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Exploring variation in susceptibility to *Phytophthora ramorum* in Japanese larch (*Larix kaempferi*)

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Abstract

Phytophthora ramorum is an invasive pathogen responsible for extensive mortality in larches in the United Kingdom. There is great interest from the forestry industry in the possibility of selection for resistance to *P. ramorum* in Japanese larch in order to retain it as a commercially viable species. This study is the first to investigate variation in resistance to *P. ramorum* among planted populations of Japanese larch in the UK. Our study uses inoculation of excised material from putatively resistant survivor trees and known susceptible trees. We found variation in susceptibility to *P. ramorum* within Japanese larch stands planted in the Galloway forest of Scotland with some trees showing significantly shorter lesion development than others, from a mean lesion length of 34.7 mm in the least susceptible clone to 135 mm in the most susceptible. Although clones from the putatively resistant and known susceptible groups were not significantly different ($p = .055$), we propose that survivor trees include a higher proportion of resistant or low susceptibility trees and would be a useful starting point for further work investigating natural resistance in larch.

KEYWORDS
host susceptibility, *Larix kaempferi*, *Phytophthora ramorum*, resistance

1 | INTRODUCTION

Phytophthora ramorum Werres, De Cock & Man in 't Veld is responsible for devastating disease outbreaks in a range of species, most prominently sudden oak death in the USA (Rizzo et al., 2002). Sudden larch death was first reported in the UK in 2009 when *P. ramorum* was found infecting and killing large numbers of Japanese larch (*Larix kaempferi* [Lamb]Carr.) in south-west England (Brasier & Webber, 2010). By 2019 at least 20,700 hectares of larch across the UK had been killed or felled in efforts to prevent further spread (R. Sketchley, 2021, personal communication). *P. ramorum* infections are especially severe on larch as the infected needles become sites of intense sporulation (Harris & Webber, 2016). High inoculum loads originating from larch needles appear to cause rapid infection of bark on the branches, with girdling lesions on the main stem resulting in subsequent mortality (Brasier & Webber, 2010).

In spring 2013, a major outbreak of *P. ramorum*, caused by the highly aggressive EU2 lineage (King et al., 2015) occurred in the Galloway forest of south-west Scotland, with an estimated 5000–6000 hectares of larch affected (Forestry Commission Scotland, 2017). There was widespread mortality in larch stands across the forest with many of these stands suffering >95% mortality (Figure 1a). However, some Japanese larch trees in heavily affected stands appeared healthy two years after the initial epidemic (Figure 1b). Such observations raised questions as to whether these trees had simply 'escaped' infection or whether they had a degree of natural resistance to *P. ramorum*.

FIGURE 1 Mortality in mature Japanese larch in the Galloway forest caused by *Phytophthora ramorum*. (a) whole stand mortality, (b) single surviving tree with foliage in the crown surrounded by the bare crowns of the dead trees

Natural variation in susceptibility to disease has been documented for many tree species (Telford et al., 2015). The ability of a tree to resist a pathogen depends on the existence of a genetic variation that confers resistance. Even in seemingly devastating disease outbreaks there have been reports of survivor trees that remain healthy and show no, or fewer, signs of infection than those around them despite high disease pressure. The prevalence of survivor trees varies between different pathosystems; from 1 in 100,000 American elms (*Ulmus americana*), which appear to be resistant to Dutch elm disease (*Ophiostoma ulmi* and *O. novo-ulmi*) (Schlarbaum et al., 1998) and the occasional American chestnut (*Castanea dentata*) surviving chestnut blight (*Cryphonectria parasitica*) infection (Hebard, 2005) to the 3% ash trees across a range of provenances that remained healthy in a common garden experiment with high levels of exposure to ash dieback (*Hymenoscyphus fraxineus*) (Plumb et al., 2020). Since it is possible that survivor trees miss infection by chance, or due to their position within the stand, it is necessary to test this putative resistance through inoculation trials. If genetic resistance is identified, it may form the basis of a resistance breeding programme (Telford et al., 2015). Inoculations of whole plant seedlings from tanoaks (*Notholithocarpus densiflorus*) surviving in *P. ramorum* infected sites developed shorter lesions than seedlings from trees in non-infected sites, suggesting that survivor trees could produce progeny for use in resistance breeding (Søndreli et al., 2019).

Hansen et al. (1989) developed inoculation methods for testing the putative resistance of Port-Orford-cedar (*Chamaecyparis lawsoniana*) to *Phytophthora lateralis* using mycelium to inoculate wounded seedlings and excised material from survivor trees. Their work showed that excised material was suitable for preliminary screening of survivor trees to submit to more intensive testing. Subsequent work has built on almost 40 years of selection and testing of survivor trees and their progeny and found both qualitative major gene and quantitative disease resistance that is sufficiently strong to now be used for restoration and reforestation (Sniezko et al., 2020). Similar testing of excised material in Juniper (*Juniperus communis*) inoculated with *Phytophthora austrocedri* indicated that survivor trees had higher levels of resistance than known susceptible controls (Green et al., 2020) and results were validated in whole plant testing showing that excised material is a useful proxy for whole plant when carrying out early screening studies (Green et al., 2020).

Variation in susceptibility to *P. ramorum* found in Californian coast live oak (*Quercus agrifolia*) populations (Nagle et al., 2011; Ockels et al., 2007) gives hope for the survival of coast live oaks in the face of *P. ramorum* spread. In contrast, resistance to *P. ramorum* in larch remains to be identified, but the concept is of great interest to the UK forest industry in order to retain larch as a viable timber species.

The usual management strategy to attempt to control the spread of invasive forest pathogens is preventative felling around infected sites, as well as felling infected trees. However, this is often counterproductive in the search for disease resistance as it removes possible survivor trees before they can be identified (Budde et al., 2016). *P. ramorum* is classed as a notifiable disease in the UK and as such is subject to statutory plant health notices. These require the mandatory felling of infected trees, along with a 250 m buffer zone of healthy trees around them (Forest Research, 2020). The creation of the Galloway forest Management Zone in south-west Scotland following the 2013 outbreak allowed the phased, rather than immediate, removal of larch inside the zone and this delay in felling allowed the identification of survivor trees and the collection of scion material for inoculation trials.

The aim of this study was to compare the response to inoculation with *P. ramorum* in grafted scions from putatively resistant and known susceptible mature Japanese larch trees located in stands in SW Scotland. The specific hypothesis tested was that grafts from putatively resistant trees will show greater resistance than grafts from known susceptible trees, allowing insight into whether natural

levels of resistance to the pathogen exist that might subsequently be investigated in a breeding programme. The inoculation methodology used in this study involved a sporangial/zoospore suspension in order to better mimic natural infection pathways. This work thus represents a novel first step in investigating natural resistance in larch to *P. ramorum*.

2 | MATERIALS AND METHODS

In December 2015 and February 2016, scions (healthy young shoots) were collected from ten mature Japanese larch with no symptoms of *P. ramorum* infection within three heavily infected stands located within 20 km of each other in the Galloway forest of SW Scotland. This 'putatively resistant' group of trees appeared healthy and had no evidence of stem cankers or epicormic recovery growth, were dominant with generally good growth form, and not located at stand edges. In January 2017, healthy scions were also collected from the lower crown of fourteen 'known susceptible' Japanese larch with top dieback due to cankers in the upper main stem and with healthy branches in the middle and lower crown, in a moderately infected stand in the area. Scion material was bagged and stored at 4°C for 1–2 months until grafting. Grafts comprised the ten most vigorous looking scions per parent tree joined on to 1+1 European larch rootstocks (Cheviot Trees, UK) in 2 L pots using the side veneer technique. Each individual ramet was labelled and the grafts were maintained in a heated polytunnel to ensure frost free conditions with natural daylight, before being moved outside after one year. To provide a duplicate set of grafts for inoculation, scions from the original grafts were grafted onto new European larch rootstocks in winter 2017/18, and maintained in the polytunnel as previously described. In October 2019 ten ramets from each of the putatively resistant and known susceptible clones were selected for inoculation.

For inoculation, 1–2 shoots of 30 cm length were excised from the leading shoot of the main stem or large branch of each ramet ensuring that selected shoots were well-developed with a basal diameter of ~1 cm. The cut shoots were labelled and each end was wrapped with cotton wool, which had been soaked in sterile distilled water (SDW). The cotton wool was secured in place with Parafilm and tin foil.

The experiment comprised 20 Japanese larch clones (ten putatively resistant and ten known susceptible) and two inoculum types (*P. ramorum* and control) with 6–8 replicate shoots per clone for the inoculation treatment and three replicate shoots per clone for the control treatment. Sporangial inoculum was prepared from a *P. ramorum* EU2 lineage isolate recently collected from Japanese larch in the area according to Denman et al. (2005) except that in order to retain sporangia the inoculum was not filtered. Colonies were grown on carrot agar and incubated in continuous light for 21 days at room temperature. Production of sporangia was confirmed by examination under a dissecting microscope. Each plate was then flooded with 3 ml of sterile water and sporangia dislodged by rubbing the surface of the culture with a sterile glass rod. The resulting suspension from 15 plates was pooled into a beaker and refrigerated at 7°C for 1 h. After refrigeration the beaker was transferred to room temperature (~20°C) for 75 min to induce zoospore release. The concentration of sporangia was determined using a haemocytometer and the suspension adjusted to achieve a final concentration of $2–5 \times 10^5$ total sporangia ml^{-1}. Released zoospores were also present in the inoculum. Inoculations were conducted within 90 min of the inoculum being produced.

Prior to inoculation shoots were pre-wounded by removing a 5 mm diameter circle of bark from the midpoint of the shoot using a scalpel. A 15 µl droplet of sporangial suspension was pipetted onto the wound and left for 15 min to allow zoospore encystment. The wounded area was then loosely wrapped in tin foil to mark the inoculation point and also to prevent inoculum cross-contamination. Control inoculations were carried out as above but using SDW. The shoots were double bagged and placed in an incubator in the dark at 15°C. The shoots were allocated to bags in a randomized complete block design with one inoculated shoot from each clone per bag and the controls were blocked separately to prevent cross-contamination of the inoculum. The experiment was conducted twice; in October 2019 and in August 2020.

The excised shoots were destructively sampled and assessed for disease at 21 days post inoculation (dpi). The needles were removed from the excised shoots, the inner and outer bark slit with a scalpel and removed from the stem, and the length of any lesions, which had developed in the phloem were measured (Figure 2a,b). DNA was extracted from bark tissue at the live-dead margins of the lesion from one ramet per clone. The presence of *P. ramorum* in the lesion was confirmed through real-time PCR amplification with primers specific to *P. ramorum* according to Tomlinson et al. (2005).

Statistical analyses were conducted using R version 4.0.4 (R Core Team, 2020) in RStudio (RStudio Team, 2020). Linear mixed models were used to make models that incorporate both fixed and random effects using the lme4 package (Bates et al., 2015). The significance of the fixed effects in each mixed model was tested using a Type-II anova in the car package (Fox & Weisberg, 2019). To test for differences between treatments a mixed model was fitted with lesion length as the outcome variable with treatment and experiment as fixed effect and clone as a random effect. To test whether there was significant difference between clones across the two trials an interaction was fitted between clone and trial. A type-II anova of the regression model showed that this was not significant (Anova, $F = 1.6$, $p = .06$) suggesting that the effect of clone was consistent across the trials. Subsequently, trial was used as a fixed effect and the interaction term was excluded from the final model. To compare the resistant and susceptible groups, a linear mixed model fit by REML was fitted to the lesion length data with group and trial repeat treated as fixed effects and clone as a random effect. To compare between inoculated clones a linear model was fitted with lesion length as the outcome variable and with trial repeat and clone as fixed effects. The estimated marginal means were then calculated from the linear models using emmeans (Lenth, 2020) and pairwise comparisons of

FIGURE 2 Examples of *Phytophthora ramorum* symptom development on Japanese larch 21 dpi, red arrows indicate site of inoculation. (a) control shoot of clone Sus10 showing slight discolouration at inoculation point but no lesion development, (b) *P. ramorum*-inoculated shoot of clone Sus2 showing extensive lesion development, the black arrows indicate the edges of the lesion (c) control shoots of clones Res6 and Res7 showing healthy needle colour. (d) *P. ramorum*-inoculated shoot of Sus13, the clone that developed the longest lesions showing needle discolouration and loss. (e) *P. ramorum*-inoculated shoot of Res7 the clone that developed the shortest lesions showing lower levels of needle discolouration and loss. Shoots shown in c–d are 30 cm long

the clones were analysed using the 'cld' function in the 'multcomp' package (Hothorn et al., 2008). 'cld' creates a compact letter display of the pairwise comparisons of marginal means so that clones, which share a letter grouping are not significantly different from one another. The Tukey method was used to adjust the p-values to control for the number of statistical tests performed.

3 | RESULTS

The control excised shoots developed very small lesions of 1–4 mm in length (Figures 2a and 3) that were of a pale colour uncharacteristic of *P. ramorum*, and were likely discolouration related to wounding (Figure 2a). Lesions were observed in all inoculated shoots (Figures 2b and 3) and ranged from 2 to 240 mm in length. Real-time PCR of a subset of lesions from each clone gave positive results for *P. ramorum*. None of the control shoots gave positive results for *P. ramorum*. There was a significant difference in lesion length between the control and inoculation treatments (Anova: $\chi^2 = 557$, $p \leq 2.2e{-}16$). The effect of experimental trial was significant (Table 1) with shorter lesions developing in the second trial (mean overall lesion length 66.5 mm, SD = 37.4) compared to the first trial (mean overall lesion length 128 mm, SD = 53.7). Comparing the two clone groups (putatively resistant vs. known susceptible), the putatively resistant clones tended to have shorter lesions (Figure 3) but the lesion lengths in both

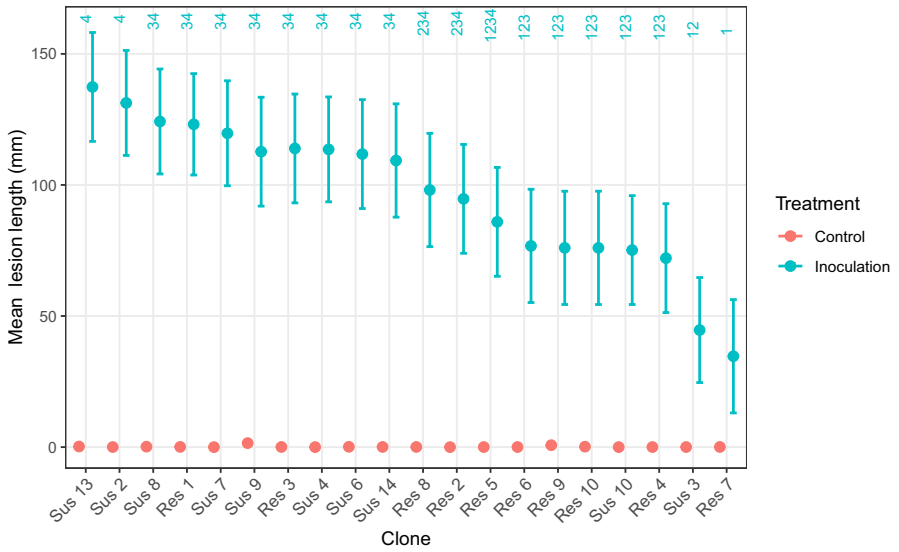

FIGURE 3 Mean lesion length for each clone inoculated with *Phytophthora ramorum* ordered by decreasing mean lesion length at 21 dpi. Clones named Sus are from the known susceptible group and Res are from the putatively resistant group. Data shown are adjusted marginal means for lesion length averaged over the levels of experiment. Error bars show the 95% unadjusted confidence intervals. Numbers denote statistical significance ($p \leq .05$ Tukey adjustment for multiple comparisons), so that clones, which share a number are not significantly different. There were no differences in mean lesion lengths among the controls and these data are not shown

TABLE 1 Type-II analysis of variance table, Wald chi-squared test for fixed effects of linear mixed model of lesion development

Fixed effect	χ^2	*p*-value
Grouping (resistant/susceptible)	3.69	.055
Trial	173.2	<.05

groups were variable and the mixed linear model showed that the main effect of group was not significant (Table 1).

Mean lesion lengths in the *P. ramorum*-inoculated clones at 21 dpi showed continuous variation from the longest, clone Sus13 at 135 mm (SD = 57.2), to the shortest, clone Res7 at 34.7 mm (SD = 22.8) (Figure 3). Pairwise comparisons showed that although there are significant differences in mean lesion length between clones at each end of the range, these overlap in the middle (Figure 3).

There was also variation between the clones in needle colour and retention. In the controls the shoots remained green and only lost a few needles (Figure 2c). The appearance of the inoculated shoots varied widely between clones. The clones with the longest lesions had more needle loss, with the majority of the needles becoming yellowed or even completely brown and desiccated (Figure 2d). In comparison, clones that developed shorter lesions had better needle retention and were greener (Figure 2e).

4 | DISCUSSION

This is the first study to investigate variation in susceptibility to *P. ramorum* in planted stands of Japanese larch. It presents data revealing that clones from the putatively resistant group tended to have shorter lesions than the known susceptible group; however, this was not statistically significant. Considering these results, our hypothesis may be revised to state that survivor trees included a higher proportion of resistant or low susceptibility trees than the trees showing infection in plantation. These findings indicate that survivor trees in high mortality stands could be a useful resource for further work investigating natural resistance in larch.

The fact that lesion lengths were on a continuum indicated that there is variation in susceptibility to *P. ramorum* within the Japanese larch population. For example, the clone with the shortest lesions, Res7, had an average lesion length four times shorter than the clone with the longest lesions, Sus13. The shorter lesions found in some of the clones tested in this experiment suggest some level of partial resistance to *P. ramorum*. The definition of tolerance or partial resistance can vary but is often considered as a reduction in disease severity rather than the absence of disease (González et al., 2012; Pagan & Garcia-Arenal, 2020), often including incomplete resistance conditioned by multiple genes of partial effect (Poland et al., 2009). The range of variation in lesion length in response to *P. ramorum* infection in the Japanese larch clones is consistent with minor gene contributions, where several quantitative trait loci (QTLs) are associated with the resistance. QTLs have been found related to other Phytophthoras; eight QTLs have been associated with resistance to *Phytophthora sojae* in Soybean (*Glycine max*) (Han et al., 2008) and multiple QTLs have been associated with resistance to *Phytophthora parasitica* in citrus (Siviero et al., 2006). Durability in pathogen resistance is expected to be greater in quantitative (as opposed to qualitative) traits that are controlled by multiple genes (Lindhout, 2002), as selective pressures in the pathogen are spread over multiple loci.

Genetic diversity within a population would provide a broader resource for the identification of resistant individuals. For example, survivor juniper trees found to be naturally resistant to *P. austrocedri*

(Green et al., 2020) were in populations of a native tree species with high genetic diversity (Merwe et al., 2000) whereas Japanese larch is an exotic plantation species in the UK. Provenance trials of Japanese larch were planted in 1934 in the UK with seeds sourced from the Nagano Prefecture in Japan (Lines, 1987) and, in 1956, with seeds from throughout the natural range (Lines & Mitchell, 1989). From these trials, Lines (1987) identified the Suwa region of Nagano Prefecture as the 'first choice' provenance for commercial planting in the UK. Two other provenances were identified as 'acceptable'; the Nikko region in Tochigi Prefecture and plantations on the island of Hokkaido. However, the provenance of the Japanese larch stands sampled in this study, and their inherent genetic diversity, is unknown. It is possible that greater genetic variation exists in the 1956 Japanese larch provenance trials, which remain standing (R. Whittet, 2020, personal communication), or in the native range of the species. Material sourced from these locations could be tested in the future for resistance to *P. ramorum*.

The material used in this study was excised from ramets that had been grafted onto European larch rootstocks. Rootstocks are known to affect the physiology of the scion and are often used to influence growth characteristics and improve response to biotic and abiotic stresses, particularly in fruit trees and grape vines (Camisón et al., 2021; Cookson et al., 2014; Habibi et al., 2022). It is possible that the rootstocks had an effect on the results shown in this work, however, without an inoculation trial of the rootstock material and inoculations of the scion material on its own root system it is impossible to predict the influence of grafting. Phenotyping of young seedlings has shown that characteristics including seed weight, speed of germination and root mass can influence disease susceptibility (Solla et al., 2011). The rootstocks used in this experiment were sourced from a commercial nursery and so we were unable to source any data on their phenotype or genotype to use as covariates in our modelling.

Currently there are four known clonal lineages of *P. ramorum* in areas of invasion (Jung et al., 2021; Van Poucke et al., 2012) with only one lineage, EU2, being used in this study as it is dominant in the study area and more aggressive on larch than the other UK invasive lineage, EU1 (Harris et al., 2021). *P. ramorum* has recently been discovered in the laurosilva forests of Shikoku and Kyushu in south-west Japan (Jung et al., 2021) and in Vietnam (Jung et al., 2020), and the high level of phenotypic and phylogenetic diversity found in these populations suggests that the pathogen is native in these regions (Jung et al., 2020, 2021). The evergreen subtropical forests where *P. ramorum* has been found in Japan are very different to the natural subalpine habitat of Japanese larch, which exists mainly at elevations between 1200 and 2400 m, on scattered volcanic mountains between 35–37°N and 137–141°E in the central island (Honshu) of Japan (Lines, 1987). It is, therefore, unlikely that Japanese larch has co-existed with *P. ramorum* in its native range, suggesting that resistance through co-evolution may not occur.

Work on other pathosystems illustrates the extensive effort required to identify and develop disease resistance in forest trees, from mass screening thousands of common ash (*Fraxinus excelsior*) for resistance to ash dieback (*Hymenoscyphus fraxineus*) (Plumb et al., 2020; Stocks et al., 2017) to multi-decade breeding programmes for resistance to *Cronartium ribicola* in North American white pines (*Pinus* L. subgenus *Strobus*) (Sniezko, 2006). In comparison, the scope of our study is limited but it is an important first step into investigating variation in resistance in the Japanese larch population.

Further work to investigate resistance to *P. ramorum* in Japanese larch is already being undertaken. A second expansion of the *P. ramorum* epidemic in SW Scotland in spring 2018 allowed the identification of an additional 50 'putatively resistant' survivor trees within affected stands from which scion material was collected (S. Green and R. Whittet, unpublished). Future resistance testing of this material should be done against multiple *P. ramorum* lineages as well as isolates within a lineage, since variation in response to inoculation with two isolates of the UK clonal lineage of *P. austrocedri* was observed in an inoculated juniper clone (Green et al., 2020). Isolate variation is also very well characterized in the relationship between *P. infestans* clonal lineages and potato (Fry, 2008). Although Hansen (1989) and Green (2020) showed the suitability of inoculating excised material as a first screen for resistance, further work should include the inoculation of whole plants to better reflect natural conditions and to remove the possible effects of grafting and rootstocks. One objective would be to test if the variation in susceptibility observed among Japanese larch clones in this study was stable against a range of *P. ramorum* isolates and lineages. Another key objective would be to determine whether this tolerance is heritable and is able to be exploited in a breeding programme, requiring sib analysis such as the establishment of a provenance progeny trial from seed.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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