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Characterizing the variation in aggressiveness and sporulation of the NA1 and EU1 lineages of *Phytophthora ramorum* in Oregon

Kelsey L. Søndreli[1] | Alan Kanaskie[2] | Sarah M. Navarro[3] | Paul Reeser[1] | Jared M. LeBoldus[1,4]

[1]Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR, USA

[2]Oregon Department of Forestry, Private Forests Program, Forest Health Section, Salem, OR, USA

[3]USDA Forest Service, State and Private Forestry, Forest Health Protection, Portland, OR, USA

[4]Forest Engineering, Resources and Management Department, Oregon State University, Corvallis, OR 97331, USA

Correspondence
Jared M. LeBoldus, Oregon State University, Department of Botany and Plant Pathology, Corvallis, OR 97331, USA.
Email: jared.leboldus@science.oregonstate.edu

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Abstract

Phytophthora ramorum, the cause of sudden oak death, is an invasive pathogen present in parts of coastal California and south-western Oregon forests. The majority of these forest infestations have been caused by the NA1 clonal lineage. In 2015, the EU1 lineage of *P. ramorum* was isolated from a tanoak (*Notholithocarpus densiflorus*) tree located in a mixed-conifer forest of Curry County, Oregon. In order to evaluate the threat to Oregon forests of the EU1 lineage relative to the established NA1 lineage, a series of experiments was conducted comparing aggressiveness and sporulation of NA1 and EU1 isolates on logs and seedlings in the growth chamber and forest. There was no significant difference in lesion size on logs inoculated with NA1 and EU1 isolates for any of the tree species tested. Across all seedling experiments differences among isolates within lineage, in terms of both aggressiveness and sporulation, were more commonly observed than differences among lineages. Site to site variation in tanoak sporulation, as measured by rain bucket baiting, appears to be correlated with the number of *P. ramorum*-positive seedlings detected at each site.

KEYWORDS
aggressiveness, EU1 lineage, *Phytophthora ramorum*, sporulation, sudden oak death

1 | INTRODUCTION

Phytophthora ramorum, the cause of sudden oak death (SOD), is an invasive oomycete pathogen that has become established in parts of coastal California and south-western Oregon mixed-conifer forests (Goheen et al., 2002; Grünwald et al., 2012; Rizzo et al., 2002). This pathogen has a broad host range including at least 130 plant species (Rizzo et al., 2002; Wesela & Randall-Schadel, 2020). The symptoms that develop vary among the hosts and plant parts infected and include necrotic stem and branch cankers, leaf spots, needle and shoot blights, and mortality (Davidson et al., 2003; Webber et al., 2010). In south-western Oregon forests, tanoak (*Notholithocarpus densiflorus*) is the most susceptible species, developing lethal stem cankers and sporulating from infected leaves and twigs (Goheen et al., 2002; Hansen et al., 2008). Although many common understorey species in this region are susceptible (Hansen et al., 2005), the epidemic in south-western Oregon is largely driven by infected tanoak in the overstorey (Hansen et al., 2008).

South-east Asia has recently been proposed as the geographic origin of *P. ramorum* with its recent discovery in the streams of Vietnam (Jung et al., 2020). Four distinct clonal lineages have been identified in Europe and North America. The lineages were named

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for the continent from which they first appeared and are numbered by order of discovery: NA1, NA2, EU1, and EU2 (Grünwald et al., 2009). Until 2015, outbreaks of SOD in forests of California and Oregon have all been caused by the NA1 lineage (Hansen et al., 2003). The NA1 outbreak in Oregon was discovered in 2001, near Joe Hall Creek in Curry County (Hansen et al., 2008), although the disease has probably been present there since 1998 (Goheen et al., 2017). Tanoak mortality resulting from a second introduction of the NA1 lineage was reported in the Hunter Creek region of Curry County in 2011 (Kamvar et al., 2015). These two sites are approximately 35 km apart.

In 2015, the EU1 lineage was recovered for the first time in North American forests from a diseased tanoak tree (Grünwald et al., 2016). This tree was located in the forest approximately 1.7 km from a nursery that had tested positive for EU1 in 2012. The Oregon Department of Forestry (ODF) and the United States Forest Service (USFS) responded to the initial 2015 infestation by removing all tanoaks within c.180 m of the infected trees. However, the EU1 lineage has since spread to at least 47 new locations occupying approximately 405 ha of forestland (Figure 1). In the spring of 2016, the EU1 lineage was isolated from symptomatic Douglas-fir (*Pseudotsuga menziesii*) and grand fir (*Abies grandis*) seedlings underneath infected tanoak (LeBoldus et al., 2017). The symptoms were similar to those reported for conifers infected with the NA1 lineage (Davidson et al., 2002, 2003). To date, both lineages have never been found together at a single site.

The EU1 lineage of *P. ramorum* was first reported in European nurseries and is one of the lineages (along with EU2 in Northern

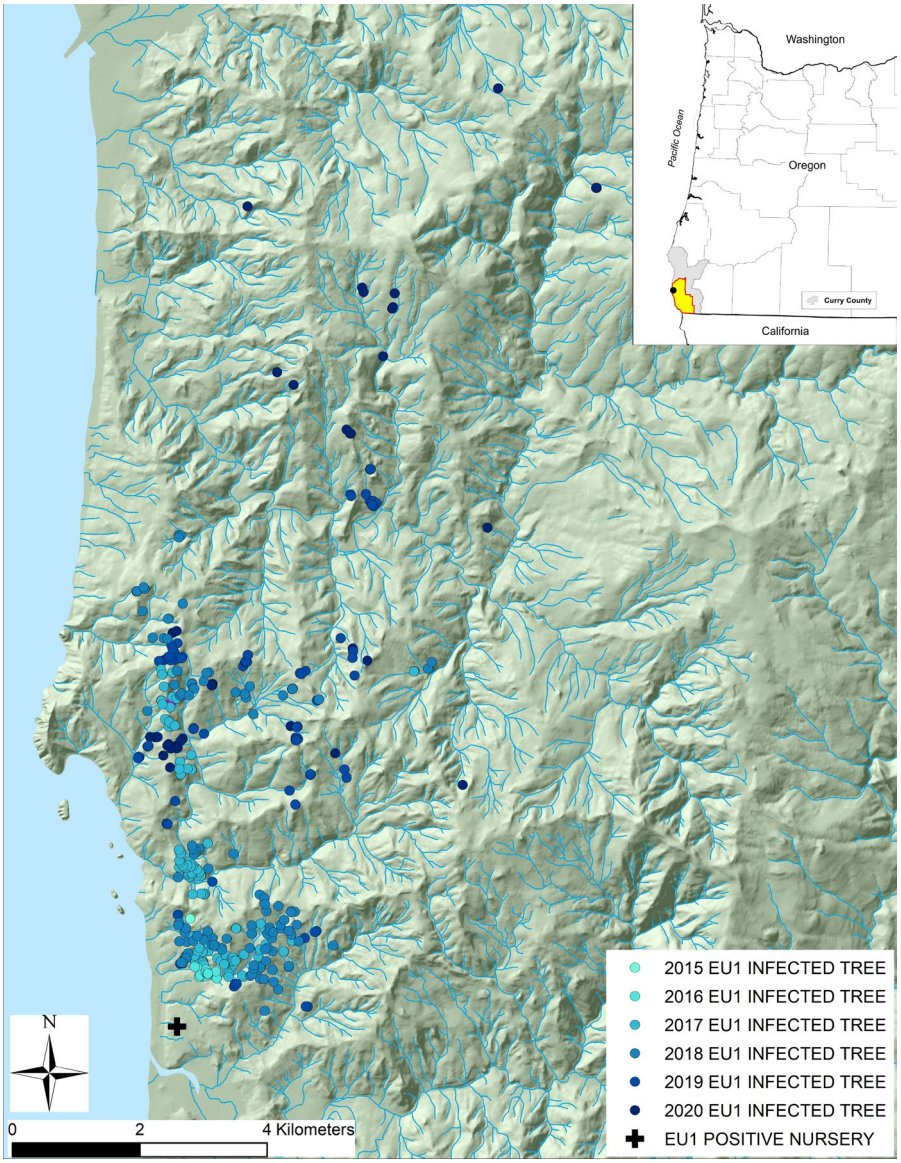


FIGURE 1 Map of EU1 outbreak in Curry County, OR, USA. Each dot represents *Phytophthora ramorum* isolated from a plant with symptoms, typically tanoak, which was subsequently determined to be EU1 or NA1 by sequencing of the lineage-specific *cbel* region. The dots are colour coded by year of collection. Inset: map of western Oregon with Curry County highlighted in yellow [Colour figure can be viewed at wileyonlinelibrary.com]

Ireland and Scotland) causing sudden larch death on *Larix kaempferi* in the United Kingdom and Irish plantations (King et al., 2015; O'Hanlon et al., 2016). The EU1 lineage has been described as more aggressive and able to produce a larger number of sporangia per unit leaf area than the NA1 lineage (Denman et al., 2005; Elliot et al., 2011; Manter et al., 2010; O'Hanlon et al., 2017). However, these comparisons have primarily been conducted on rhododendrons under tightly controlled conditions. No comparisons of aggressiveness, the amount of disease induced by a pathogenic isolate on a susceptible host, or sporulation of EU1 and NA1 isolates on common forest tree species of Oregon have been published.

A recent economic analysis of the EU1 outbreak predicted that if the pathogen were to reach the neighbouring Coos County, the timber export terminal would be under threat with a potential loss of 1,200 jobs and $58 million dollars a year in wages and benefits (Highland Economics and Mason, Bruce & Girard, Inc., 2019). Understanding the potential of the EU1 lineage, in terms of aggressiveness and sporulation on Oregon tree species, may help predict the future impact of SOD in the mixed-conifer forest of southwestern Oregon.

The overall goal of this project was to better characterize the variation in behaviour of the EU1 and NA1 lineages of *P. ramorum* in Oregon forests. The objectives were to (a) determine whether EU1 isolates are more aggressive than NA1 isolates on common Oregon tree species; (b) determine whether there are differences in sporulation between the two lineages among hosts; and (c) compare the incidence of seedling infection under field conditions at NA1- and EU1-infested sites.

2 | MATERIALS AND METHODS

2.1 | Field collections and pathogen identification

During the winter and spring of 2016, *N. densiflorus* and *A. grandis* with symptoms were identified during systematic surveys for SOD in Curry County, Oregon (Goheen et al., 2017). Leaf and stem segments were aseptically removed from plants with symptoms and *P. ramorum* was isolated from bark and shoot tissue using semiselective medium (corn meal agar amended with 20 ppm Delvocid (50% natamycin), 200 ppm Na-ampicillin, and 10 ppm rifamycin SV; CARP). Samples were plated in the field in Curry County, as well as shipped to Oregon State University (OSU) overnight on ice and plated additionally in the laboratory at OSU and incubated at 19 °C in the dark. *P. ramorum* was identified by the presence of characteristic chlamydospores, hyphae, and sporangial morphology and was isolated. DNA was extracted from hyphae using a magnetic particle extraction performed with the Mag-Bind Plant DNA DS kit (Omega Bio-tek Inc.,) and the KingFisher Flex automated extraction platform (Thermo Fisher Scientific) at the Center for Genome Research and Biocomputing at OSU. A portion of the *cbel* gene was amplified and sequenced using the CBEL5 U and CBEL6L primers as described by Gagnon et al. (2014). The sequences of the isolates of unknown lineage were aligned to sequences of *cbel* for reference isolates of NA1, NA2, EU1, and EU2 using the Staden package in GAP v. 4.11.2. The alignments were used to assign each isolate to a lineage (Table 1).

The isolates used in the inoculation experiments described below were each collected from a different tree. The NA1 isolates were randomly selected from all available NA1 isolates collected in 2016 and came from three sites located in Curry County. Because the EU1 outbreak was initially restricted to a single site, isolates were randomly selected for use in inoculation studies from all EU1 isolates collected at that site in 2016 (Figure 1, see 2016 tree locations).

2.2 | Log inoculations

Two separate experiments were conducted to compare the variation in aggressiveness of forest isolates of the NA1 (n = 3) and EU1 (n = 3) clonal lineages of *P. ramorum* (Table 1). Inoculations were conducted on logs freshly cut from bole sections of three forest-grown tree species: Douglas-fir (*P. menziesii*), Oregon white oak (*Quercus garryana*), and tanoak (*N. densiflorus*). For each of the two separate experiments, logs came from the same trees. The logs ranged in size from 10 to 20 cm in diameter and were cut to a length of approximately 1 m. The ends of the logs were sealed with Anchorseal wax emulsion to slow drying. Three logs of each tree species were inoculated with each of the six isolates. A cork-borer was used to make 0.5 cm diameter holes through the bark to the cambium. A plug of agar colonized by *P. ramorum* was placed into each hole and the piece of bark was replaced. Each inoculation point was covered with damp cheesecloth and a square of aluminium foil held in place with duct

TABLE 1 Isolates of *Phytophthora ramorum* used in this study, their host of origin, date of collection, lineage of the isolate, and GPS coordinates for the host tree

Isolate	Host[a]	Date isolated	Location	Lineage	GPS coordinates (lat., long.)
1	Tanoak	2016/05/25	Curry County, OR	NA1	42.11364, −124.27864
2	Tanoak	2016/06/09	Curry County, OR	NA1	42.29202, −124.32580
3	Tanoak	2016/08/10	Curry County, OR	NA1	42.29976, −124.38480
4	Tanoak	2016/08/11	Curry County, OR	EU1	42.29169, −124.39720
5	Grand fir	2016/08/11	Curry County, OR	EU1	42.29163, −124.39736
6	Tanoak	2016/08/09	Curry County, OR	EU1	42.29116, −124.39548

[a]Tanoak (*Notholithocarpus densiflorus*); grand fir (*Abies grandis*).

tape. Control inoculations were conducted in an identical manner except that sterile agar was used. Isolates were randomly assigned to three inoculation points equally spaced around the circumference of the log and each isolate was replicated three times on different logs. Logs were incubated in a growth chamber in plastic bags for 5–6 weeks in the dark. Subsequently, the wrappings were removed, and the outer bark was scraped away with a drawknife to expose the margins of any necrotic area. The length and width of each lesion was measured, and the area estimated as an ellipse minus the area of the inoculation wound (0.2 cm^2). In order to verify *P. ramorum* was the cause of the lesion, reisolations were conducted by removing pieces of tissue at four sides of the margin of each lesion and plating them onto CARP plates. The log experiment was repeated approximately 1 month later using the same method with additional tree species included (Table S1).

2.3 | Seedling inoculations

2.3.1 | Conifers

In total, 140 dormant seedlings of Douglas-fir and western hemlock were provided by ODF in the autumn of 2016. These seedlings were grown at the IFA Nursery Inc. in Elkton, Oregon. Seedlings were planted into Cone-tainers (Ray Leach SC10 Super Cone-tainers, Stuewe and Sons, Inc.) measuring 3.8-cm in diameter and 21-cm deep, filled with growing medium (SunGro Professional Mix #8; SunGro Horticulture Ltd). The medium was amended with 38 g slow-release fertilizer (15-9-12, N-P-K; Scotts Osmocote Plus, Scotts Company Ltd). Plants were placed in a greenhouse with an 18-hr photoperiod supplemented with 600 W high-pressure sodium lamps and a temperature regime of 20/16 °C (day/night). Plants were watered as needed.

Seedling inoculations were conducted when the new apical shoot had elongated to a length of approximately 5 cm. Each seedling was inoculated at two separate positions with the same isolate. Inoculations at position 1 were conducted by removing all needles from 1 cm of new growth immediately adjacent to the apical bud scale scars. A 5 mm-diameter plug of agar colonized by *P. ramorum* was placed over the area where the needles had been removed. The inoculum was secured to the stem by wrapping it with Parafilm. Inoculations at position 2 were conducted by removing a small lateral branch with a sterile scalpel approximately 10 cm from the soil surface. A 5 mm-diameter plug of agar colonized by *P. ramorum* was placed over the wound and wrapped in Parafilm. Ten seedlings of each species were inoculated with the six isolates used above (Table 1), plus an agar control, at each position. Seven days after inoculation, the Parafilm was removed and lesion length and stem girdling were recorded. All inoculation experiments were conducted in a growth chamber with an 18 hr light photoperiod under daylight F32 T8 fluorescent bulbs (Phillips), at day/night temperatures of 20/18 °C. The experiment was repeated the following year as described above with one exception. Trees were inoculated at a single position at the midpoint of the seedling. The change in methodology was a result of the lack of difference in lesion size and sporulation between the inoculation positions.

2.3.2 | Tanoak

In the autumn of 2017, 70 tanoak seedlings from one open-pollinated parent tree were obtained from USDA Forest Service Dorena Genetic Resource Center, Cottage Grove, Oregon. Seedlings were planted in Cone-tainers measuring 3.8-cm in diameter and 21-cm deep filled with growing medium. The growing medium consisted of peat:composted fir bark:perlite:pumice (40:20:20:20) with Apex 16 N:95P_2O_5:10K_2O control-release fertilizer (5–6 month) (J. R. Simplot Company) added prior to sowing. Seedlings were grown in an open greenhouse (not climate controlled) for one year. Irrigation was carefully monitored to avoid overwatering. Slow-release fertilizer was supplemented once per week with a rotation of 17 N:5P_2O_5:24K_2O, 20 N:9P_2O_5:20K_2O, or 20 N:18P_2O_5:18K_2O, depending on weather conditions and physiological state of the seedlings.

The tanoak seedlings were inoculated with the six isolates of *P. ramorum*, described above (Table 1), and an agar control (10 trees for each isolate and 10 mock-inoculated controls; total = 70 trees). The trees were inoculated at a single position by removing a leaf at the midpoint of the seedling, placing the *P. ramorum* agar plug over the wound, and wrapping it in Parafilm. Seven days after inoculation, the Parafilm was removed and the lesion length and stem girdling were recorded. All inoculation experiments were conducted in the same manner. This experiment was repeated two more times in the autumn of 2018; thus, all inoculations were conducted at a similar time of year and when trees were in a similar phenological state. Each experiment was conducted with 70 tanoak seedlings grown from acorns of one open-pollinated parent tree. In each experiment the parent tree was different.

2.4 | Seedling sporulation

2.4.1 | Conifers

A sporulation experiment was conducted using the same conifer seedlings that had been inoculated in the experiment described above. Following measurement, the lesion was removed and placed in a 100 × 25 mm Petri dish above damp filter paper, sealed with Parafilm for 5 days, and incubated at room temperature (approximately 20 °C). Subsequently, sporangia were harvested from infected plant material by washing the two lesions from each treatment together in 100 ml deionized water. Washing was conducted in a 250 ml Erlenmeyer flask on an orbital shaker at approximately 200 rpm for 10 min. Wash water was poured through a 180 μm sieve and then through a vacuum filter funnel containing a 47 mm diameter, 14 μm pore size, polycarbonate filter, on which the spores were

collected. Filters were transferred to an agar plate containing antibiotics to inhibit sporulation and bacterial growth (1 L distilled water, 15 g agar, 300 mg cyclohexamide, 100 mg streptomycin, 50 mg tetracycline.HCl). The entire area of spore deposition was covered with a few drops of lactoglycerol and a cover slip. Sporangia on the membrane were counted along two transects (37 mm long, 0.4 mm wide) across each filter, using a Zeiss Standard microscope with 40× objective. The count of sporangia in each transect was divided by a factor derived from the area of transect relative to area of deposition to estimate the total number of sporangia deposited on the membrane. This count was considered to be the number of sporangia per cm of the two lesions. Reisolation of *P. ramorum* following sporangial harvest was conducted by plating a subset of randomly selected lesions (n = 26) and controls (n = 4) on CARP. This experiment was repeated the following year but with only one inoculation position on each conifer seedling. The lesions in the second experiment were each washed separately.

2.4.2 | Tanoak

For each of the three tanoak inoculation experiments described above, sporulation was induced by spraying the seedlings with water 7 days after inoculation and placing clear polyethylene bags over each tree and sealing them to the Cone-tainer. The bags were removed 4 days later and the length of each lesion was measured. Following measurement, the lesion was cut out from the stem, and sporangia were harvested by washing in 100 ml deionized water. Sporangia were washed and counted as described above for conifer seedlings. Reisolation of *P. ramorum* following sporangial harvest was conducted by plating all necrotic lesions (n = 60) and controls (n = 10) on CARP.

2.5 | Field planting

A field experiment was conducted in order to evaluate the relative susceptibility of Douglas-fir, Sitka spruce (*Picea sitchensis*), western hemlock, and western larch (*Larix occidentalis*) seedlings to the two clonal lineages, NA1 and EU1, of *P. ramorum* under field conditions. The experiment was conducted in 2017, and 2018/2019. In 2017, two sites with active infestations of NA1 (latitude 42.29371, longitude −124.39964) and EU1 (latitude 42.19450, longitude −124.35078) were selected. At each site, dormant seedlings provided by the ODF nursery described above (Douglas-fir, Sitka spruce, western hemlock) or purchased from the Franklin H. Pitkin Forest Nursery at the University of Idaho (western larch) were planted under infected tanoak on 12 April 12 2017. In December 2018, two NA1 sites (Site 1 = latitude 42.19455, longitude −124.35076; Site 2 = latitude 42.18632, longitude −124.34298) and two EU1 sites in Curry County (Site 1 = latitude 42.29321, longitude −124.3941; Site 2 = latitude 42.29326, longitude −124.39243) with active infestations were selected and planted. The experimental design was similar for both experiments, with 20 seedlings of each species planted at each site in a completely randomized design. In 2017, one site for each of NA1 and EU1 was selected because there was only one active untreated EU1 site available at that time. As the outbreak expanded, two sites were selected in the subsequent year.

Two 8 L buckets containing approximately 1 L of distilled water with two tanoak and two rhododendron leaves were placed among the seedlings as bait to monitor each site for sporulation (i.e., rain baiting buckets). The bait leaves were removed and replaced four times between April and June 2017 and 11 times between December 2018 and June 2019. The leaves were taken to the OSU lab in Corvallis, and sections of the bait leaves that showed symptoms were excised and plated onto CARP. Developing colonies were monitored for 2 weeks and *P. ramorum* infection was confirmed based on microscopic colony morphology.

After 10 weeks in the 2017 sites and 6 months in the 2018/2019 sites, seedlings were collected by cutting them at the ground line and tissue with symptoms was washed in 5% bleach for approximately 30 s, rinsed twice in deionized water, and blotted dry. The petiole and portions of necrotic tissue from lesions were excised and plated onto CARP. Up to 10 needles with symptoms and stem segments totalling 10 cm in length, encompassing the bud scales, were removed from each seedling and plated. If no tissue with symptoms was observed, the top 10 cm of the seedling was plated. *P. ramorum* infection was confirmed by hyphal morphology and the presence of characteristic sporangia and chlamydospores.

2.6 | Statistical analysis

2.6.1 | Log inoculations

Two separate log inoculation experiments were conducted with Douglas-fir, tanoak, and Oregon white oak (Table 2). In both cases the experimental design was a completely randomized design with three replicates for each species by isolate combination. The two experiments were analysed together. A mixed model in the lme4 (Bates et al., 2007) package in R (R Core Team, 2016) was used to test the effect of lineage, isolate nested within lineage (isolate [lineage]), experiment, and replicate log on lesion area of the inoculated logs. Lineage was considered a fixed effect while isolate (lineage), experiment, and replicate log were considered random effects. Statistical significance was assessed at α = .05. A two-step model selection process was used to test significance of both fixed and random effects. The order of testing was determined based on the magnitude of the default z test in the lme4 package; the parameter with the largest value was tested first. The likelihood ratio chi-square test was used to test the significance of each parameter. Following model selection, lineage means were compared using the lmer Test package in R (Kuznetsova et al., 2015). The effect of each parameter (lineage, isolate [lineage], experiment, log) on lesion area for each tree species was analysed using a separate model.

TABLE 2 Variance estimates (σ^2) and *p* values for the random effects of Log, Experiment, and Isolate (lineage) from the mixed effects models comparing lesion area (cm^2) on three common southwestern Oregon tree species following inoculation with isolates of *Phytophthora ramorum* of the clonal lineages NA1 (*n* = 3) and EU1 (*n* = 3)

	Douglas-fir		Tanoak		Oregon white oak	
Source	σ^2	*p*	σ^2	*p*	σ^2	*p*
Log	235	.197	0	1	64	1
Experiment	307	.138	4,105	<.001	2,547	.071
Isolate (lineage)	350	.164	1,113	.143	0	1
Total	1,343	—	4,009	—	8,246	—

2.6.2 | Seedling inoculations

For both the conifer and tanoak seedling inoculations, the experimental design was a completely randomized design with each isolate by tree species combination occurring 10 times. A model using the mixed procedure in SAS v. 9.4 (SAS Institute) was used to analyse the random effects of replicate and isolate (lineage) and the fixed effects of lineage and inoculation placement on lesion length and sporangia per mm (Littell et al., 2006). Statistical significance was assessed at $\alpha = .05$. Model selection was conducted as described above. The significance of the fixed effects was evaluated using the *F* test for the fixed effects in the mixed model output of the final model. When necessary, unequal variances were modelled using the repeated statement in SAS. Following model selection, isolate (lineage) means were compared using the best linear unbiased predictors (BLUPs) and the 95% prediction intervals that were generated by the estimate statement in SAS v. 9.4 (Littell et al., 2006). The LSMEANS statement was used with a Tukey's correction to test the hypothesis that isolates from the EU1 lineage produce larger lesions and more sporangia than isolates from the NA1 lineage. Data for both lesion length and sporulation were analysed in a similar manner. In addition, the proportion of trees girdled by the two lineages were compared using the fischer.test function in R (R Core Team, 2016).

2.6.3 | Field planting

The proportion of infected trees for each species at EU1- and NA1-infested sites was reported. The number of positive seedlings from each year and site were reported separately. No statistical analysis was performed.

3 | RESULTS

3.1 | Log inoculations

The appearance and development of lesions were similar to those reported previously in the literature. Necrosis within the vascular cambium was observed immediately beneath the bark, typically centred on the inoculation point, and was longitudinally greater than tangentially. Comparisons between NA1 and EU1 isolates indicated no differences in mean lesion area between the two lineages (Figure 2), although the range of lesion sizes was twice as large for those caused by the EU1 lineage than the NA1 lineage (Figure 2). There was no effect of isolate (lineage), experiment, or log on the variation in lesion area reported for any of the species tested, with the sole exception of tanoak, where there was a significant effect of experiment on the variation in lesion area (Table 2). Lesions on additional species, not included in the analysis, were comparatively smaller (Table S1). Bigleaf maple (*Acer macrophyllum*) and Pacific madrone (*Arbutus menziesii*) differed in mean lesion area between the two lineages (Table S1). There were no significant differences in mean lesion area among isolates, for any of the species. *P. ramorum* was reisolated from 42 of 54 lesions in each experiment. All reisolations made from the mock-inoculated controls were negative for *P. ramorum*.

3.2 | Seedling inoculations

3.2.1 | Conifers

Symptom appearance was similar to previous reports in the literature for both Douglas-fir and western hemlock with dark sunken cankers on the main stem and wilting of the expanding shoots. In experiment 1, 72% and 53% of the Douglas-fir seedlings were girdled 7 days after inoculation at the upper and lower inoculation positions, respectively. A similar pattern was evident with western hemlock, with 72% and 57% of the seedlings girdled at upper and lower inoculation points, respectively. There were no significant differences in girdling between lineages for either species in experiment 1. In experiment 2, significantly more of the Douglas-fir seedlings inoculated with EU1 isolates were girdled (63%) than the NA1-inoculated seedlings (37%) ($p = .0006$). A similar pattern was apparent for western hemlock where 63% of EU1-inoculated trees were girdled compared to 40% of NA1-inoculated trees ($p = .0028$).

There were no significant differences in lesion length between the EU1 and NA1 lineages when either Douglas-fir or western hemlock seedlings were inoculated (Table 3). This pattern was consistent across the inoculation points and experiments, with the sole exception of western hemlock in experiment 1, where larger lesions

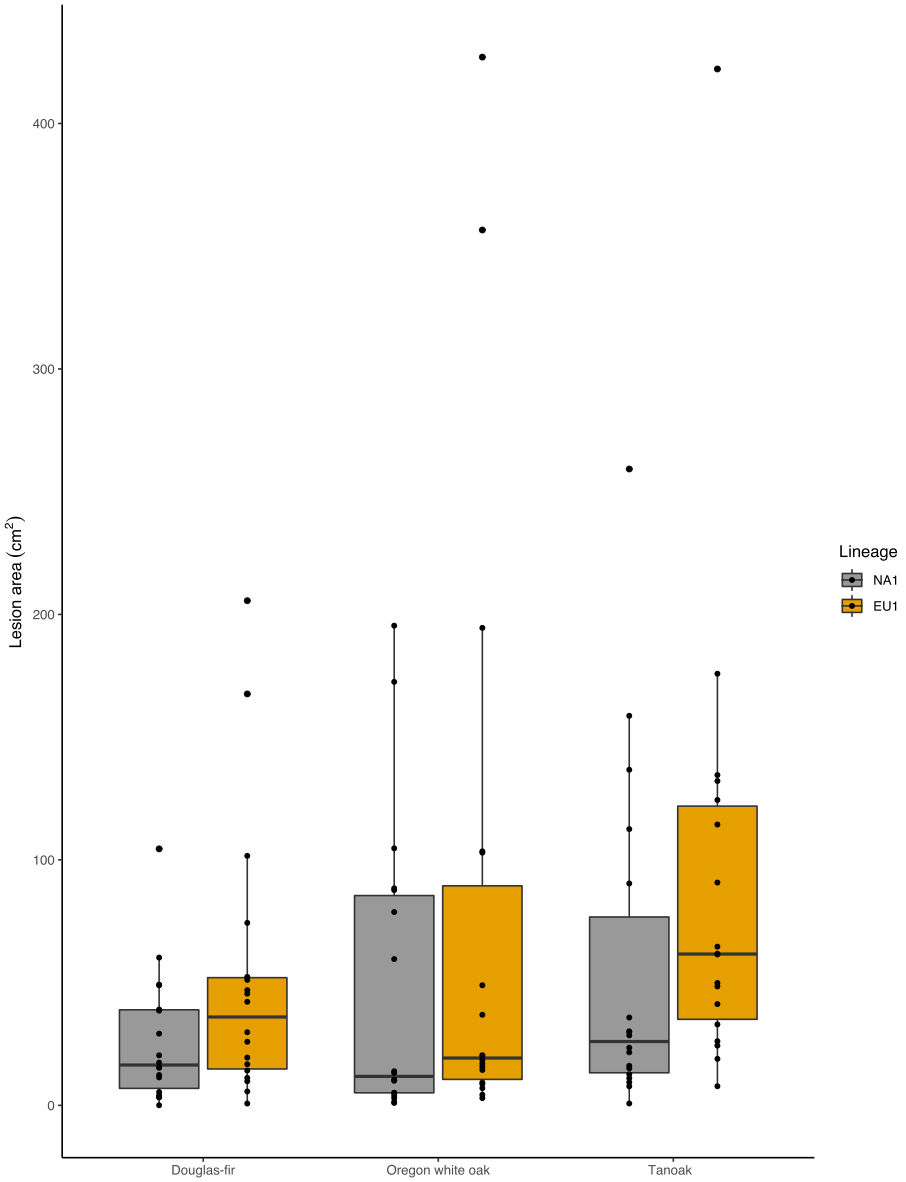


FIGURE 2 Dot plots superimposed on box plots comparing mean lesion area (cm^2) and range of observations among three common southern Oregon tree species following inoculation of logs with isolates of the NA1(n = 3) and EU1 (n = 3) clonal lineages of *Phytophthora ramorum*. The results from the two experiments were combined. Each individual point is a data point, the line in the middle of the box is the median value; the upper and lower hinges of the box represent the 25th and 75th percentiles; and the whiskers approximately represent the 95% confidence interval [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Comparisons of mean lesion length (cm) for Douglas-fir, western hemlock, and tanoak seedlings inoculated with isolates of the NA1 and EU1 clonal lineages of *Phytophthora ramorum* for each experiment

		Lineage				
		NA1		EU1		
Species	Experiment	Mean	Range	Mean	Range	*p*
Douglas-fir	1	2.80	0.0–7.4	4.11	0.0–10.0	.36
	2	1.82	0.2–5.1	3.52	0.2–6.9	.17
Western hemlock	1	2.23	0.0–7.6	2.50	0.0–5.7	.39
	2	1.94	0.1–3.5	2.11	0.2–5.1	.38
Tanoak	1	3.96	0.0–7.5	6.27	2.8–10.7	**.03**
	2	5.10	0.4–8.9	5.91	3.1–8.6	.14
	3	2.48	0.3–5.1	3.43	0.2–6.1	.23

Note: Values in bold indicate significant p values with $p < .05$ using t test.

consistently developed on the lower stem position (i.e., older tissue) irrespective of lineage. Despite the lack of differences in lesion length between the two lineages there were, nonetheless, differences among isolates in terms of lesion length on Douglas-fir seedlings in experiment 1 and 2, and western hemlock seedlings in experiment 1 but not experiment 2 (Figure 3).

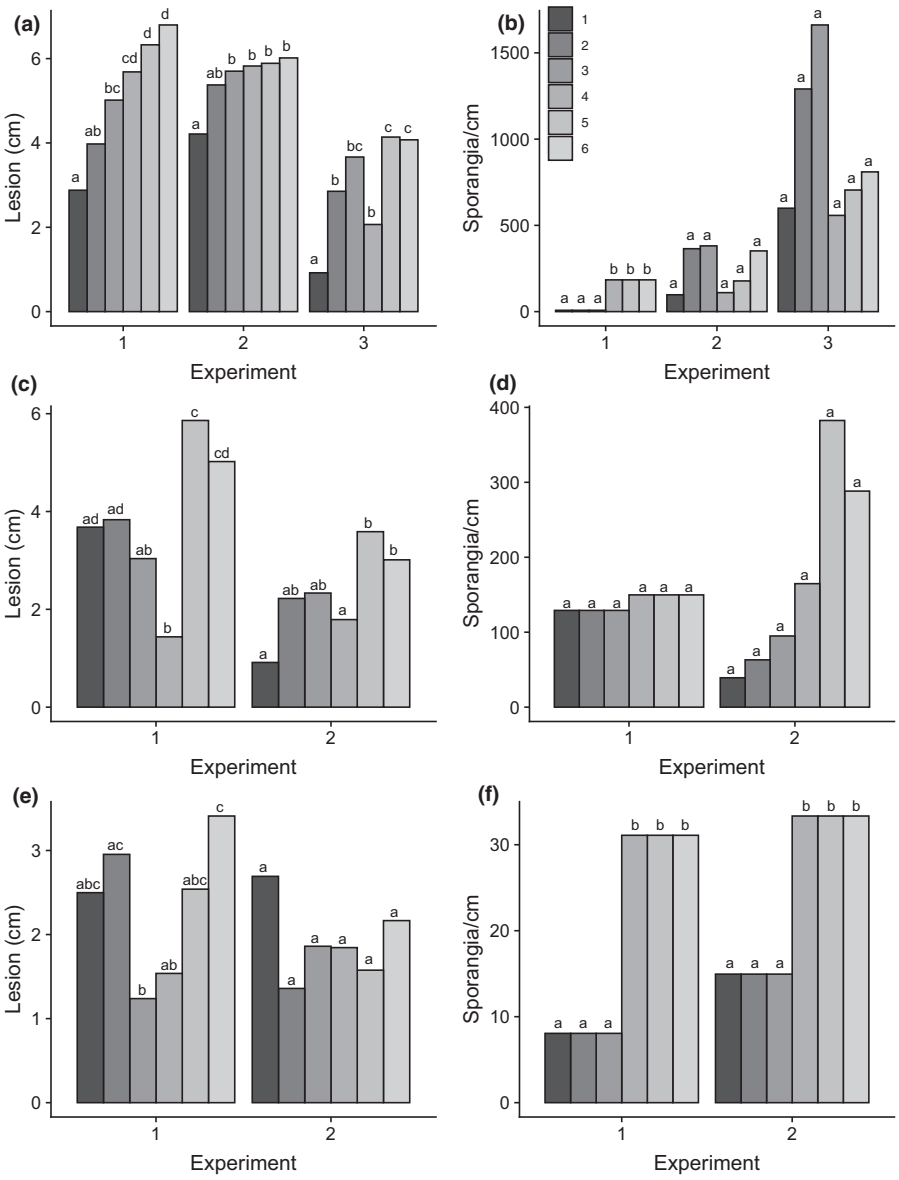


FIGURE 3 Comparison of infections by six isolates of NA1 and EU1 lineages of *Phytophthora ramorum* on tree seedlings; isolates 1–3 are NA1 and isolates 4–6 are EU1. The bar charts show lesion length (cm) and number of sporangia per cm for tanoak (a, b), Douglas-fir (c, d), and western hemlock (e, f). Each cluster of bars is a different experiment and bars with different letters are significantly different (α = .05)

3.2.2 | Tanoak

In experiment 1, significantly more tanoak seedlings inoculated with EU1 isolates were girdled (83%) than tanoak seedlings inoculated with NA1 isolates (30%) ($p < .0001$). A similar difference was observed in experiment 2 where 77% and 30% of seedlings inoculated with EU1 and NA1 isolates, respectively, were girdled ($p < .0001$). The pattern was different in experiment 3 where 20% of the seedlings inoculated with EU1 isolates were girdled and 23% of seedlings inoculated with NA1 isolates were girdled ($p = .718$). When comparing the lineages in terms of lesion length, EU1 isolates on average produced larger lesions in experiment 1 than NA1 isolates (Table 3). In contrast, lesion lengths were similar between EU1-inoculated seedlings and NA1-inoculated seedlings in experiments 2 and 3 (Table 3). There were significant differences among isolates within the same lineage in terms of lesion length in all three experiments (Figure 3).

3.3 | Seedling sporulation

3.3.1 | Conifers

There were no differences in terms of sporangia/cm produced by NA1- and EU1-inoculated Douglas-fir seedlings (Table 4; Figure 3d). This pattern was consistent across inoculation positions. In contrast, with western hemlock, in experiment 1, inoculation with EU1 produced a larger number of sporangia per cm than inoculation with NA1; however, this was not observed in experiment 2 (Table 4). There were no significant differences among the six isolates for inoculated Douglas-fir in terms of sporangia/cm (Figure 3d). When reisolations were carried out from Douglas-fir and western hemlock, 24 of 26 lesions were positive for *P. ramorum* and 0 of 4 controls were positive in experiment 1. In experiment 2, 55 of 60 Douglas-fir lesions and 0 of 10 controls were positive, and 41 of 60 western hemlock lesions and 0 of 10 controls were positive.

TABLE 4 Comparisons of mean sporangia per cm of lesion for Douglas-fir, western hemlock, and tanoak seedlings inoculated with isolates of the NA1 and EU1 clonal lineages of *Phytophthora ramorum* for each experiment

		Lineage				
		NA1		EU1		
Species	Experiment	Mean	Range	Mean	Range	*p*
Douglas-fir	1	129.10	0.0–77.4	149.79	0.0–85.0	.38
	2	65.76	0.0–274.0	278.52	0.0–508.9	.09
Western hemlock	1	8.06	0.0–7.3	31.10	0.0–13.0	**.01**
	2	14.95	0.0–127.9	33.34	0.0–219.2	.05
Tanoak	1	7.73	0.0–61.5	184.16	0.0–768.1	**<.01**
	2	281.15	0.0–1678.9	213.30	6.9–1564.3	.35
	3	1184.01	0.0–8687.5	690.90	0.0–5886.3	.24

Note: Values in bold indicate significant *p* values with $p < .05$ using *t* test.

TABLE 5 The infection of conifer tree seedlings by *Phytophthora ramorum* in field planting trials in 2017 and 2018/2019

	2017		2018/2019			
	NA1	EU1	NA1		EU1	
Species	Site 1	Site 1	Site 1	Site 2	Site 1	Site 2
Douglas-fir	2/20	11/30	0/20	0/20	4/20	0/20
Western hemlock	0/22	2/28	0/20	0/20	0/20	0/20
Sitka spruce	0/21	11/20	0/20	1/20	15/20	0/20
Western larch	2/15	27/30	5/20	2/20	16/20	1/20

Note: Each number refers to the number of *Phytophthora ramorum*-positive seedlings determined after plating of tissues, divided by the total number of trees planted.

3.3.2 | Tanoak

In experiment 1 the EU1-inoculated tanoak had 10 times the number of sporangia/cm than NA1-inoculated tanoak. In contrast, there were no differences among lineages in experiment 2 and 3. However, there were differences among isolates in all three experiments for lesion length (Figure 3a). When reisolations were made from tanoak, 60 of 60 lesions were positive for *P. ramorum* in all experiments. All 10 controls in all three experiments were negative for *P. ramorum*.

3.4 | Field planting

In 2017, the numbers of *P. ramorum*-positive seedlings were higher at the EU1 site compared to the NA1 site for Douglas-fir, western hemlock, western larch, and Sitka spruce (Table 5). In 2018/2019 there were differences in the number of *P. ramorum*-infected seedlings among species and sites (Table 5). When examining the number of positive seedlings for each species on a site-by-site basis, the largest differences were among sites. In 2017, there were a total of 10 rain baiting bucket collection intervals containing bait leaves exposed at each site from April to June. At both sites, eight out of 10 buckets of bait yielded leaf baits that were positive for *P. ramorum*. Beginning in December 2018 and ending in June 2019, a total of 22 bucket rain bucket collection intervals of bait leaves were collected at each site. At the NA1 sites, 8 (site 1) and 13 (site 2) collections of bait leaves yielded *P. ramorum*. In contrast at the EU1 sites, 16 (site 1) and 5 (site 2) collections of bait leaves yielded *P. ramorum*. These differences in *P. ramorum*-positive seedlings at each site appeared to be correlated to the inoculum exposure at each site (Tables S2 and S3).

4 | DISCUSSION

Symptoms of the NA1 lineage of *P. ramorum* have been characterized for most of the common susceptible plant species found in the mixed-conifer forests of south-western Oregon and California (Davidson et al., 2003; Hansen et al., 2005). Lesion development in the log inoculations described above was consistent with previous descriptions, regardless of clonal lineage, for all three species (Hansen et al., 2005). Although not statistically significant, all lesions produced by EU1 isolates were larger, regardless of host species, than lesions produced by NA1 isolates. This is consistent with the literature, where the EU1 lineage of *P. ramorum* is generally considered more aggressive (Denman et al., 2005; Elliot et al., 2011; Manter et al., 2010; O'Hanlon et al., 2017). However, the variation in lesion size caused by the EU1 isolates was similar to the variation in lesion size caused by the NA1 isolates. This large variation in size

within each host species by lineage combination, and the residual variation, potentially masked the ability to detect statistical differences among lineage.

The variance components for log, experiment, and isolate (lineage) varied depending on host species. For both tanoak and Oregon white oak, the variance estimates were largest for experiment, suggesting that there were probably differences in host resistance (De Dobbelaere et al., 2010; Grunwald et al., 2008; Huberli et al., 2012; Søndreli et al., 2019), chemistry (Nagle et al., 2011), time of year of collection of the trees (Harris & Webber, 2016), and bark moisture (Oßwald et al., 2014), which contributed to the observed variation. Although efforts were made to ensure that logs were all of uniform size, harvested from a single tree for each experiment with a similar canopy position and overall health, future work should incorporate a larger number of logs, improving the ability to partition the variance among sources and detect differences among the lineages.

In contrast to the log inoculation, the variation associated with seedling inoculations was less a result of the residual variation, but rather related to differences among lineages and isolate (lineage). Across conifer species, lesion length differed among isolates. There was no effect of lineage on lesion length on Douglas-fir and western hemlock. However, differences among isolates within lineage were noted. In most of the seedling experiments, significantly more EU1-inoculated trees were girdled than NA1-inoculated trees. This result is significant because girdling typically results in seedling mortality. In terms of sporulation, the only difference in counts of sporangia between NA1- and EU1-inoculated conifers was observed for western hemlock in experiment 1. Overall, the number of sporangia produced on Douglas-fir and western hemlock in the sporulation experiment was low compared to the number produced on inoculated tanoak seedlings, indicating that infected Douglas-fir and western hemlock would probably only contribute a small amount to inoculum production in south-western Oregon forests.

Similar differences among experiments were apparent when comparing the inoculated tanoak seedlings. For example, tanoak developed larger lesions when inoculated with EU1 isolates relative to NA1 isolates in experiment 1, but not experiments 2 and 3. This difference in experiment 1 was consistent with the measures of sporulation, with more sporangia being produced by EU1 lesions in experiment 1. The combination of larger lesions and a greater number of sporangia per unit of lesion length observed for EU1 in experiment 1 is consistent with reports for rhododendron (Denman et al., 2005; Elliot et al., 2011; Manter et al., 2010; O'Hanlon et al., 2017). This suggests a potentially greater impact on susceptible forest species within the area of the EU1 outbreak. This is particularly alarming given observations from the UK that Douglas-fir boles can become infected when exposed to the massive amounts of sporangia produced by *P. ramorum* on Japanese larch (*L. kaempferi*); currently, field observations of infections by the NA1 lineage in Oregon and California have shown that Douglas-fir infection symptoms are limited to wilting and twig dieback (King et al., 2015).

The lack of differences in sporulation between NA1- and EU1-inoculated tanoak in experiments 2 and 3 is noteworthy. Several possible explanations exist for this difference from the results of experiment 1. The simplest explanation is genetic variation among the host genotypes used in each experiment. The mother trees from which the acorns were collected were different for all three experiments, and seedlings were all half-sibs with the pollen contribution coming from an unknown number of donors. This hypothesis is supported by Søndreli et al. (2019), who reported a family by isolate interaction when evaluating the variation in susceptibility of 30 tanoak families to NA1 and EU1 isolates. A second possible explanation for the differences among experiments was proposed by Kasuga et al. (2016), who hypothesized that environmental and host differences can trigger genome level changes in *P. ramorum*, affecting pathogenicity and virulence. However, although one of the six isolates was collected from grand fir rather than tanoak, it did not appear to impact the isolates' ability to infect and cause disease. Finally, the three experiments were conducted at different times of the year, which may have contributed to the observed differences (Harris & Webber, 2016).

The impact of sporulation on the epidemiology of SOD can be seen when comparing the number of *P. ramorum*-positive seedlings planted at the EU1 and NA1 sites in 2017. Rain baiting buckets indicated that the seedlings at both sites were exposed to *P. ramorum* inoculum for approximately the same period of time in 2017. The number of *P. ramorum*-positive seedlings was much higher for all species at the EU1 site than at the NA1 site. The probable explanation for this difference is an increase in sporulation on EU1-infected tanoak relative to NA1-infected trees under the environmental conditions of the 2017 field season. In contrast, the differences in infection observed in the 2018/2019 experiment appear to be dependent on both site and lineage. Specifically, trees planted at EU1 site 2 appear to have been exposed to fewer windows of inoculum than trees planted at EU1 site 1. The reduced inoculum exposure potentially resulted in fewer *P. ramorum*-positive seedlings. Although sites were selected in order to have similar basal area of infected tanoak, differences in either sporulation or the environmental conditions driving sporulation probably resulted in the different number of positive seedlings. The importance of rain and warm temperatures as drivers of sporulation and infection for the NA1 lineage has been well documented (Eyre et al., 2014; Garbelotto et al., 2017).

The EU1 lineage was first reported in Oregon forests in 2015 on a single infected tanoak. By 2020, the EU1 outbreak had occupied 405 ha of forestland at 47 separate locations. This pattern of spread is similar to that initially reported for the NA1 lineage (Hansen et al., 2008; Kamvar et al., 2015). It is important to note that EU1 now occurs in proximity to trees infested with NA1. The two lineages of *P. ramorum* are different mating types. Although unlikely, as mating success is low between isolates of *P. ramorum* (Boutet et al., 2010; O'Hanlon et al., 2017), the presence of the two mating types in the same area of forest raises the risk of sexual reproduction, which has the potential to produce more virulent genotypes (Boutet et al., 2010; Grünwald et al., 2016).

In addition, two important caveats to the experiments discussed above are the method of inoculation used and the physiological differences in the response of logs versus the response of intact seedlings.

Wound inoculation, although useful for comparing aggressiveness and sporulation among isolates is not representative of natural infection and disease development under field conditions. In addition, the response of cut logs compared to intact seedlings is also likely to be different, potentially impacting the conclusions. As a result, care should be taken when extrapolating the results from wound inoculations on cut logs to susceptibility of trees under field conditions.

Three lines of evidence support the hypothesis that the EU1 clonal lineage of *P. ramorum* has the potential to be more aggressive and could pose a greater risk to Oregon forests than the NA1 lineage. Cankers produced on all logs inoculated with EU1 were, on average, twice the size of cankers produced by the NA1 lineage; however, the variation in lesion size was similar for both lineages. Across all experiments a greater number of EU1-inoculated trees became girdled and died than those inoculated with NA1; this is a key factor in determining seedling survival. Tanoak seedlings inoculated with EU1 isolates also produced more sporangia than NA1-inoculated tanoak. Finally, under conducive environmental conditions Douglas-fir, western hemlock, western larch, and Sitka spruce seedlings appear to have a greater risk of infection at EU1-infested sites compared to NA1-infested sites. Management of SOD in south-western Oregon has recently emphasized the detection and eradication of all EU1-infected trees. Given the limited resources available for monitoring and treatment, targeting the EU1 infestation, with its potential for a greater impact to the mixed-conifer forests of south-western Oregon, is justified. However, continued research across a wider range of sites, environmental conditions, and species is essential to better understand the relative impacts of the two lineages on the coastal mixed-conifer forests of south-western Oregon and California.

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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.

ORCID

Jared M. LeBoldus https://orcid.org/0000-0003-3303-4269

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SUPPORTING INFORMATION

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

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