the pathogen to go undetected. Delays in diagnoses could lead to increased levels of infection and a build-up of inoculum. Therefore, high throughput molecular methods such as real time PCR which have a high success rate in detecting the pathogen such be used.

7.5 Concluding Remarks

The aggressive nature of *P. ramorum* infection on all three species of larch (high levels of sporulation and pathogenicity) and the difficulty in containing the disease suggest that the future of larch forestry in the UK is limited. Disease management strategies to contain the pathogen have struggled to cope with the sheer number of infected trees and as a result have been modified in Wales and south west Scotland. Consequently it would be unwise to invest in new plantings of any of the three commercially grown larch species as spread is likely to continue due to the UK's highly favourable environmental conditions (Ireland, Hardy & Kriticos, 2013) and the distribution of available sporulating hosts such as larch and rhododendron (Jones, 2013; Purse *et al.*, 2013). Inevitably this change in practice will have a number of consequences, as larch, in addition to being an important timber crop in the UK, is the only species of deciduous conifer and provides value for landscapes, recreational purposes and enhances biodiversity (Brasier & Webber, 2010). The sudden larch death epidemic may therefore also have a detrimental effect on local economies and the UK's strategic reserve of timber (Brasier & Webber, 2010). It is also clear that the EU2 lineage is significantly more pathogenic on larch bark compared with other tree hosts for unknown reasons and justifies the need to continue disease management.

7.6 Future work

Whilst this study has addressed a series of fundamental epidemiological questions on sporulation, pathogenicity, persistence and adaptively of *P. ramorum* on larch, further work is needed to gain greater understanding of the pathogen's biology and to facilitate disease management.

7.6.1 Bark infectivity

Studies exploring the infective potential of *P. ramorum* zoospores on host bark would be beneficial to add to our understanding of the disease on larch species. In the field, bark infections are initiated by sporangia and zoospores landing on the bark surface, germinating and penetrating the bark tissues. Susceptibility assays used in this study established the ability of *P. ramorum* to colonise the inner bark of larch hosts through wound inoculation and bypassed the capacity of *P. ramorum* to penetrate intact bark. The outer bark of a tree is typically the first line of defence against invading pathogens and this therefore needs to be investigated. Thick bark with few fissures may provide a host with greater disease resistance, as it acts as a barrier limiting pathogen entry into vulnerable underlying tissues such as phloem and cambium. Findings from such a study may aid in the understanding of larch bark susceptibility and explain field observations which suggest that European larch is more resilient in the field.

7.6.2 Genetic epidemiology

Despite the near clonal nature of the EU1 population of *P. ramorum* (Smith & Gilbert, 2003; Goss, Carbone & Grünwald, 2009; Grünwald *et al.*, 2009), this study along with others, has demonstrated the presence of variation in both sporulation levels and pathogenicity within the population. Due to the limited genetic variation in the EU1 population and variability in these factors expressed in genetically identical isolates, the differences are thought to be caused by epigenetic modifications regulating gene expression (Kasuga *et al.*, 2012). Studies on the population genetics of the EU1 lineage have been carried out across Europe (Ivors *et al.*, 2006; Vercauteren *et al.*, 2010) and on the nursery population in the UK (Fera, 2012a). None of these studies, however, have looked at the UK forest population of *P. ramorum*, which is unique as its main host is larch. It would therefore be interesting to establish whether the differences observed in the adaptive behaviours of isolates in this study could be attributed to genomic differences or whether they were due to differences in gene regulation brought about by their historic interactions with hosts.

Microsatellite analysis of the UK *P. ramorum* forest populations could also assess whether the pathogen's 'jump' to its new host, larch, in 2009, was due to the emergence of a new genotype (the UK has already been shown to have unique genotypes not present in continental Europe) and could help to identify pathways of spread by monitoring the appearance of rare genotypes, thus providing information for disease management. Finally, microsatellite analysis could also establish whether the pathogen is evolving on larch due to

the high turnover of inoculum on the host's needles. Studies in North America have shown increasing genotypic diversity in the NA1 lineage with significant genetic differentiation amongst populations at different sites (Prospero *et al.*, 2007). Genetic changes in the pathogen population may lead to subgroups of the pathogen becoming differentially adapted to different hosts, and could pose additional threats to forestry and plant health and merits further investigation.

7.6.3 Extended persistence

Continued assessment of *P. ramorum* in the litter and soil of felled larch sites would be beneficial and could further assist in disease management as the extent of persistence of *P. ramorum* in the litter is still not known. Such knowledge is not only important in aiding our understanding of the biology of *P. ramorum* and related pathogens, but is vital for disease containment. Replanting on infected sites after felling is not recommended for at least three years, and then not with larch or other susceptible species. Despite replanting with resistant plants there is a need to know how long the pathogen can persist in the litter as its presence threatens any regenerating susceptible plants with infection. Susceptible plants will need to be continually removed as long as the risk of infection remains. Additionally, management of restocked sites by contractors may spread the disease via vehicle tyres, boots and equipment to new uninfected sites. Knowledge of the pathogen persistence on felled sites could inform land users, stakeholders and policy makers of the length of time sites with SPHNs need to be monitored before biosecurity measures relaxed

7.6.4 EU1 and EU2 lineages

Whilst the work carried out in this study has addressed a series of fundamental questions regarding the comparative fitness characteristics of the two European lineages of *P. ramorum*, the extent of any increased threat from the EU2 lineage to forestry and plant health is still unclear. Evidence from this study suggests that the EU2 lineage may not pose an increased risk to forestry, despite its faster growth rate at a wider range of temperatures and greater pathogenicity on host bark, due to its lower level of sporulation on larch foliage in comparison to the EU1, suggesting that spread may be limited. EU2 risk, however, may also depend on factors such as sporangia size, the number of zoospores per sporangia, spore viability and infectivity. Therefore further work investigating such factors is required if a greater understanding of the differences between the two lineages is to be established, and

consequently whether the EU2 lineage needs to be assigned a more specific quarantine status to prevent its spread into Europe.

Additional work looking at the interactions between the two European lineages would also be extremely informative. Current evidence suggests that the distribution of the two European lineages of *P. ramorum* do not overlap but are likely to do so in the near future (King, Harris & Webber, 2014). Both lineages occupy the same ecological niche and therefore may not be able to coexist. It would therefore be interesting to establish which lineage has the competitive advantage and is likely to replace the other.

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Appendix

I. Tables of analysis of symptom development on non larch host

Parameters for factors are differences compared with the reference level:

Factor - Reference level

Time of year - May

Host - Bay

Isolate - BRC01

Estimates of parameters for the fitted model for symptom development on non larch hosts

Parameter	Estimate	Standard error	t (95)	t pr.	Antilog of estimate
Constant	-4.29	1.16	-3.70	<0.001	0.014
Time of year - August	-2.33	2.94	-0.79	0.430	0.098
Time of year - October	3.64	1.23	2.97	0.004	38.23
Host - sweet chestnut	3.99	1.16	3.44	<0.001	53.79
Host - rhododendron	2.40	1.18	2.02	0.046	10.98
Isolate PLY72	0.67	0.49	1.36	0.176	1.953
Time of year - August .Host – sweet chestnut	4.14	2.96	1.40	0.165	62.81
Time of year - August .Host - rhododendron	3.89	2.94	1.32	0.189	48.90
Time of year - October .Host - Chestnut	-2.19	1.27	-1.71	0.090	0.110
Time of year - October .Host - rhododendron	-0.27	1.30	-0.20	0.838	0.767
Time of year - August .Isolate - PLY72	0.74	0.76	0.98	0.330	2.100
Time of year - October .Isolate - PLY72	-2.03	0.65	-3.11	0.002	0.131

Accumulated analysis of deviance for the fitted model for symptom development on non larch hosts

Change	d.f.	Deviance	Mean deviance	Deviance ratio	Approx. F pr.
+ Time of year	2	682.83	341.41	11.8	<0.001
+ Host	2	3142.12	1571.06	54.5	<0.001
+ Isolate	1	1.67	1.67	0.06	0.81
+ Time of year .Host	4	754.63	188.66	6.54	<0.001
+ Time of year .Isolate	2	559.02	279.51	9.70	<0.001
Residual	95	2738.65	28.83		
Total	106	7878.91	74.33		

II. Tables of analysis of in host symptom development on foliage by different isolate of *P. ramorum*

Estimates of parameters for the fitted model for variation in host symptom development on foliage by different isolate of *P. ramorum*

Parameter (Isolate)	Estimate	Standard error	t (95)	t pr.	Antilog of estimate
BOC07	0.615	0.525	1.17	0.251	1.85
BRC01	0.088	0.502	0.17	0.863	1.091
P1349	1.906	0.742	2.57	0.016	6.728
P1376	-1.633	0.742	-2.2	0.036	0.1954
P1403	1.267	0.605	2.1	0.045	3.552
P2540	-2.296	0.868	-2.64	0.013	0.1007

Accumulated analysis of deviance for the fitted model for variation in host symptom development on foliage by different isolate of *P. ramorum*

Change	d.f.	Deviance	Mean deviance	Deviance ratio	Approx. F pr.
- Constant	-1	-7.27	7.27	0.19	0.664
+ Isolate	6	1337.23	222.9	5.92	<0.001
Residual	29	1092.42	37.67		
Total	34	2422.38	71.25		

III. Range in lesion size caused by isolates of *P. ramorum* in larch bark

Range in lesion size caused by isolates of *P. ramorum* in larch bark in spring 2013

Species	Isolate	Tree	Range (cm ²)	Species	Isolate	Tree	Range (cm ²)	Species	Isolate	Tree	Range (cm ²)
European	P1376	1	17.9 - 164.5	Hybrid	P1376	1	17.6 - 27.4	Japanese	P1376	1	4.1 - 4.9
European	P1376	2	35.3 - 66.9	Hybrid	P1376	2	4.9 - 5.0	Japanese	P1376	2	2.7 - 5.5
European	P2470	1	15.8 - 82.2	Hybrid	P2470	1	8.7 - 21.6	Japanese	P2470	1	2.8 - 3.9
European	P2470	2	35.7 - 58.6	Hybrid	P2470	2	2.4 - 5.4	Japanese	P2470	2	4.5 - 6.4
European	P2738	1	13.8 - 72.4	Hybrid	P2738	1	15.4 - 28.1	Japanese	P2738	1	3.5 - 11.2
European	P2738	2	48.3 - 52.9	Hybrid	P2738	2	4.8 - 6.0	Japanese	P2738	2	4.2 - 10.1
European	BRC01	1	11.1 - 123.7	Hybrid	BRC01	1	15.8 - 23.0	Japanese	BRC01	1	3.7 - 5.6
European	BRC01	2	31.8 - 62.7	Hybrid	BRC01	2	6.2 - 6.6	Japanese	BRC01	2	7.2 - 42.1
European	BOC07	1	14.5 - 96.7	Hybrid	BOC07	1	12.2 - 39.2	Japanese	BOC07	1	3.1 - 5.6
European	BOC07	2	44.2 - 62.0	Hybrid	BOC07	2	5.4 - 5.8	Japanese	BOC07	2	2.9 - 8.5
European	PLY72	1	16.7 - 105.7	Hybrid	PLY72	1	14.3 - 23.9	Japanese	PLY72	1	4.8 - 6.8
European	PLY72	2	56.7 - 62.6	Hybrid	PLY72	2	4.1 - 6.6	Japanese	PLY72	2	4.1 - 17.5
European	P1403	1	10.0 - 86.9	Hybrid	P1403	1	15.2 - 30.3	Japanese	P1403	1	4.5 - 9.8
European	P1403	2	36.9 - 49.0	Hybrid	P1403	2	4.3 - 62.8	Japanese	P1403	2	14.5 - 15.2

Appendix

Range in lesion size caused by isolates of *P. ramorum* in larch bark in summer 2012

Species	Isolate	Tree	Range (cm ²)	Species	Isolate	Tree	Range (cm ²)	Species	Isolate	Tree	Range (cm ²)
European	P1376	1	86.6 - 163.3	Hybrid	P1376	1	102.3 - 147.8	Japanese	P1376	1	39.2 - 69.0
European	P1376	2	88.8 - 209.4	Hybrid	P1376	2	47.6 - 50.4	Japanese	P1376	2	199.0 - 216.1
European	P2470	1	43.3 - 188.4	Hybrid	P2470	1	95.7 - 138.8	Japanese	P2470	1	27.3 - 48.8
European	P2470	2	83.2 - 189.2	Hybrid	P2470	2	40.4 - 55.6	Japanese	P2470	2	134.8 - 207.5
European	P2738	1	64.5 - 188.2	Hybrid	P2738	1	81.9 - 176.4	Japanese	P2738	1	36.1 - 79.3
European	P2738	2	104.8 - 197.4	Hybrid	P2738	2	42.9 - 43.8	Japanese	P2738	2	152.8 - 220.4
European	BRC01	1	64.2 - 124.3	Hybrid	BRC01	1	53.8 - 119.0	Japanese	BRC01	1	1.4 - 29.1
European	BRC01	2	60.4 - 158.7	Hybrid	BRC01	2	30.3 - 36.7	Japanese	BRC01	2	142.0 - 278.7
European	BOC07	1	85.1 - 171.7	Hybrid	BOC07	1	77.7 - 189.5	Japanese	BOC07	1	39.5 - 69.3
European	BOC07	2	76.8 - 231.5	Hybrid	BOC07	2	42.8 - 99.5	Japanese	BOC07	2	117.0 - 227.1
European	PLY72	1	72.9 - 147.5	Hybrid	PLY72	1	79.8 - 190.2	Japanese	PLY72	1	38.7 - 51.1
European	PLY72	2	80.1 - 230.5	Hybrid	PLY72	2	55.5 - 57.7	Japanese	PLY72	2	167.9 - 220.2
European	P1403	1	68.1 - 113.3	Hybrid	P1403	1	102.3 - 206.9	Japanese	P1403	1	30.1 - 67.5
European	P1403	2	56.1 - 203.2	Hybrid	P1403	2	27.3 - 38.0	Japanese	P1403	2	234.4 - 236.0

