

RESEARCH ARTICLE

Genetic resistance to *Phytophthora lateralis* in Port-Orford-cedar (*Chamaecyparis lawsoniana*) – Basic building blocks for a resistance program

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Societal Impact Statement

Non-native pathogens and pests cause high mortality to tree species globally and may imperil the future viability of associated forest ecosystems. *Phytophthora lateralis*, an oomycete, causes Port-Orford-cedar root disease and is a major cause of mortality in the ecologically and economically important conifer species *Chamaecyparis lawsoniana* (Port-Orford-cedar). The *P. lateralis* resistance program shows promise to help stabilize *C. lawsoniana* in its native range of northwestern California and southwestern Oregon, USA, and serves as a leading example of disease resistance breeding in forest trees

Summary

- A non-native, invasive pathogen, *Phytophthora lateralis*, has caused extensive mortality within the native range, northern California and southern Oregon USA, of *Chamaecyparis lawsoniana* (Port-Orford-cedar), as well as in horticultural and amenity plantings in the USA and Europe. Restoration of affected sites is contingent upon development of populations with genetic resistance. Naturally occurring genetic resistance has been identified in *C. lawsoniana*, and an active selective breeding program seeks to characterize and increase resistance levels.
- Two seedling root dip inoculation trials, assessed for mortality for nearly three years each, are used to examine the types and levels of genetic resistance in *C. lawsoniana*. Most seedlings utilized in these trials are progeny of crosses from parent trees that exhibited apparent resistance to the disease in earlier trials.
- Seedling trials suggest that both qualitative major gene and quantitative disease resistance occurs in *C. lawsoniana*. Both types of resistance to *P. lateralis* appear to be present at levels high enough to be immediately useful for restoration and reforestation. The data suggest that the qualitative resistance is conditioned by a

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single major gene (designated here as *Pla*), but nothing is known about the number of genes involved in quantitative disease resistance.

- Seedling progeny from resistant parent trees in containerized seed orchards are now being used for restoration and reforestation. Resistant seedlings or clones could also be used to re-establish *C. lawsoniana* in urban forests.

KEYWORDS

major gene resistance, non-native pathogen, *Phytophthora lateralis*, Port-Orford-cedar root disease, qualitative resistance, quantitative disease resistance

1 | INTRODUCTION

Non-native pests and pathogens have negative impacts on forest health globally, especially where there is a high degree of susceptibility resulting from a lack of co-evolution alongside the disease or pest (Aukema et al., 2010; Britton & Liebhold, 2013; Fei, Morin, Oswalt, & Liebhold, 2019; Jung et al., 2018; Santini et al., 2013; Showalter et al., 2018). Despite the lack of co-evolution with these pests and pathogens at least some forest species will have genetic resistance (Snieszko & Koch, 2017; Snieszko, Smith, Liu, & Hamelin, 2014). In general non-coevolved resistance arises when a trait that evolved for a different function provides some resistance to a novel pest or pathogen; known as exaptation (Gould & Vrba, 1982). Knowing the type(s), frequency, and geographic distribution of resistance existing across the range of a tree species will provide valuable information useful to applied resistance programs interested in restoration and reforestation.

Phenotypic patterns of resistance are usually divided into two categories, qualitative and quantitative (Poland, Balint-Kurti, Wissner, Pratt, & Nelson, 2009). This is a simplistic division and we must acknowledge that there is a resistance continuum, and in many cases little can be inferred about the underlying inheritance. In the simplest case, qualitative resistance can be conditioned by a single major gene (e.g. Kinloch & Dupper, 2002), and at the other extreme, quantitative disease resistance (QDR), which can have a complex pattern of inheritance, is often the result of several to many genes interacting (King, David, Noshad, & Smith, 2010; Poland et al., 2009; St Clair, 2010). Durable resistance will be a necessity in long-lived forest trees, and QDR is generally thought to provide a higher likelihood of being durable than qualitative resistance (Cowger & Brown, 2019; Poland et al., 2009; Snieszko, Johnson, & Savin, 2019). However, there are only a few examples documenting QDR in forest tree species, and studies are needed to guide applied forestry and conservation programs (see Snieszko et al., 2019 for an example). Because there is growing recognition that in many forest trees other management activities will have limited impact on slowing the progress of a disease or pest epidemic, there may be a need for more resources to be directed toward development of resistant populations useful for restoration or reforestation (Showalter et al., 2018). We also note that plants can marshal defenses to pathogens

that fall into two categories: resistance and tolerance. Tolerance, in contrast with resistance is not thought to impact the pathogen directly, but instead influences traits that can limit the health and fitness costs of being infected (Martins et al., 2019). Though both tolerance and resistance aid in improved survival of infected individuals it can be difficult to untangle the difference using phenotyping approaches alone.

Chamaecyparis lawsoniana (Port-Orford-cedar) is an ecologically and economically important conifer species native to northwestern California and southwestern Oregon (Zobel, Roth, & Hawk, 1985). The species has been extensively impacted by the non-native pathogen *Phytophthora lateralis*, which is responsible for the Port-Orford-cedar root disease (Betlejewski et al., 2003, 2004; Hansen, Goheen, Jules, & Ullian, 2000). The pathogen, which was first discovered in a North American nursery in 1923, has subsequently led to high mortality of *C. lawsoniana* in portions of its native range (Figure 1) (Hansen et al., 2000; Zobel et al., 1985). *P. lateralis*, an oomycete, is generally a soil and water-borne root pathogen and can kill trees of all sizes, with riparian areas most heavily affected (Hansen et al., 2000; Zobel et al., 1985). There are also examples of aerial infection, most notably in plantings in Europe (Robin et al., 2011). *C. lawsoniana* has also been a valuable tree for horticultural use in North America and Europe (often known as Lawson's cypress), but the impact of the pathogen has greatly curtailed its use (Zobel et al., 1985). Recent findings of *P. lateralis* on *C. obtusa* in Taiwan suggest Asia as the origin of this pathogen (Brasier, Vetraino, Chang, & Vannini, 2010; Webber et al., 2012). Although its main impact has been on *C. lawsoniana*, mortality of *Taxus brevifolia* (Pacific yew) has also been documented (DeNitto & Kliejunas, 1991; Hansen et al., 2000; Murray & Hansen, 1997), and a few additional conifers, including *Juniperus communis* and *Microbiota decussata* also appear to be very susceptible (Ebba Peterson, personal communication).

In many of the affected areas, it is difficult to eliminate the pathogen directly, and land managers are reluctant to plant *C. lawsoniana* without access to seedlings with identified genetic resistance to the disease. The first testing of *C. lawsoniana*, using rooted cuttings from several hundred surviving trees in areas of high disease incidence, concluded that there was little or no resistance to *P. lateralis* or that refinements in testing techniques would be needed to uncover resistance (Hansen, Hamm, & Roth, 1989; Zobel et al., 1985).



FIGURE 1 *Chamaecyparis lawsoniana* (Port-Orford-cedar) killed by *Phytophthora lateralis* (a) in South Slough National Estuarine Research Reserve (SSNERR), southern Oregon; (b) mortality along Munger Creek, southern Oregon; (c) dying tree with characteristic *P. lateralis* stain above root collar in SSNERR; (d) mortality in Redwood National Park, northern California; and (e) seedling with lesion at the root collar typical of phenotype in root dip and raised bed tests

A subsequent trial suggested that there was variation in susceptibility among parent trees and their progeny, and that the resistance was due to a slowing in the rate of advance of *P. lateralis* and was not immunity (Hansen et al., 1989). This information and concerns about population viability and conservation status of *C. lawsoniana* raised awareness and helped provide the support needed to undertake a resistance program (Betlejewski et al., 2004; Farjon, 2013; Potter, Escanferla, Jetton, Man, & Crane, 2019). Large operational resistance screening trials began in the 1990s in which more detailed results, including some evidence for both QDR and MGR disease resistance emerged (Sniezko, 2004, 2006). These trials were part of a comprehensive breeding program that was established at the USDA Forest Service's Dorena Genetic Resource Center (DGRC), Cottage Grove, Oregon, to aid in restoration and reforestation of *C. lawsoniana* (Sniezko et al., 2012b).

To improve success in restoration and reforestation, it is important to determine the type(s) of genetic resistance (MGR, QDR, or

both) that exist so that breeding efforts can maximize the chance to deploy resistance that is durable. In this paper, we report on the use of two seedling inoculation trials from DGRC to examine the phenotypic range of *P. lateralis* resistance available in *C. lawsoniana*. Using the data from these trials, which incorporate a more extended assessment period than used previously and many parent trees and their progenies, we seek to clarify whether progeny phenotypes display a qualitative or quantitative resistance response. The two screening trials, established nine years apart and with different isolates of the pathogen, provide the most comprehensive examination of resistance in the species to date.

We show here that there is evidence of both QDR and MGR resistance to the disease. These data will provide managers and breeders information that can be used to assign parent trees to seed orchards or breeding populations, make forward selections from within seedling families, and provide managers with estimates of usable resistance from the initial field selections (Sniezko et al., 2019).

2 | MATERIALS AND METHODS

The two greenhouse inoculation trials used here were initiated in 2005 and 2014 and are part of an annual series of trials for an applied resistance breeding program that is evaluating progenies of over 1,000 parents selected in an earlier stage of screening. The parents used in these trials included some of the earliest field selections from the resistance program as well as more recent field selections. The early selections had been used for more than 10 years in numerous trials (of one-year or less in duration) and were the progenitors of most of the resistant and susceptible controls in these two trials. The susceptible controls used are typical of the level of resistance that might be found in most parent trees in wild *C. lawsoniana* populations. The other parents in these trials were a subset of the >14,000 trees that had been evaluated to a lesser extent (generally in one or two earlier trials). Prior to the 2005 trial, none of the parents had been evaluated using a 3-year testing period.

The trials include seedling families from full-sib and self-pollinations as well as open-pollinated families from wild stands and containerized orchards. Self-pollinated families were used because of the ease of making the cross (although they had somewhat lower seed yields). The self-pollinated families showed little or no evidence of inbreeding depression in traits that could cause these families to show higher and faster mortality (R. Sniezko, unpublished). A self-pollinated family from a susceptible control parent was included in each trial.

2.1 | Experimental design

Trial one included 125 families (88 parents represented) of *C. lawsoniana*, including 87 full-sib families, 28 self-pollinated families, and 10 open-pollinated half-sib families (four from field selections from southern Oregon forests and six from the clone bank at DGRC) (Dataset S1). Many of the parents were field selections (69 parents) from sites varying in degree of exposure to *P. lateral*is. The remaining 19 parents were progeny (forward) selections from prior testing. Thirty-five of the 88 parents were represented in only one cross or half-sib family, while the other 53 parents were represented in two to 12 crosses (full-sibs or selfs) or half-sib families. Twenty-eight of the parents were self-pollinated to generate 27 S_1 and 1 S_2 families. The two susceptible controls included a self-pollinated family of parent CON1 and a full-sib family involving two parents identified in previous seedling testing as highly susceptible (R. Sniezko unpublished data). Six half-sib families served as resistant controls.

Trial one was sown at DGRC in spring 2004. Seed were sown in 164 ml Ray Leach supercell cone-tainers (3.81 cm wide $\times$ 20.955 cm height), and the seedlings were cultured in an unheated greenhouse until January 2005. In fall 2004, seedlings were placed into a randomized complete block (RCB) design with seven blocks and two to seven seedlings per family per block (21–49 total seedlings per family, with an average of 43.8 seedlings per family), arranged in row plots. Seven families had insufficient seedlings (11–16) for inclusion in all blocks and were arranged only in four of the seven blocks.

Prior to sending the seedlings to Oregon State University (OSU) for inoculation, seedling tops were cut in Blocks 1, 2, and 3 for rooting at DGRC for potential later inclusion in a breeding orchard. In addition, for eight families some of the tops were cut in the remaining blocks for a different study. The seedlings were treated with a foliar application of Chlorothalonil and Benomyl approximately two months prior to inoculation to discourage foliar disease.

Seedlings were inoculated at OSU in early 2005 using the root dip inoculation method (Hansen et al., 2012). Inoculum consisted of a mixture of zoospores from isolates 366 and 368 of *P. lateral*is collected previously from diseased trees; cultures for block 1 were grown in pea broth while those for blocks 2–7 were grown in carrot broth. Before inoculation, any roots growing out of the bottom of the containers were clipped. The bottom 1 cm of each tube was immersed in the *P. lateral*is zoospore suspension for 48 hr. Inoculation of the seedlings was spread out over four weeks, beginning on February 2, 2005, with one or two blocks inoculated per week.

Seedlings were maintained in the OSU greenhouse and assessed for symptoms and mortality 17 times over a 1008-day post-inoculation (p.i.) period. The first assessment occurred when wilted or off-color foliage was noted, 103–124 days after inoculation, depending on the block. Seedlings that were wilted or off-color were scraped to observe presence of the lesion characteristic of *P. lateral*is infection and were classified "dead" if the girdling lesion was present (Figure 1e). A few dead trees did not have the characteristic lesion; these were noted as dead from other causes and excluded from analyses (five observations). Subsequent assessments occurred at varying intervals (3–15 weeks), depending on the detection of additional wilted or off-color seedlings. At the end of the trial, surviving seedlings were scraped at the root collar, and those with lesions present and no wilting were considered dead (none in this trial).

Trial two included 145 families (Dataset S1), including 14 full-sib families, 123 self-pollinated families (116 S_1 , 7 S_2), seven open-pollinated (half-sib) families, and one low resistant open-pollinated bulk lot from susceptible parents in the DGRC clone bank. Five families were common between trial one and trial two (Table 1). Parent CON1, a susceptible control, was also common to both trials but represented in different crosses. The 14 full-sib families included eight crosses among field selections archived in the DGRC clone bank as well as six involving

TABLE 1 Comparison of survival percent and days to mortality (DTM), post inoculation, with *Phytophthora lateral*is, of five seedling families of *Chamaecyparis lawsoniana* common between two root dip trials

Family	% Survival		DTM	
	Trial 1	Trial 2	Trial 1	Trial 2
117490 $\times$ CF1	100	97.6	na	141
SIS-41427 $\times$ PRI-60974	24.5	19	526.4	443.2
117490 OP	100	97.6	na	968
118463 OP	4.1	9.9	242.6	176.2
510015 OP	59.2	63.5	153	205.9

forward selections (five of them crossed with the susceptible CON1 parent). The trial had two susceptible and two resistant controls (Dataset S1). Most of the parents used in full-sib crosses or selfs had been rated resistant in either previous stem dip trials or both a stem dip and root dip trials that used rooted cuttings (R. Sniezko unpublished data).

Seed were sown in 164 ml Ray Leach supercell cone-tainers during early Spring 2013, and the seedlings were cultured in an unheated greenhouse at DGRC. Seedlings were treated with beneficial nematodes to reduce potential root weevil damage. Seedlings were placed in a RCB design, with six blocks of up to seven seedlings per family, arranged in row plots (19–42 total seedlings per family, an average of 37.2 seedlings per family). In early December 2013 before transport to OSU for inoculation, seedling tops were cut to reduce the risk of foliar disease and to produce rooted cuttings for potential inclusion in seed orchards.

Inoculation of seedlings was carried out over a six-week period from February 4 to March 11, 2014, with one block inoculated each week. Inoculation was like trial one, except that *P. lateralis* isolates PL3 and PL4 were used. The trial was assessed for disease symptoms and mortality 23 times using the same methods as described in trial one; the first assessment was on April 22, 2014 (42–77 days p.i., depending on block), and the final occurred on October 20, 2016 (954–989 days p.i.). At the final assessment, 51 living, non-wilted, seedlings (from 38 different families) were found to have lesions at the root collar and were considered dead for this trial.

The trials were terminated when plant health was no longer tenable in the pot system being used.

3 | DATA SUMMARY AND ANALYSIS

3.1 | Survival and Days-to-mortality

Survival was calculated by assessment date, and survival curves were generated for each family. DTM was calculated for each dead seedling as the number of days between its inoculation date and the date it was rated as dead. For families with no survival, the mean DTM included all seedlings in a family, but for families with survival >0%, it included only those dead (censored population). DTM provides an indicator of the presumed rate of progression of the *P. lateralis* pathogen in each seedling following inoculation. We used the survival curves to help characterize the variation in resistance both within and among families and interpreted the variation in survival and DTM as a reflection of the broad range in resistance of parent trees and their progeny in *C. lawsoniana*.

3.2 | Classifying resistance type

The Mendelian segregation ratios 3:1, 1:1, and 1:3 for alive:dead seedlings were tested for each family using exact binomial tests in R version 3.5.0 (R Core Team, 2018). Parent trees whose families did not significantly differ from the 3:1 and 1:1 ratios potentially possess MGR, controlled by a single dominant gene, while those with 1:3 ratios may

possess a single recessive gene for resistance. For those parents represented in more than one cross or family, examination of all families involving that parent further aided determining the likelihood of whether that parent had MGR, particularly if the parent had been crossed with a known susceptible parent. In some cases, some crosses with a given parent suggested that it might be MGR, but if additional crosses with the same parent showed high mortality, the parent was not designated MGR. Parents involved in only one cross (or not crossed with a susceptible parent) and showing a segregating ratio that might indicate MGR, but without further supporting data are designated MGRp. For non-MGR families, if either DTM or percent mortality varied significantly from the susceptible controls, we designate the family as QDR. In cases where there are several to many crosses per parent, and they all fit the DTM criteria, we designate the parent as QDR (Dataset S1). Statistical differences among families in percent survival for four different time intervals (at approximately 6, 12, 24 months p.i. and the final assessment) were tested with likelihood ratio tests using binomial models with survival as the response and block (reduced model) or family, block, and associated interactions (full model). For DTM at the final assessment, determination of which families differed from the two susceptible controls was tested with a Mann-Whitney-Wilcoxon rank sum test in R version 3.5.0 (R Core Team, 2018).

4 | RESULTS

Both seedling root dip inoculation trials were successful, with the susceptible controls reaching 0% survival and averaging less than 130 and 100 DTM in trials one and two respectively (Table 2, Dataset S1). In trial one, there was no statistical difference in survival or DTM in the three blocks where tops had been cut, compared to the three blocks where seedling tops were intact. In both trials, the range of survival among the many families spanned nearly a continuous distribution from 0 to 100 (Figures 2a, 3a), and family variation in DTM occurred across the range of survival in both trials (Figure 4). The five families in common to the two trials showed very similar performance, despite the difference in isolates used and the 9-year difference in initiation of the trials (Table 1). There were significant differences among families for survival ($p < .05$) in both trials during the four time intervals tested and for DTM (Table 3, Dataset S1), with the family means varying widely (Figures 2, 3, 4 for both survival and DTM). In some families, seedling survival plateaued early, but for others survival continued to decrease over the duration of the two trials (Figures 2, 3). By the end of both trials, only 8 and 29 families had 0% survival in trial one and two respectively.

4.1 | Trial summary

4.1.1 | Trial one

At first assessment, 103–124 days post-inoculation (p.i.) for the seven blocks, the trial averaged 89.9% survival, and mean family survival

TABLE 2 Mean % survival and days to mortality (DTM) by cross type for two *Chamaecyparis lawsoniana* root dip inoculation trials

Cross Type ^a	Trial 1			Trial 2		
	#Families	% Survival	DTM	#Families	% Survival	DTM
OP	4	2.6 (0–4.1)	159.6 (137.3–217.6)	6	39.8 (9.9–79.2)	251.2 (176.2–378.4)
CC	86	33.3 (0–100)	325.7 (136.8–700.0)	13	46.2 (0–100)	256.8 (103.7–517.9)
S ₁	26	35.8 (0–100)	291.4 (122.7–680.2)	115	19.5 (0–91.7)	260.8 (86.2–671.9)
S ₂	1	2.0 (–)	136.3 (–)	7	30.1 (0–100)	353.5 (119.7–598.8)
Resistant controls	6	52.4 (4.1–100)	272.3 (153.0–498.1)	2	58.3 (19.0–97.6)	705.6 (443.2–968.0)
Susceptible controls	2	0 (0–0)	127.5 (127.3–127.6)	2	0 (0–0)	93.7 (88.9–98.6)
Trial averages	125	33.0 (0–100)	305.9 (122.7–700.0)	145	23.5 (0–100)	267.9 (86.2–968.0)

Note: Range of family means for each trait indicated in parentheses. Where the four open-pollinated (OP) families in trial one were from field selections from southern Oregon forests, and the six OP families in trial two were orchard open-pollinated. The full-sib (CC) families include crosses among parents that showed differing levels of resistance and susceptibility in prior resistance testing. The S₁ and S₂ are first- and second-generation self-pollinated families, respectively, from parents that were either among the top 10% selections in previous stem dip trials or showed some resistance in prior root dip trials. The resistant controls included families that have major gene resistance (MGR), quantitative disease resistance (QDR) or both, and all but one were orchard open-pollinated seedlots; the remaining resistant control was one controlled cross (CC) in trial two. The susceptible controls showed high mortality and low DTM in previous resistance screening trials, and include two self-pollinated lots, one controlled cross, and one bulked collection from fast-dying susceptible parents in the Dorena Genetic Resource Center clone bank.

varied from 28.6% to 100%. At approximately 1-year p.i. mean survival was 47.8%, but only the two susceptible controls had reached 0% survival (which occurred by 10 months p.i.). By the final assessment (987 to 1,008 days p.i.), mean survival decreased to 32.9% (mean 312 DTM), and family means ranged from 0% to 100% (Figure 2a), with family means for DTM ranging from 122.7 to 700 (Figure 4, Dataset S1). The susceptible control parent (CON1) had poor survival (0 to 10.2) in eight of ten crosses (Figure 2c, Table 4). The four open-pollinated seedlots from southern Oregon forests were highly susceptible, averaging 0 to 4.1% survival by the end of the trial and 137.3–217.6 DTM (Dataset S1); these four families showed little resistance in an earlier stem dip inoculation trial (R. Sniezko, unpublished), and are good proxies for the level of resistance found in the majority of native Port-Orford-cedar forests.

All families had higher mean survival and/or DTM than the susceptible controls (Figures 2, 4, Dataset S1). The two susceptible controls had 0% survival and averaged 127.3 and 127.6 DTM, respectively, while the other six families with 0% survival at the end of the trial had mean DTM ranging from 169 to 277.1 (Figures 2a, 4; Dataset S1). Thirty of the 35 families with lowest survival (0–10%) varied significantly ($p < .05$) in mean DTM compared to the two susceptible controls, with means ranging from 136.3 to 438.9 (Figures 2a, 4, Dataset S1). Thirty-five of the 125 families had 50% or greater survival, including five families with 100% survival (Figures 2a, 4, Dataset S1). On a parental mean basis (1–12 crosses per parent), 18 of the 88 parents represented in the 125 seedlots averaged 50% or greater survival, and 21 of the parents averaged 400 DTM or greater.

The eight controls spanned the range of resistance, from no survival and fast mortality (DTM 127.3) to 100% survival (Figure 2b, Table 2). The within-family pattern of mortality varied widely; in some families mortality quickly peaked at a moderate level and then plateaued with little or no additional mortality over the remaining

duration of the test, while for other families mortality continued for the duration of the trial (Figure 2b).

4.1.2 | Trial two

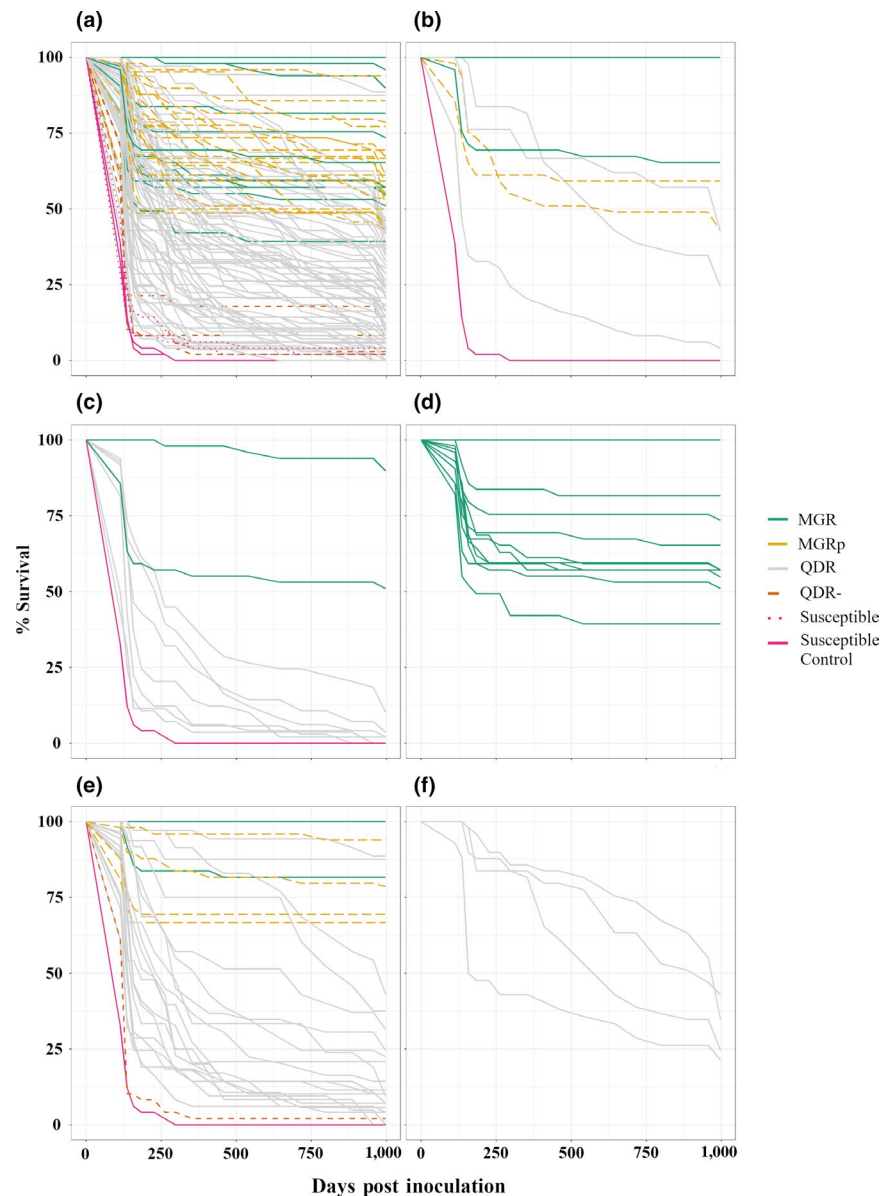
The two susceptible controls reached 100% mortality rapidly (<100 DTM); the putative QDR control (SIS-41427 × PRI-60974) had 19% survival and averaged 443.2 DTM, and the fourth control, an open-pollinated family of parent 117490, had 97.6% survival (Dataset S1). Overall, survival of the 145 families was similar to trial one and ranged continuously from 0% to 100% (Figure 3a). DTM also varied widely at each level of survival (Figure 4b, Dataset S1). In addition to the two susceptible controls, 27 families in this trial had 0% survival; five of these had rapid mortality (DTM < 100), while the seven other families took nearly twice as long (DTM > 190) (Figure 4b, Dataset S1). At the final assessment, the trial averaged 23.5% survival and 267.9 DTM (Table 2).

5 | CLASSIFYING RESISTANCE TYPE

5.1 | Major gene resistance

In trial one, three of the CF1 families showed no significant difference from a 3:1 Mendelian segregation ratios of live:dead seedlings. This finding suggests that they could be a cross between two parents heterozygous for the resistance gene. Seven CF1 families showed no significant difference from a 1:1 ratio suggesting a cross among a parent heterozygous for the resistance gene and a susceptible parent (Figure 2d, Tables 4 & S1) which provides phenotypic evidence that parent CF1 is heterozygous for MGR. The pattern of very high survival for families of 117490 suggests that it is likely homozygous

FIGURE 2 Variation in *Chamaecyparis lawsoniana* survival over time (days post-inoculation) following root dip inoculation with *Phytophthora lateralis* in Trial 1 (a) among all 125 seedling families (family mean survival varies from 0% to 100% and days to mortality (DTM) varies from 123 to 700); (b) among a susceptible checklot (0% survival, <130 DTM), six resistant control families, and a full-sib family with DTM 526.4 (survival 24.5); (c) expression of major gene resistance (MGR) and quantitative disease resistance (QDR) among nine full-sib crosses and a self-pollinated family involving susceptible control parent (CON1); (d) expression of resistance among 10 full-sib crosses, one self-pollinated family (S1 family) and an orchard open-pollinated family of parent CF1, putatively heterozygous for MGR (e) among the 28 self-pollinated families displaying wide variation in resistance levels and types, including MGR, low to high levels of QDR, and high susceptible CON1 S1; (f) moderate to high levels of QDR (moderate survival and DTM from 315 to 700) in the four full-sib families involving parent SIS-41427. Color and line types are used to specify type of genetic resistance: MGR (solid sea green), possible MGR (MGRp) (long dashed gold line), QDR (solid gray line), low level QDR (QDR-) (dashed orange line), Susceptible (dotted magenta line), and Susceptible control (solid magenta line)



for MGR (Table 4 further illustrates the survival of parents 117490 and CF1 in several crosses, relative to crosses involving the susceptible control). We designate this locus *Pla*.

Parents CF1 and 117490 are the most studied MGR candidates, but in trial one, 44 of the 125 families (including 10 with parent CF1) showed no significant difference from alive:dead segregation ratios, either 3:1 (12 families) or 1:1 (33 families, including one family which also showed no difference for 3:1) (Dataset S1, Figure 5), suggesting that there is not enough evidence to preclude the possibility that one parent (1:1 ratio) (Figure 5b) or both parents (3:1) (Figure 5a) might be heterozygous for MGR. Multiple crosses with these parents are needed to help determine the MGR status of the parents. There were 15 parents involved in the crosses of families that failed to show differences from 3:1 segregation, and some of those parents were involved in other crosses. In the simplest case, we would expect a parent heterozygous for *Pla* to show no difference from a 3:1 or 1:1 alive:dead ratio in crosses with other heterozygotes or susceptible parents respectively.

Examination of 11 parents that were represented in more than one family showed that four parents had moderate to high survival in all crosses (including CF1, Figure 2d), providing further support that those four parents were heterozygous for MGR, while the seven other parents had at least one cross with 2.0 to 28.6% survival (Dataset S1), indicating that these parents were not MGR. The S_1 family of parent SIS-40051 had 93.9% survival (DTM 388.7) which differs from a 3:1 ratio ($p < .05$) (survival exceeds expectation), but in another cross it had 53.6% survival (DTM 260.1), suggesting that it could be heterozygous for *Pla* and could also have QDR.

Forty-four of the families failed to show significant differences from a 1:3 segregation ratio and nine of these also failed to show significant differences from a 1:1 ratio (Dataset S1, Figure 5c). A Mendelian segregation ratio of 1:3 may signify that both the pollen parent and the seed parent are heterozygous for a recessive gene; additional crosses will be needed to determine if a recessive gene is responsible in any of these cases.

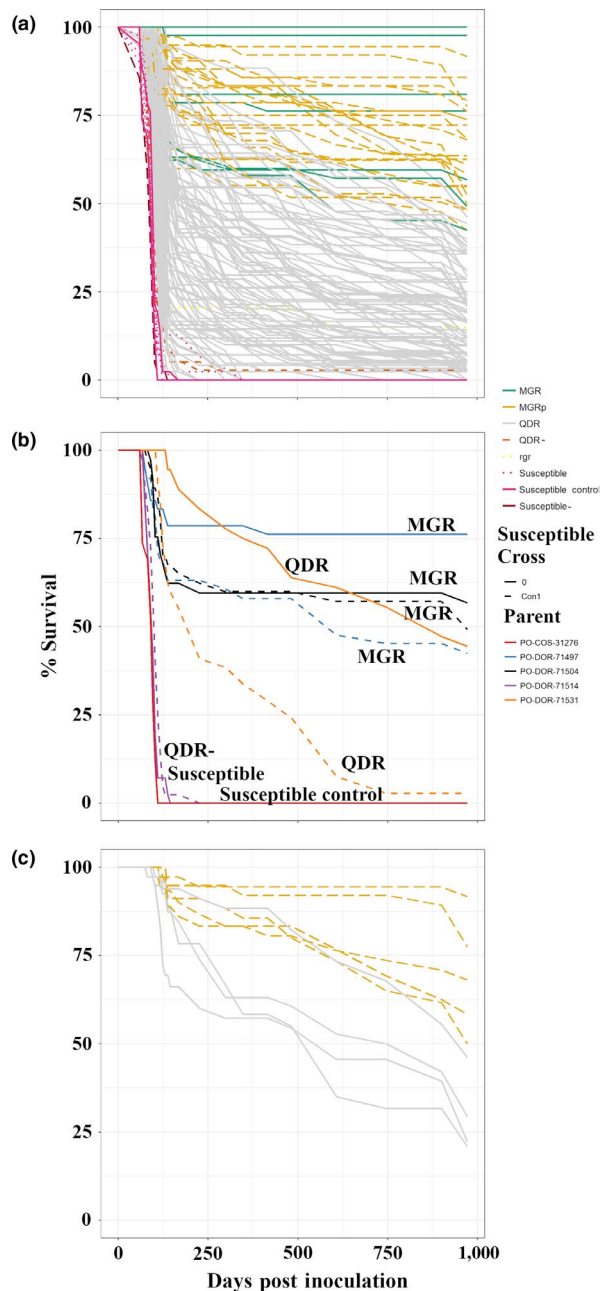


FIGURE 3 Variation in *Chamaecyparis lawsoniana* survival over time (days post-inoculation) following root dip inoculation with *Phytophthora lateralis* in Trial 2 among (a) 142 families and one bulked seedlot (two families with 100% survival not shown); (b) four parents represented by both a self-pollinated S1 family (solid lines) and a full-sib cross involving susceptible CON1 parent (dotted lines), and compared to susceptible control (COS-31276 S1 family). Two of the four parents show putative major gene resistance (MGR), one parent shows only quantitative disease resistance (QDR), and the fourth parent has little resistance; and (c) the 10 families with the highest days to mortality (DTM 517.9 to 968.0), including several with possible major gene resistance (MGRp), and others with QDR. Color and line types are used to specify type of genetic resistance: MGR (solid sea green line), possible MGR (MGRp) (long dashed gold line), rgr (dotted yellow line), QDR (solid gray line), low level QDR (QDR-) (dashed orange line), Susceptible (dotted magenta line), Susceptible control (solid magenta line) and families worse than susceptible controls (long dashed dark red line)

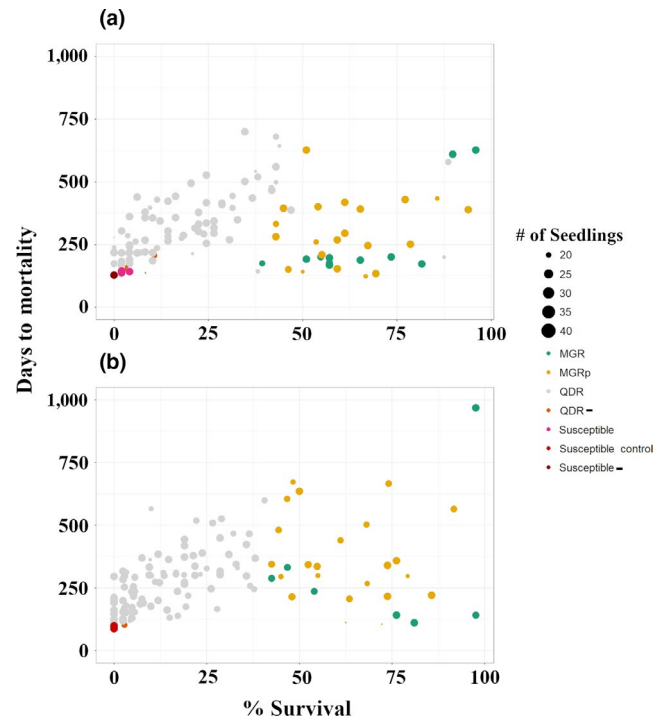


FIGURE 4 Variation in genetic resistance in *Chamaecyparis lawsoniana*, indicated by scatterplot of percent survival and days to mortality (DTM), to Port-Orford-cedar root disease among seedling families in two trials inoculated with *Phytophthora lateralis* (a) 120 families in Trial 1 (excludes 5 families that have 100% survival); and (b) 142 families and 1 bulked open-pollinated susceptible seedlot in Trial 2 (excludes two families with 100 percent survival). Size of circle indicates approximate number of seedlings in family; circle color indicates resistance type. Families with major gene resistance (MGR)(sea green), families with probable major gene resistance (MGRp) (gold), families with quantitative disease resistance (QDR) (gray), families with very low level of QDR (QDR-) (orange), the susceptible controls (Red), families that are susceptible (light red), and families that are worse than susceptible controls (dark red)

For trial two, 14, 29, and 49 of the 145 families failed to show significant differences from 3:1, 1:1, and 1:3 alive:dead segregation ratios respectively. Six families that failed to show differences from a 3:1 ratio also failed to show differences from 1:1, while 10 families that failed to show significant differences from 1:1 also failed to show significant differences for 1:3 (Dataset S1). Four other families had 97.6–100% survival, including the three families involving parent 117490, indicating that at least one of their parents may be homozygous for *Pla*. A fifth family (71501 × self) had 91.7% survival (and DTM 564.5), and further investigation is needed to determine if it is a MGR parent. As with trial one, additional crosses will be needed to confirm which of the parents show MGR inheritance.

5.2 | Quantitative disease resistance

Compared to the susceptible controls, most of the other families in both trials displayed higher survival and/or longer DTM (Figures 2–6, Tables 4, 5, S1). Although as noted above, resistance in some

TABLE 3 Statistical significance of *Chamaecyparis lawsoniana* (Port-Orford-cedar) seedling families (Sow) at four time intervals (post inoculation) in two root dip trials of *Phytophthora lateralis*

Trial	Sow Year	6 month			1 year			2 year			Final		
		Sow	Block	Sow*Block	Sow	Block	Sow*Block	Sow	Block	Sow*Block	Sow	Block	Sow*Block
Trial 1	2005	0.000	0.004	0.008	0.000	0.014	0.013	0.000	0.014	0.015	0.000	0.009	0.167
Trial 2	2014	0.000	0.000	0.000	0.000	0.009	0.010	0.000	0.608	0.455	0.000	0.945	0.839

Note: Significance (*p*-values from Likelihood Ratio Tests) of binomial models fit to trial 1 and trial 2.

families appears to be due to a single major gene, *Pla*, that leads to high survival, but for most other families showing resistance MGR does not appear to be present. The range of DTM found within and between these non-MGR families (Figures 2–6, Tables 4, 5, Table S1) suggests a more complex inheritance. This finding also suggests that resistance is under quantitative genetic control and presumably influenced by several to many genes, although a single gene could also be responsible for such a phenotypic pattern (Poland et al., 2009). It is also notable that in many cases, mortality does not reach 100% in these QDR families. Several examples discussed below illustrate the level and quantitative pattern of resistance.

The progeny of some parents were particularly notable for the extended length of time in which the mortality occurred relative to the susceptible control (Figure 2f, Tables 5, Table S1). Four full-sib families involving parent SIS-41427 had moderate survival and high DTM (315.2, 526.4, 560.1, and 700.0) relative to the two susceptible controls (DTM <130). In three of these families, most mortality occurred more than a year after inoculation (Figure 2f, Dataset S1). In another case, involving a 2 × 3 parent mating design, several parents, notably 70016 and 70072, showed very high DTM in the crosses in which they were involved, as well as moderate to high levels of survival, while crosses involving parent 70064 showed lower survival and much lower DTM when crossed with the same two other parents (Table 5). Parent 70016 appears to be a strong candidate for having MGR, but also QDR; while parent 70072 displays only QDR, (note from Table 4 that it shows only 3.6% survival when crossed with a susceptible control CON1 parent that has little evidence of QDR).

The eight crosses involving parent SIS-41924, a parent previously characterized in the earlier trials as relatively fast-dying and highly susceptible (Sniezko, unpublished), showed a range of resistance much higher than the two susceptible controls (Figure 6a, Dataset S1); they averaged 17.9% survival and 297.8 DTM (seven of the eight crosses had DTM >215). This parent appears to possess a relatively low level of QDR, in contrast with some of the parents in which QDR appears to be present at a higher level.

Of the 10 families with highest DTM in trial one, the two families with the highest survival, involved MGR parent 117490 (Figure 6b, Dataset S1), noted above as a putative homozygous dominant parent for *Pla*. The *S*₁ family of parent 117650 also had high survival (88.6%) but based on its pattern of segregation in two other crosses (including 10.2% survival in a cross with CON1), it is not a MGR parent. The other seven families showed moderate survival (24.5–51.0%) and a pattern of slow, continuous within-family mortality over time. Five of these families also failed to deviate from a 1:1 alive:dead segregation, which could suggest that they may have both MGR and QDR; however, seven of the eight parents were involved in other crosses, and in each case, some crosses showed survival too low (0 to 21.4%) for a heterozygous MGR parent, suggesting that QDR alone is present. In the remaining case, the parents were involved in only one cross, and more data are needed to ascertain if these parents possess both MGR and moderate to substantial QDR or just QDR. A similar trend exists in trial two for the top families for DTM (Figure 3c). In another

Parent	Survival (%)			Days to mortality		
	117490 PlaPla	CF1 Plapla	CON1 plapla	117490	CF1	CON1
70280	–	57.1	0		176.4	169
70138	–	57.1	2		196.4	303.9
70169	–	54.8	–		199.9	
70234	100	–	2	na		216
117650	–	57.1	10.2		197.5	355.6
70262	–	57.1	2		167.4	181.8
70070	95.9	39.3	0	626.5	174.6	174.9
70072	–	73.5	3.6		199.6	284.7
117490	100	100,100	89.8	na	na,na	609.7
CF1	100, 100	81.6	51	na,na	172.1	191.4
CON1	89.8	51	0	609.7	191.4	127.6
OP	100	65.3	–	na	187.2	–

Note: The cross of 117490 × CF1 and its reciprocal are both represented, S₁ families are in bold font. See text for calculation of DTM (no value is available for families with 100% survival, shown as na in table). The Pla gene represents the simplest hypothesis for inheritance and further work is needed to confirm if other loci are also involved.

example, an examination of four parents which had been both selfed (S₂ families) and crossed with the susceptible CON1 control parent, suggest that two of the parents are MGR, one parent is QDR, and the fourth parent is nearly as susceptible as the susceptible controls (Figure 3b). The selfed families generally show similar or higher levels of resistance than the crosses with CON1.

Families with similar levels of final survival can reach that level at different rates, depending on whether the parents have MGR or on the level of QDR inherited from the parents. For example, the full-sib family SIS-41182 × COS-30771 had high early mortality (10.7% survival 10 months p.i. and 3.6% survival at the end of the trial and DTM 215.1); in contrast the full-sib family COS-30882 × COS-30735 had moderate early survival (59.2% at 10 months p.i.) followed by a generally steady decrease over time, averaging 6.1% survival (DTM 438.9) at the end of the trial (Figure 6c & e). Another paired example: full-sib family COS-30678 × COS-30771 had 48.6% survival at five months p.i., which decreased very slightly to 46.2% by the end of the trial (DTM 150.3), while full-sib family SIS-41924 × SIS-41427 had high early survival 89.8% at five months p.i., followed by a general linear decline in survival, reaching 42.9% survival (DTM 560.1) at the end (Figure 6d & f). Parent COS-30771, involved in two of these crosses and five other crosses, appears to have a low to moderate level of QDR but averaged higher survival when crossed with COS-30678 and PO-SIS-40051 (putative MGR parents). The seven full sib crosses with parent COS-30771 averaged 22.5% survival and 332.4 DTM (six of the seven crosses had DTM >215).

6 | DISCUSSION

The phenotypic distributions of the data from the seedling families suggest that both MGR and QDR are present in *C. lawsoniana* and

TABLE 4 Percent survival and Days-to-Mortality (post-inoculation, DTM) with *Phytophthora lateralis* in root dip trial one of full-sib, half-sib (OP), and S₁ *Chamaecyparis lawsoniana* families for a (1) 117490, a parent putatively homozygous for major gene resistance (Pla Pla), (2) CF1, a parent putatively heterozygous for major gene resistance (Pla pla), and (3) CON1, a susceptible control parent (pla pla)

further illustrate that by extending the screening trial duration to 3 years it is possible to more clearly delineate several types of genetic resistance. Here, we designate a parent (or family) QDR if it differs in survival and/or DTM from the susceptible control and if there is no evidence to support that it is MGR. Many families in these two trials show QDR and careful study will be needed to dissect it further and see if the inheritance, and basis for the resistance, varies by family, and whether factors such as modifier genes or recessive genes are also involved in at least some cases. We have shown that the range of QDR varies widely between parent trees, and at least for some parents a level of QDR is present in all crosses involving that parent. The frequency of parents with MGR appears to be rarer than those with QDR. Many of the putative QDR families had surviving seedlings at the end of the greenhouse trials, and those individuals would be good candidates for forward selection for breeding to increase the level of resistance and for inclusion in seed orchards. As with many other species, we acknowledge that the underlying inheritance of QDR is still unknown (Poland et al., 2009). Nevertheless, the phenotypes presented here provide the basis for future studies, including using genomic resources, to examine inheritance in greater detail. The individuals in this study, and the many others being tested in this breeding program, also provide information on many parent trees to select for inclusion in seed orchards or breeding to increase resistance, while maintaining a high level of genetic diversity in populations used in restoration or reforestation.

We suggest that the large differences in DTM within and among families indicate variation in the ability to slow the progress of the *P. lateralis* pathogen in the 20.6 cm length from the bottom of the seedling container to the root collar, since only the bottom 1 cm of root tissue was exposed to inoculum. Logistical constraints, foremost the general vigor of the seedlings in the small tubes, precluded continuing the greenhouse trials for longer periods; yet, since the

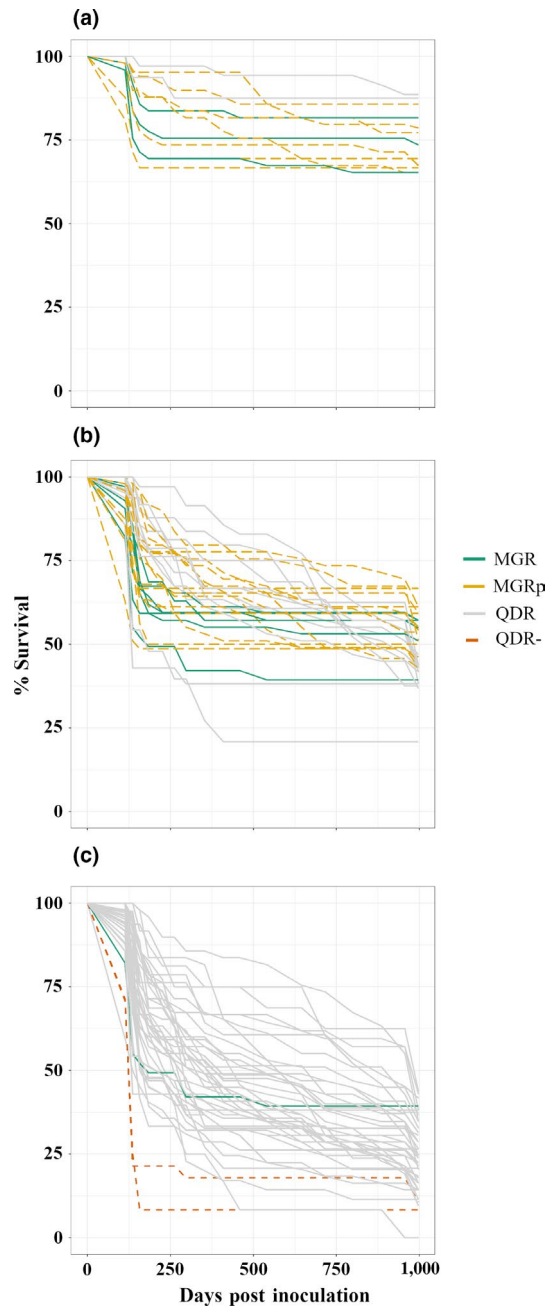


FIGURE 5 Survival over time for *Chamaecyparis lawsoniana* seedling families showing failure to differ significantly ($p = .05$) from within family segregation ratios of (a) 3:1 (12 families), (b) 1:1 (33 families), and (c) 1:3 (44 families) for alive:dead in *Phytophthora lateralis* inoculation Trial 1. The 3:1 and 1:1 ratios provide evidence of major gene resistance (MGR), but data from other crosses with some parents suggest some of these families have quantitative disease resistance (QDR) and not MGR. One possible interpretation of the 1:3 ratio is the presence of a single recessive gene but see text for further discussion. Note: One family shows both 3:1 and 1:1 ratio; nine families did not differ significantly for both 1:1 and 1:3 ratios. Color and line types are used to specify type of genetic resistance: MGR (solid sea green line), possible MGR (MGRp) (long dashed gold line), QDR (solid gray line), and low level QDR (QDR-) (dashed orange line)

susceptible controls had 0% survival within the first year, this had little or no impact on rating families as having MGR or QDR.

In trial one, 28 of the parents were self-pollinated, and examination of the survival of their progeny (Figure 2e) suggests four broad patterns: (1) 0% survival and fast mortality of all seedlings in a family, (2) low or no survival but within-family variation in time of mortality and higher DTM relative to the two susceptible controls, (3) moderate level of survival and considerable within-family variation in time to mortality and relatively high DTM, and (4) moderate to very high survival, with relatively early within-family plateau of survival and generally relatively low or moderate DTM (Figure 2e).

Relatively little is known about the underlying basis of the chemical and structural defenses against pathogens in forest trees (Kovalchuk et al., 2013). In another study of *C. lawsoniana*, some seedlings or rooted cuttings from resistant parents died, but the dead seedlings or rooted cuttings from some MGR parents often showed sunken, necrotic bark lesions at the root collar, often marked by resin flow, and many of those trees also died later relative to those of susceptible parents (Oh, Hansen, & Snieszko, 2006). In stem-wound inoculations of the materials from the same parents, stems of resistant parents had shorter lesions, often marked by resin, while in foliage inoculations, expanding patches of necrotic scale leaves were apparent for the susceptible parents and one resistant parent but reduced on foliage of a different resistant parent, which also exuded resin (Oh et al., 2006). A histopathology investigation indicated that *P. lateralis* hyphae in roots of rooted cuttings or seedlings from MGR parents grew slower in cortical cells and did not seem to penetrate the vascular tissues. Additionally, infection in the roots of resistant parents was marked by thickening of the cortical cell walls, collapsed cells, wall thickening, and apposition of electron-dense materials and crystals in cell walls were some of the structural changes common in resistant stems (Oh & Hansen, 2007). At least some differences between the two MGR parents or their progeny were noted, including some limited xylem colonization and indications of differences in zoospore attraction to roots (Oh & Hansen, 2007). This may suggest that different genes are responsible for resistance between them, but further investigations on the nature of resistance are warranted in MGR families as well as the QDR families noted in the trials reported here.

The presence of a single major gene, designated *Pla*, is just the simplest of several hypotheses to explain the segregation pattern of the families denoted MGR here, we also acknowledge that there could be more than one locus involved. Most MGR parents identified thus far appear to be heterozygotes for the *Pla* gene; however, 117490, a field selection made in 1989 on the Rogue River-Siskiyou National Forest in southern Oregon, appears to be homozygous for *Pla*, showing little or no mortality in seedling families in both trials. The low percentage of mortality in some families involving parent 117490 may be due to factors such as incomplete penetrance, modifier genes or microsite influences on stability of the resistance that have been suggested in other species where some deviations from expected ratios have occurred (Blaker & MacDonald, 1981;

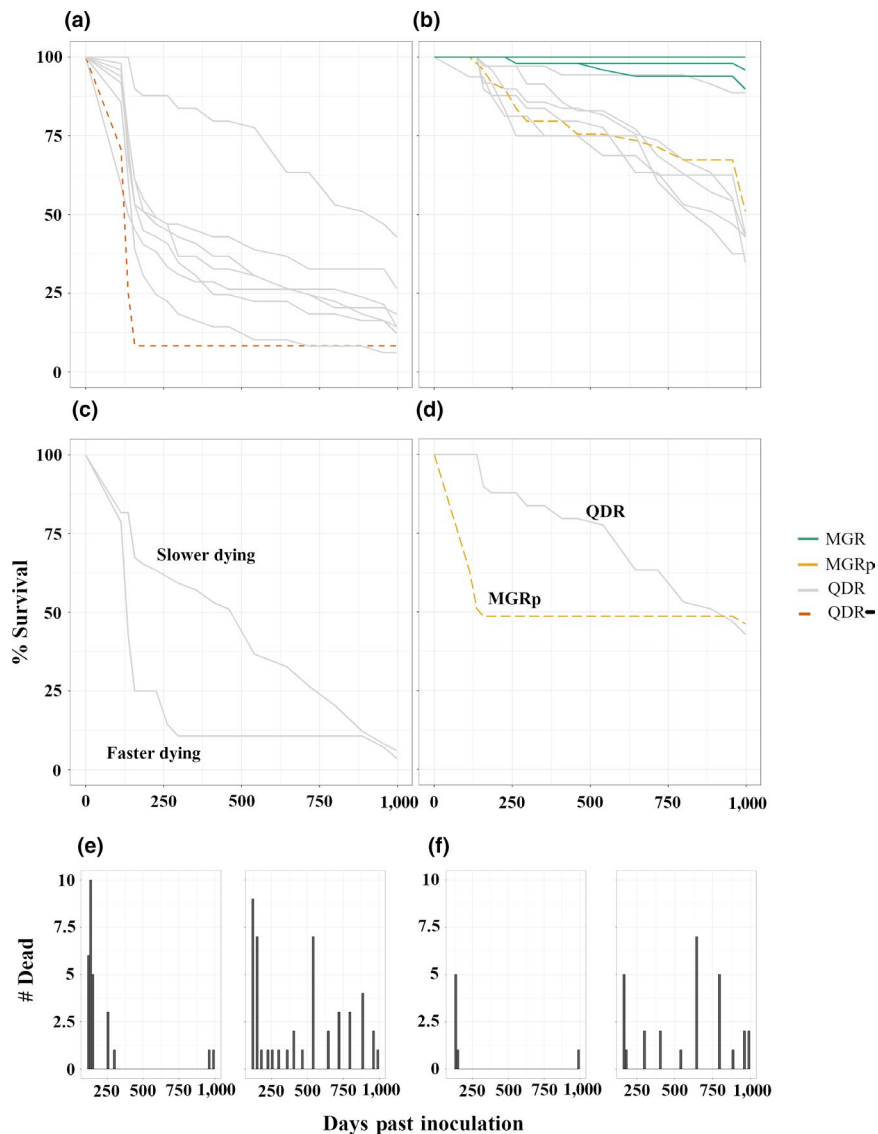


FIGURE 6 Variation in *Chamaecyparis lawsoniana* (Port-Orford-cedar) survival over time (days post-inoculation) following root dip inoculation with *Phytophthora lateralis* in Trial 1 for (a) 8 full-crosses with parent SIS-41924 showing low to moderate levels of quantitative disease resistance (QDR), with days to mortality (DTM) from 137 to 560; (b) the 10 families with the highest DTM (526.4 to 700); (c and e) contrasting mortality pattern between two families with similar low final survival (but DTM of 215 and 439), suggesting differential levels of QDR; (d and f) contrasting mortality pattern over time between two families with similar moderate levels of final survival (but DTM = 150 and 560), suggesting much higher QDR in one family. Color and line types are used to specify type of genetic resistance: MGR (solid sea green line), possible MGR (MGRp) (long dashed gold line), QDR (solid gray line), and low level QDR (QDR-) (dashed orange line)

TABLE 5 Survival percent and Days-to-Mortality (DTM), post-inoculation with *Phytophthora lateralis* of *Chamaecyparis lawsoniana* for six crosses of a 2×3 parent factorial mating. Note the high DTM contrasts with that of the two susceptible controls with <130 DTM (and 0% survival) in trial one and are an indicator of quantitative genetic resistance

Parent	Survival			Days to mortality		
	70016	70064	70072	70016	70064	70072
70234	77.2	4.1	46.9	428.8	247.0	387.1
70070	54.1	2.0	22.4	400.7	165.1	378.0

Junghans et al., 2003; Kinloch, Sniezko, Barnes, & Greathouse, 1999). In trial two, the S_2 family from the 71560 parent also showed 100% survival, and thus parent 71560 is likely homozygous for *Pla*. This parent was a forward selection from the S_1 family of parent SIS-42770 that had shown 92.5% survival in a previous trial (R. Sniezko, unpublished). The combination of MGR and QDR appears to exist in some parents; for example, the crosses involving MGR parent CF1 also showed an increase in DTM (ranging from 31.2% to 54%) over the susceptible controls. The presence of both types of resistance in

some genotypes should increase the likelihood of durable resistance in those trees. The extent to which MGR and QDR operate independently or show epistatic effects in various crosses needs further investigation.

No information is currently available on the number of genes controlling QDR in *C. lawsoniana*. The variation among families in survival and DTM were large in both trials, and this along with the within-family variation in DTM within some families suggests that several-to-many genes may be involved. In some cases, such as the

full-sib family SIS-41924 x SIS-41427, the level of survival is high enough (42.9% with DTM 560.1) to suggest that one of the parents might be heterozygous for *Pla*, but several other crosses with each of these two parents show low survival (6–25%) indicating that high QDR, rather than MGR, is more likely. The underlying basis of QDR is not well-understood, but in other plant species, there are several hypotheses, including the production of metabolites that aid in defense response (Kovalchuk et al., 2013; Poland et al., 2009; St Clair, 2010).

In addition to MGR and QDR, some evidence suggests that there may also be resistance controlled by a recessive gene, notably, the failure to reject the hypothesis of a 1:3 segregation ratio. Recessive genes for resistance have been documented in several crop species (St Clair, 2010), and it has also been documented in at least one tree species, although the number of genes involved was not resolved (Newcombe & Ostry, 2001). Another more likely explanation is that the efficacy of QDR in some families could lead to survival levels that result in significant 1:3 segregation like that expected for a single recessive gene.

7 | CONCLUSION

In the system investigated here three overarching observations can be derived: (1) QDR appears to be present in *C. lawsoniana* and parents or families with QDR can vary widely in survival and/or DTM from each other; (2) MGR appears to be present in *C. lawsoniana* as a dominant major gene (*Pla*) and potentially in a second case as a recessive gene. We have provided multiple lines of evidence suggesting the presence of MGR through several inoculation tests of candidate parents and crosses with susceptible genotypes and tests of Mendelian segregation ratios, all of which suggest that MGR occurs at a low frequency among all the parental genotypes tested; and (3) although we have shown that both QDR and MGR appear to be present in *C. lawsoniana*, and potentially occur in combination, we still do not know how durable the resistance is over the scale of decades in the field. It is important to note that in this study we are not attempting to be definitive on patterns of inheritance (as described in Poland et al. (2009); that is still unknown in many systems with QDR. However, we are illustrating that of those *C. lawsoniana* parent trees identified with resistance we can expect a wide range of survival and time to mortality of progeny of parents with QDR as well as a low frequency of parents with MGR and relatively high survival. Future studies must untangle the many questions relating to the number of genes and their interactions leading to QDR.

The results we have reported here are encouraging. Although most of the trees in forests are highly susceptible, we show here that a large concerted applied program involving over 14,000 initial field selections has located hundreds of parent trees with resistance that can be incorporated into seed orchards and future breeding efforts. The large number of confirmed resistant selections will help maintain the genetic diversity needed in future forests. Ongoing breeding will use this information for forward selections to increase

resistance in individuals that will be used in seed orchards to produce seed for reforestation and restoration. The resistance should also be valuable to re-establish horticultural use of *C. lawsoniana*. Field trials are ongoing to assess the stability, durability, and usability of resistance in *C. lawsoniana* and to further develop the capacity to restore a species impacted by a non-native invasive pathogen. Reforestation and restoration efforts with resistant seed has been underway for over a decade and the knowledge that there are several types of resistance provides us with cautious optimism that resistance will be durable.

The *C. lawsoniana* program to develop resistance to *P. lateralis* offers a successful and concrete example for those contemplating resistance programs in other species. Thousands of field selections were made initially, breeding zones established, several types of screening methodologies developed, tested, and utilized, field trials and seed orchards established (Hansen et al., 2012; Sniezko et al., 2012a; Sniezko et al., 2012b). The development of the program involved the participation of interdisciplinary teams of tree breeders, pathologists, foresters, and organizations, as well as the sustained support from several government agencies. The concerted effort, aided by some biological aspects of this species, has made this ongoing resistance program one of the fastest to develop resistance populations of trees. The work reported here on the types and magnitude of genetic resistance will be incorporated into the breeding program to help ensure that genetically diverse populations of *C. lawsoniana* with resistance to *P. lateralis* are available for restoration and reforestation. Recent testing of some resistant *C. lawsoniana* with other lineages and sources of *P. lateralis* (Robin et al., 2014), and in the field (Sniezko et al., 2012a) provide some support for future durability of resistance, but as with all resistance programs, ongoing monitoring will be needed.

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AUTHOR CONTRIBUTIONS

RAS designed the trials, provided inputs to data summaries, and wrote the initial draft. JSJ assisted in analysis and writing the manuscript. AK and DS provided data summaries and analyses. EH, PR, WS implemented and collected data for the two greenhouse trials and provided pathology support. All authors contributed to writing and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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