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Intraspecific variation in host susceptibility and climatic factors mediate epidemics of sudden oak death in western US forests

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Umbellularia californica is one of the key infectious hosts of the exotic *Phytophthora ramorum*, which causes sudden oak death (SOD) in California and Oregon forests. This study provides a comprehensive analysis of the epidemiologically relevant parameters for SOD in California and southern Oregon, including potential differences between the two states. Experimental infection of *U. californica* leaves was optimal when leaves were wet for 6–12 h, temperature was approximately 19°C and pathogen concentration was approximately $2{\cdot}7 \times 10^4$ zoospores mL^{-1}. Seasonal variation in host susceptibility and disease incidence was examined for two populations by inoculating detached leaves at 12 dates and by monitoring naturally infected leaves, respectively. Susceptibility of *U. californica* and disease incidence varied significantly in time and the variation was highest for both in spring. Susceptibility of trees from 17 natural populations from California and southern Oregon was assessed in detached leaf inoculations. One California and three southern Oregon populations had significantly and repeatable lower average susceptibility in artificial inoculations, but differences among three selected California and Oregon populations were not significant in inoculations of seedlings grown from seed in a common garden. This study concludes that *U. californica* susceptibility has a large environmental component, yet still predicts potential disease severity in different sites especially where infestations are young or the pathogen has not yet arrived. The accuracy and utility of predictive risk models for *P. ramorum* will be enhanced by the inclusion of both the environmental and host susceptibility components.

Keywords: disease risk spread, foliar necrosis, oomycete, plant–pathogen interaction, seasonal variation

Introduction

Phytophthora ramorum, the causal agent of sudden oak death (SOD), presents a significant and costly problem to the western USA and Europe, where it has been recently introduced through the nursery trade (Ivors *et al.*, 2006; Mascheretti *et al.*, 2008) from an unknown origin (Werres *et al.*, 2001; Rizzo *et al.*, 2002). In California, it has killed many thousands of oaks (*Quercus agrifolia* and *Q. kelloggii*) and tanoaks (*Notholithocarpus densiflorus*) (Rizzo *et al.*, 2002; Meentemeyer *et al.*, 2008). It is unusual as a forest pathogen in that the epidemic in California is driven primarily by one foliar host, California bay laurel (*Umbellularia californica*), which supports abundant foliar sporulation but is tolerant of infection (Davidson *et al.*, 2005, 2008). Within this complex framework, regional differences have been reported for the west coast of North America. For instance, in forest stands of Oregon and California where tanoaks are the dominant species, *N. densiflorus* is believed to be the main contributor to sporulation (Davidson *et al.*, 2008; Hansen *et al.*, 2008).

Survival and establishment of invasive pathogens depends on the presence of susceptible hosts and on a disease-conducive environment, while invasion is mediated by ecological and biological interactions between the hosts and the pathogen (Burdon *et al.*, 1989; Gilbert, 2002). In the case of *P. ramorum*, pathogen populations are comprised of genetically similar individuals, clonally derived from a few founding individuals (Mascheretti *et al.*, 2008, 2009). Three distinct lineages of *P. ramorum* are known worldwide of which only the NA1 lineage is involved in the forest epidemic in the western USA (Ivors *et al.*, 2006; Grünwald *et al.*, 2009). Genotypic variability appears limited within

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wild populations of the pathogen where a single lineage is present (Ivors *et al.*, 2006). Although phenotypic differences among the different clonal lineages (Elliott *et al.*, 2011) and isolates from different hosts (*Q. agrifolia* and *U. californica*) have been observed (Hüberli & Garbelotto, 2011), until further variability develops within individual lineages of the pathogen, or additional lineages are introduced in the wild, disease will most likely be driven by host and environmental factors.

Both temperature and moisture play a key role in any *Phytophthora* disease interaction with a susceptible host. Whilst the parameters of sporulation are known for *U. californica* (Davidson *et al.*, 2005, 2008), the parameters required for infection of this or any other native hosts are unknown. Foliar infection and necrosis precede sporulation (Davidson *et al.*, 2005, 2008) and, in the case of *U. californica*, there appears to be no significant trade-off between severity of the disease and transmission (i.e. sporulation), with the two being positively correlated (Anacker *et al.*, 2008). Recently, Tooley *et al.* (2009) investigated the requirements for infection of *Rhododendron* cv. Cunningham's White by *P. ramorum* and found that disease was greatest at 20·5°C and moisture periods of 24–48 h. This information is currently not available for *U. californica*.

Host density, in particular for *U. californica*, has been reported to be positively correlated to the spread of *P. ramorum* in California (Meentemeyer *et al.*, 2004, 2008, 2011; Swiecki & Bernhardt, 2008). Differences in susceptibility within a host population may additionally influence the pathogen's capacity to establish, sporulate and spread across the landscape. Varying susceptibility among individuals and/or populations of epidemiologically relevant hosts may help to drive the well-documented patchiness in disease distribution (Meentemeyer *et al.*, 2004, 2008, 2011). As the authors fully acknowledged, these models did not consider intraspecific host susceptibility, which, if present, could increase the models' predictive accuracy.

Umbellularia californica is expansive in its range of habitats encompassing diversity in climate, soil structure and associated forest species. Its native range extends from Umpqua River Valley of Douglas County in Oregon to southern San Diego County in California, and 257 km inland to southern Sierra Nevada (Stein, 1990; Fig. 1). It is likely that some adaptive genetic variation exists among populations. Earlier work by Anacker *et al.* (2008) over a small spatial scale within Sonoma County suggested there was a genetic basis for susceptibility observed in detached leaf inoculations, but that local environmental factors mediated disease expression in the forest populations.

The goal of this study was to provide a comprehensive analysis of the epidemiologically relevant parameters for

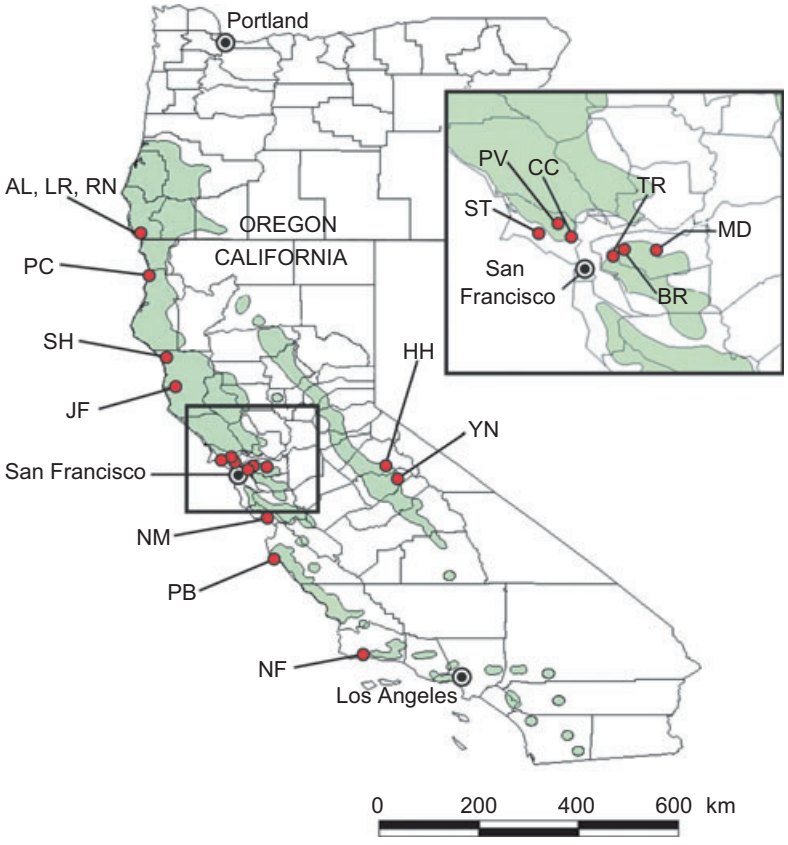

Figure 1 Seventeen California and Oregon populations from which 15 trees of *Umbellularia californica* were sampled for detached leaf inoculation with *Phytophthora ramorum* zoospores (see Table 1 for location names). Distribution of *U. californica*. (■).

the plant host documented to be driving the California SOD epidemic. If outbreaks of *P. ramorum* are determined by climate and host susceptibility, then it could be predicted that (i) leaf infection should occur substantially within a limited range of environmental or climatic parameters, (ii) season should affect susceptibility of the host and the observed pattern of susceptibility should be synchronous with the pathogen's life cycle, and (iii) individual hosts should vary in susceptibility within and among populations. Finally, the findings reported in this study on host susceptibility were used to compare predictions of disease severity based on field observations (where the disease was present in 2005), climatic parameters (incidentally also determined by the work described here) and host availability (as modelled by Meentemeyer *et al.*, 2004 and Václavík *et al.*, 2010).

Materials and methods

Isolates and inoculum production

Isolate Pr-52 (CBS 110537, ATCC MYA-2436) of the NA1 lineage (Ivors *et al.*, 2006; Grünwald *et al.*, 2009), originally isolated from a *Rhododendron* sp. in Santa Cruz County during 2000, was used in all inoculations. It is the most pathogenic isolate on detached leaves of *U. californica* compared to 10 isolates of *P. ramorum* from diseased native and ornamental plant species from California and Oregon (Hüberli & Garbelotto, 2011). Prior to commencement of all experiments, Pr-52 was passaged through *U. californica* leaves and reisolated on P_{10}ARP, a *Phytophthora*-selective agar medium modified with 25 mg of pentachloronitrobenzene (PCNB) (Rizzo *et al.*, 2002), to prevent loss in pathogenicity.

Zoospores were produced as described in Hüberli *et al.* (2003) and were diluted to 2×10^4 zoospores mL^{-1} (unless stated otherwise) using a haemocytometer. Prior to contact with zoospore solutions, labware was acid-washed (5 M HCl) for 24 h and then washed three times with deionized (DI) water to reduce zoospore attraction to these surfaces.

Plant material

Branches (15–20 cm lengths) with symptomless leaves were collected from *U. californica* trees, placed into water and transported back to the laboratory in cooler boxes with ice (approximately 15°C). Leaves were selected for this experiment if they were judged to be mature based on cuticle thickness, darker colour (compared to lighter coloured juvenile leaves), and size and position on the branch. Leaves were inoculated 1–4 days after collection either attached to a branch placed in water or detached. In a preliminary study prior to these collections, it was determined that storage of leaves in cool conditions (15°C) for up to 4 days before inoculation did not affect lesion size significantly ($P > 0{\cdot}05$; D. Hüberli *et al.*, unpublished data). Additionally, no significant ($P > 0{\cdot}05$) difference in susceptibility was found between inoculations of detached leaves and inoculations of leaves on branches (D. Hüberli *et al.*, unpublished data). All leaves were surface sterilized with 70% ethanol prior to inoculation.

Optimal environmental parameters for host infection

The optimal environmental parameters (temperature, exposure time to inoculum, and inoculum concentration) required for infection were determined in three separate inoculation studies. Branches with symptomless leaves were collected from one tree at the University of California, Berkeley. The following day, the first mature leaf still attached to the branch was placed into an individual flask containing sterile DI water. Flasks were placed into a clear plastic humid chamber which was misted with DI water daily.

To determine the optimal time of exposure to inoculum suspension, 10 leaf tips were immersed in 300 μL of zoospore (10^4 zoospores mL^{-1}) solution for 6, 12, 24, 36 or 48 h, after which the inoculum vessel (500 μL modified microcentrifuge tubes; Hüberli *et al.*, 2003) was removed and leaves were incubated in the clear humid chamber for a total of 14 days at 20°C with ambient light. After removal of the inoculum vessel, the leaf tip was allowed to dry at room temperature before plants were returned to the humid chambers. Control leaves ($n = 10$) were immersed in sterile DI water rather than zoospore suspensions.

Temperatures at inoculation time were tested by immersing leaf tips for 18 h in a 10^4 zoospore mL^{-1} solution and incubating in humid chambers at 15, 19, 23 or 28°C for 14 days. Ten leaves were inoculated for each of four trees from Solano County, California, as well as the tree used above.

Optimal zoospore concentration for inoculation of leaves was tested using leaves collected from five trees at the University of California, Berkeley, including the tree used in the two above experiments. Five leaves per tree were immersed for 18 h in aqueous suspensions of 1×10^2, 1×10^3, 1×10^4 or $2{\cdot}7 \times 10^4$ zoospores mL^{-1} and incubated 14 days at 20°C in humid chambers.

For all three experiments, outlines of the lesions were traced onto film and lesion areas were calculated using 1-mm^2-graph paper. To confirm the presence of *P. ramorum*, two leaf pieces (5 mm^2) from each lesion margin were plated onto P_{10}ARP. The leaf tips of symptomless leaves, including the control leaves, were also plated onto P_{10}ARP and plates were monitored for *P. ramorum* growth for 2 weeks.

Analyses of variance (ANOVA) using the general linear model in the software STATISTICA 5·0 (Statsoft) were carried out on each of the experimental factors for infection trials, including duration of exposure to inoculum suspension, infection temperature and zoospore concentration (independent variables). In each analysis, the dependent variable was lesion area, which was log-transformed prior to analysis to ensure assumptions of normality were met. The proportion of lesion and leaf size

was not used as a dependent variable because the entire leaf was not exposed to the inoculum, but only 11 mm of the leaf tip.

Seasonal effects on host susceptibility and disease incidence

Effect of season on survival of the pathogen on leaves was assessed by isolating from naturally infected trees, and the effects on variation in host susceptibility was assessed by artificial inoculations of healthy detached leaves. For both studies, 15 trees each from sites CC and ST (Fig. 1) were randomly selected at 20 m intervals along a transect. The same 30 trees (15 × two sites) were sampled 12 times during 2003 and 2005 (Fig. 2). At each sampling time, a total of 20 leaves from each tree was inoculated within 4 days from collection by placing the tip of each leaf into a 50 mL Falcon tube containing 300 μL zoospores (2×10^4 zoospores mL^{-1}) of isolate Pr-52 (see above). Two control leaves per tree were mock-inocu-

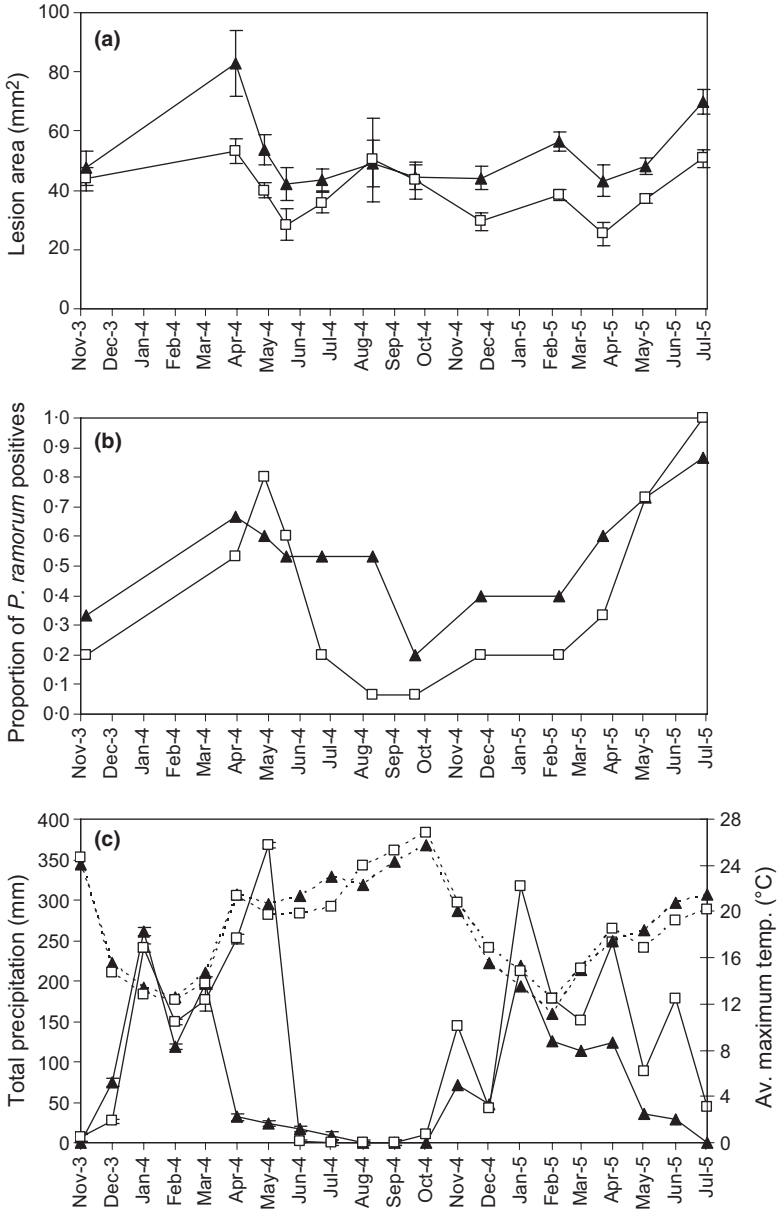

Figure 2 (a) Mean lesion size (±2 SE) on detached symptomless *Umbellularia californica* leaves collected from each of 12 trees in China Camp (CC, ▲) and Samuel P. Taylor (ST, □) State Park, Marin County, California, after inoculation with zoospores of *Phytophthora ramorum* at different sampling times from 2003 to 2005. Note that these values are based on a single sampling date in the indicated month. (b) Proportion of recoveries of *P. ramorum* on *Phytophthora*-selective agar medium (P_{10}ARP) from leaves with symptoms collected from the trees prior to each of the inoculations. Note that these values are based on a single sampling date in the indicated month. (c) Total precipitation (solid lines) and average maximum temperature (dashed lines). These are averages of daily readings for the month.

lated with sterile DI water. After an overnight incubation at 20°C, leaves were removed from the zoospore solution or sterile DI water and incubated in moist chambers for a further 8 days at 20°C. At harvest, leaf images were digitized with a flatbed scanner (Epson Perfection 1650) and lesion area was determined using ASSESS 1·01 (APS Press). For each tree, two leaf pieces from the lesion margin of five randomly selected leaves with symptoms and from leaf tips of all symptomless leaves, including controls, were plated onto P_{10}ARP and monitored as above.

In order to assess natural seasonal variation of disease incidence, up to four leaves with symptoms from each of the 15 trees at the two sites were also collected and plated onto P_{10}ARP as described above. Growth of *P. ramorum* from plated leaves was taken as confirmation of infection. Additionally, a PCR assay (Hayden *et al.*, 2006) was performed on DNA extracts from bulked tissue to confirm the presence of *P. ramorum* in symptomatic leaves. Culture-negative but PCR-positive leaves were counted as infected.

Climatic data for 2003–2005 were obtained from weather stations at Point San Pedro (approximately 3·7 km from site CC, at sea level; http://www.cimis.water.ca.gov/cimis/data.jsp), and at Barnaby (approximately 3 km from site ST; http://www.raws.dri.edu).

A repeated measures nested ANOVA was used and log-transformed lesion area was analysed as a function of the independent fixed factor population and random factor individual tree, nested within populations. The repeated measures fixed factor of sampling time and leaf area was included as a changing covariate (i.e. different covariates at each sampling time). The statistical model employed requires a balanced design. At some sampling times data were missing from some trees and leaves, so sample sizes were equalized to 12 trees/population and eight leaves/tree per sampling time by removing extraneous data points at random. Spearman's rank order correlation analyses were used to determine the effects on lesion area and recovery rates of the following climatic variables recorded over the 2, 7 and 28 days periods prior to sampling: the daily minimum, mean and maximum temperature (°C); the daily minimum, mean and maximum relative humidity (%); and the daily cumulative rainfall (mm). Benjamini–Hochberg's (BH) correction for multiple tests was used to adjust the threshold levels of significance of correlation coefficients; this alternative method to Bonferroni's correction offers increased statistical power (Waite & Campbell, 2006).

In light of the fact that linear regressions may not capture threshold effects of environmental variables on natural field infection, a series of comparisons among frequencies of successful pathogen isolation at different times of year were additionally run. Because frequency of successful isolations differed between the two sites (CC > ST, one-tailed Fisher's exact test $P = 0{\cdot}01$), analyses were performed independently for each site. Based both on an understanding of the biology of the pathogen, and on the determination of the optimal environmental parameters for host infection provided by this study, chi-square analyses were run to compare frequency of successful isolations among months (using Pearson's test), between dry and wet months (using Fisher's exact test on data pooled for all wet and all dry months), and between warm-wet months and cool-wet months (using Fisher's exact test on data pooled for all wet and warm months as opposed to data from cool wet months). Wet months included all months with any rainfall (Fig. 2), while warm-wet months were those characterized by the presence of any rainfall with average maximum temperatures above 16°C (Fig. 2).

Variation in susceptibility of host populations from California and Oregon

Leaves from 15 trees of *U. californica* were sampled along transects with approximately 20 m between each tree of 17 mixed forest populations in Oregon and in California (Fig. 1). The great geographic distance between sites (approximately 800 km), space limitations and the difficulty of producing huge volumes of inoculum with equal concentration of zoospores made it impossible to compare all populations at the same time. Hence, four to six populations were sampled in each of six separate trials conducted from November 2003 to September 2004 (Table 1). Two populations (CC and ST) were sampled at each trial to serve as reference populations between trials, one population in Oregon (AL) was sampled three times and two California populations were sampled twice (LR and JF); the same trees were sampled on each occasion. In order to estimate disease incidence at each site (see below) four symptomatic leaves per tree were plated onto selective media as described earlier. Inoculations of healthy leaves were performed and evaluated on 20 leaves per tree as described above for the study of seasonality.

Variation in susceptibility among populations was assessed using a separate nested ANOVA for each of the seven trials. The log-transformed lesion area was the dependent variable. Population was a fixed effect while individual trees, nested within populations, were treated as a random effect. Leaf size was a covariate. In all trials the design was unbalanced because of missing data and/or trees for which leaves were all contaminated after inoculation and incubation, so data for trees and leaves/tree were randomly selected and removed from larger groups to ensure that the nested design was balanced. After removal of data (if required), there were always 13–15 trees and 10–18 leaves/tree in each trial. If main effects were significant, Fisher's least significant difference (LSD) tests were used to determine which populations within each of the trials were significantly different.

To ascertain the heritability of variation in susceptibility, at least 40 drupes were collected in October to November 2004 from each seed-producing tree (parent) that had been previously sampled for leaf inoculations at sites CC, ST and AL and some trees which had not been previously sampled. In the laboratory, the fruit and outer seed coat were removed and the seed was washed in

Table 1 Details of 17 *Umbellularia californica* study sites. Actual disease in 2005 and predicted disease, by risk models and detached leaf assay by this study (see Fig. 4), are shown for each site

							Predicted disease severity/spread[d]	
Trial[a]	Location	County, State	Site	GPS coordinates	Forest type[b]	Observed disease[c]	Host–climate models[e]	Leaf susceptibility[f]
1–6	China Camp State Park	Marin, CA	CC	38°00′14·74″N, 122°29′48·72″W	234	4	4	4
1–6	Samuel P. Taylor State Park	Marin, CA	ST	38°01′46·99″N, 122°44′08·41″W	234	3	3	3
1, 2, 5	Alfred A. Loeb State Park	Curry, OR	AL	42°06′45·86″N, 124°11′14·45″W	234	0	3–4	1
1, 5	Siskiyou National Forest (Little Redwood Trail)	Curry, OR	LR	42°08′59·22″N, 124°08′44·34″W	232	0	3–4	1–2
1	Siskiyou National Forest (Redwood Nature Trail)	Curry, OR	RN	42°07′05·92″N, 124°11′50·76″W	234	0	3–4	1
1	Pacheco Valley Open Space Preserve	Marin, CA	PV	38°02′29·95″N, 122°33′10·21″W	255	2	3	4
2, 5	Jackson State Forest	Mendocino, CA	JF	39°21′08·40″N, 123°33′26·58″W	232/ *Notholithocarpus densiflorus*	0	3	3
2	Standish Hickey State Park	Mendocino, CA	SH	39°52′35·43″N, 123°43′30·56″W	232/*N. densiflorus*	0	4	3–4
2	Redwoods State Park (Prairie Creek)	Humboldt, CA	PC	41°21′50·64″N, 124°01′21·78″W	232/*N. densiflorus*	0	3	3–4
3	The Forest of Nisene Marks State Park	Santa Cruz, CA	NM	36°59′33·93″N, 121°54″22·92″W	232/255/*N. densiflorus*	3	3	3
3	Pfeiffer Big Sur State Park	Monterey, CA	PB	36°15′01·91″N, 121°46′52·24″W	232/255/*N. densiflorus*	4	3	3–4
3	Nojoqui Falls County Park	Santa Barbara, CA	NF	34°31′50·01″N, 120°10′34·36″W	255	0	1	3–4
4	Tilden Regional Park	Alameda, CA	TR	37°52′58·28″N, 122°13′35·17″W	255	3	2	3
4	Briones Regional Park	Contra Costa, CA	BR	37°55′35·04″N, 122°09′27·98″W	255	3	2	3
4	Mount Diablo State Park	Contra Costa, CA	MD	37°54′51·54″N, 121°55′21·11″W	255	0	1	3
6	Yosemite National Park	Mariposa, CA	YN	37°43′33·73″N, 119°33′20·93″W	211/*Quercus wislizeni*	0	0	1
6	Hetch Hetchy State Park	Tuolumne, CA	HH	37°57′01·68″N, 119°47′23·08″W	*Q. wislizeni*	0	0	3–4

[a]Trials 1–6 were inoculated 6 November 2003, 30 March 2004, 27 April 2004, 18 May 2004, 10 August 2004 and 21 September 2004.
[b]Society of American Foresters' forest type: 211 = *Abies concolor* (white fir); 232 = *Sequoia sempervirens* (redwood); 234 = *Pseudotsuga menziesii* (Douglas-fir), *N. densiflorus* (tanoak), *Arbutus menziesii* (Pacific madrone); and 255 = *Quercus agrifolia* (California coast live oak) (Eyre, 1980).
[c]*P. ramorum* disease on *U. californica* (based on field observations, confirmed either by isolation or by DNA-based detection) and on the canker hosts *Q. agrifolia* and *N. densiflorus* (based on field observations) during this study. (For current update see http://www.oakmapper.org.)
[d]Disease severity/spread risk levels: 0 nil, 1 low, 2 moderate, 3 high and 4 very high.
[e]Risk model prediction of *P. ramorum* disease spread risk based on infection parameters (temperature and moisture as defined by Davidson *et al.* (2005, 2008) and this paper) and the presence of susceptible hosts of disease (*U. californica* had the highest potential to spread inoculum) as determined by Meentemeyer *et al.* (2004) for California and Václavík *et al.* (2010) for Oregon.
[f]Prediction of *P. ramorum* disease severity/spread risk based on the relative susceptibility of *U. californica* at each site (Fig. 4).

bleach (1:500 solution) for 30 s, followed by a 30 s rinse in sterile DI water. Seeds were stratified for 6 months at 4°C in individual plastic zip-lock bags containing moistened perlite and vermiculite (50:50) and were examined periodically for germination. Germinating seeds were transferred to trays containing perlite in the glasshouse, and seedlings that successfully established were transferred to 10 cm (diameter) × 35 cm plastic pots. At the end of this process, there were five or six parent trees represented by more than five seedlings from each of the three sites. Vegetative propagation of cuttings from adult trees was unsuccessful.

In August 2006, when seedlings were approximately 1-year-old, five mature leaves from five seedlings per par-

ent were inoculated (25 inoculations per parent), yielding 125 leaf inoculations per site. Four leaves were inoculated with zoospores in tubes as described previously, while the fifth leaf from each seedling was inoculated with sterile water. Incubations and harvests were carried out 9 days later as described previously. The experiment was repeated 1 year later using the same seedlings.

Because of the low seed set in experimental trees in some populations, there was not data for all of the parents used in the study, so offspring–parent regressions were not possible. The correlation among offspring of a shared mother was calculated for the two trials, with variance components estimated by modelling log-transformed lesion area as a function of population (fixed effect) and parent tree within population and seedling within parent tree within population (random effects). Leaf area had no significant effect and so was not included as a covariate. The design was unbalanced, so leaves were removed at random to ensure a balanced design of four replicates. The same five parent trees for each site were used in both trials.

Narrow-sense heritability, h^2, is the proportion of total variance in lesion size that is due to additive genetic effects. For sibling studies, h^2 is calculated as the variance due to the shared parent as a proportion of total variance, divided by a parameter that describes the probability of siblings inheriting identical alleles at any locus; for half-siblings, this parameter is ¼ and for full-siblings the parameter is ½ (Falconer & Mackay, 1996). The families in this study were expected to be a mix of half- and full-siblings, so the convention of calculating $h^2 = V_{parent}/V_{total}/1/3$, i.e. $h^2 = 3 \times V_{parent}/V_{total}$ was used. It should be noted that this study follows the common convention of reporting this value as h^2, narrow-sense heritability, but that the shared maternal parent and the probable inclusion of some full-siblings will cause the estimate to be inflated by some degree from any maternal and dominance effects.

Actual and predicted disease severity

Actual and predicted disease severities were determined for each site using the scale 0 (nil), 1 (low), 2 (moderate), 3 (high) and 4 (very high). Actual disease severity was determined from field observations along each of the site transects (approximately 300 m long) during the course of this study based on: (i) symptoms on *U. californica* leaves and collected and confirmed either by isolation or by DNA-based detection of the pathogen, and (ii) mortality of any canker hosts (*Q. agrifolia* and *N. densiflorus*). Scores were assigned as follows: 0, no disease evident; 1, some foliar disease (<5% trees) with no mortalities evident; 2, average foliar disease (6–25% trees) with no mortalities evident; 3, high foliar disease (>25% trees) with minor mortality and cankers obvious in canker hosts (<1% trees); and 4, high foliar disease and mortality and cankers obvious in canker hosts (>25% trees). Predicted disease spread risk was estimated from (i) the predictive risk models and (ii) our detached leaf assay. The predictive risk models developed for California (Meentemeyer *et al.*, 2004) and Oregon (Václavík *et al.*, 2010) included environmental parameters favourable for infection (from the data produced by this study) and availability of susceptible and infectious hosts. The maps produced from these models showed the predicted spread risk on the above scale of 0–4; these maps were used to determine the risk of spread in the study sites for California (Fig. 6 in Meentemeyer *et al.*, 2004) and Oregon (Fig. 3 in Václavík *et al.*, 2010). Detached leaf assay disease severity/spread risk predictions were determined by comparing susceptibility levels of populations with the two reference populations (CC and ST) which are both from areas with

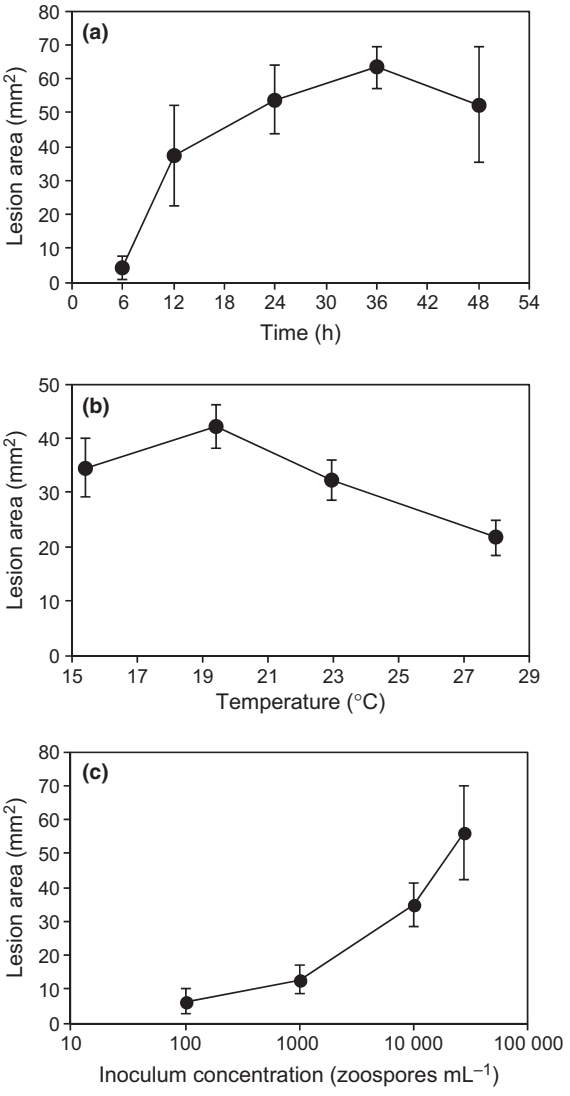

Figure 3 Optimal environmental parameters for infection of *Umbellularia californica* leaves in detached inoculations with *Phytophthora ramorum* zoospores. Mean lesion area (±2 SE) on leaves after (a) varying times of exposure to zoospores, (b) incubation at four different temperatures, and (c) inoculation with different concentrations of zoospores.

established SOD and were included in each trial. The homogeneous groups produced in LSD tests as described earlier were used to score each site.

Results

Optimal environmental parameters for host infection

All inoculated leaves formed water-soaked lesions after 1–2 days, developing to tan or brown leaf tip lesions as described by Davidson *et al.* (2005) after the incubation. Some lesions did not coalesce but were spotty in nature in the inoculation area. *Phytophthora ramorum* was reisolated from all leaves with symptoms.

Duration of exposure to inoculum suspension, incubation temperature and inoculum concentration all had significant effects on *P. ramorum* lesion size in *U. californica* leaves (Fig. 3). For duration of exposure (Fig. 3a; ANOVA: $F_{4,15} = 10{\cdot}85$, $P = 0{\cdot}0002$), all leaves exposed to zoospore suspensions for 6 h or more were significantly different (LSD test: $P < 0{\cdot}05$) from the control. Lesions were significantly smaller (LSD test: $P < 0{\cdot}0001$) when leaves were exposed to zoospores for 6 h than when leaves were exposed for 12 h or more (Fig. 3a). There was no significant difference (LSD test: $P > 0{\cdot}30$) in lesion area among leaves exposed to zoospores for 12–48 h.

Temperature likewise had a statistically significant effect on mean lesion areas of leaves (Fig. 3b; ANOVA: $F_{3,174} = 14{\cdot}69$, $P < 0{\cdot}0001$). Lesions in all trees were significantly (LSD test: $P < 0{\cdot}03$) larger when incubated at 19°C than at all other temperatures except at 23°C (Fig. 3b). At 19°C there was a significant difference ($P < 0{\cdot}05$) in lesion area amongst the five trees.

The average lesion area increased exponentially with higher inoculum concentrations (Fig. 3c; ANOVA: $F_{3,96} = 14{\cdot}01$, $P < 0{\cdot}0001$). Significantly larger lesions (LSD test: $P < 0{\cdot}001$) were produced by $2{\cdot}7 \times 10^4$ zoospores mL^{-1} compared to the lowest two concentrations, but the lesions were not significantly different ($P = 0{\cdot}23$) from those produced by 1×10^4 zoospores mL^{-1}.

Seasonal effects on host susceptibility and disease incidence

Fluctuations in the susceptibility of *U. californica* trees within populations and individual trees for sites CC and ST were observed over a period of 1·5 years. Population, individual tree within a population, sampling time, and their interactions all had highly significant effects on lesion area (Table 2). While the covariate of leaf area was not significant ($P > 0{\cdot}06$ in all cases), the *P*-value did approach significance in some cases and further investigation is warranted.

Maximum susceptibility occurred in late March 2004 and late June 2005 (Fig. 2a). Susceptibility declined and remained low from late April 2004 to May 2005 for both sites. Only between August and late September 2004 did the two populations converge in susceptibility (Fig. 2a).

The recovery proportion of *P. ramorum* from leaves with symptoms collected on site was higher than 50% for both sites in March 2004, and was highest for the ST site in April 2004 (Fig. 2b). After May 2004, recoveries declined rapidly for the ST site and remained below 50% until April 2005, while for the CC site recoveries were stable above 50% until September 2004 when they declined below these levels. Recoveries for both sites began increasing after October 2004 and reached levels of above 80% by July 2005. Following summer 2004, rainfall commenced after October and continued for both sites beyond June 2005 (Fig. 2c). The rainfall season in 2005 was extended beyond that in 2004 (Fig. 2c).

Experimental lesion area and recovery rate from the wild were not significantly correlated (Spearman's *R*: $P > 0{\cdot}05$ in all cases) with any of the climatic variables (daily minimum, mean and maximum temperature; daily minimum, mean and maximum relative humidity; and the daily cumulative rainfall at each of 2, 7 and 28 days periods prior to sampling) tested. Experimental lesion area and recovery rate were also not correlated across sites (Spearman's *R*: $r = 0{\cdot}25$, $P = 0{\cdot}44$ for CC; $r = 0{\cdot}007$, $P = 0{\cdot}98$ for ST). Significant variation in pathogen recovery as a function of time was found for both sites by comparing recovery frequencies for each month (Pearson's: $P = 0{\cdot}02$ and $P < 0{\cdot}0001$ for CC and ST, respectively). At both sites, recovery was significantly greater in wet (66% and 63% for CC and ST, respectively) than in dry (30% and 11% for CC and ST, respectively) months (Fisher's exact test: $P = 0{\cdot}002$ and $P < 0{\cdot}0001$ for CC and ST, respectively), but recovery in wet-warm months (46%) was significantly higher than in wet-cool months (16%) only for ST (Fisher's exact test: $P < 0{\cdot}0001$).

Table 2 Repeated measures analysis of variance of detached leaf lesion area within and among populations of *Umbellularia californica* from China Camp (CC) and Samuel P. Taylor (ST) State Park, California, in response to inoculation with *Phytophthora ramorum* at 12 different inoculation dates from November 2003 to June 2005

	SS	MS	d.f.	*F*	*P*	Epsilon[a]	Corrected *P*[a]
Population	11·08	11·08	1, 167	65·7	<0·001		
Individual tree (Pop.)	20·18	0·92	22, 167	10·0	<0·001		
Sampling time	105·61	9·60	11, 1837	13·1	<0·001	0·73	<0·001
Population × sampling time	4·45	0·40	11, 1837	2·5	0·004	0·73	0·01
Individual tree (Pop.) × sampling time	102·75	0·42	242, 1837	2·3	<0·001	0·73	<0·001

[a]Epsilon and the *P*-value correction calculated with the Greenhouse–Geisser correction.

Variation in susceptibility of host populations from California and Oregon

Response to inoculation

After inoculation and 9 days' incubation, symptoms on leaves collected from the 17 populations in California and Oregon were qualitatively the same. Controls never had lesions and *P. ramorum* was never isolated from these leaves.

The recovery rate of *P. ramorum* from experimentally infected leaves was significantly correlated with experimental lesion area in all trials ($r > 0{\cdot}36$; $P < 0{\cdot}001$), except trial 3. Trees that formed smaller foliar lesions also had fewer infected leaves. Henceforth, only lesion area data are presented.

Variation among populations

In all trials, significant variation in lesion area was detected among populations (Table 3). Reference site CC always had the largest lesions, whilst reference site ST always had lesions that were significantly smaller than CC (Fig. 4). Populations from sites AL, RN and YN had significantly smaller lesions than both CC and ST populations. Trees at site AL formed the smallest lesions in all three trials in which it was included. Lesions were significantly smaller in site LR than both reference populations in trial 1, but in trial 5, site LR was only significantly smaller than reference population CC. In trial 2, sites SH and PC were significantly smaller and larger than sites CC and ST, respectively. For all other populations, lesion areas were not significantly different from one or both of the reference populations.

Variation among individual trees within a population

In all trials, except trial 4, lesion area varied significantly among individual trees within a population (Table 3). The greatest differences were observed in trials 1 and 5, in which mean lesion areas for trees at site CC were more than threefold larger than for trees at site AL (Fig. 4, trial 1 and 5). In fact, 10 of 15 trees (trial 1), 10 of 13 trees (trial 2) and 13 of 14 trees (trial 5) from site CC were more susceptible than all 15 trees from site AL (Fig. 5 from trial 1 data).

Within all populations sampled, some individual trees were consistently less susceptible than the rest of those tested. To test for repeatability of successive trials, Spearman's rank order correlations within individual trees were calculated across trials 1 and 5 for sites represented in both trials, and across trials 2 and 5 for sites represented in both trials. Within-tree correlations were highly significant for both comparisons: trials 1 and 5 ($r = 0{\cdot}56$, $P < 0{\cdot}0001$) and trials 2 and 5 ($r = 0{\cdot}61$, $P < 0{\cdot}0001$).

Heritability of susceptibility in a common garden

In both trials conducted 1 year apart, no significant differences in experimental lesion size were found among the three populations or among parents within a population from which seeds were collected (Table 4). Individual seedlings varied significantly in lesion area for both trials. In each trial, there was a non-significant trend towards smaller lesions in seedlings from the AL population than ST and CC (Fig. 6). There was also a trend towards a greater effect of parent for inoculations at 2 years compared to 1 year (Table 4). For 1-year-old seedlings, parental effect was calculated as $P = 0{\cdot}36$ and $h^2 = 0{\cdot}03$, while for 2-year-old seedlings, parental effect approached statistical significance at $P = 0{\cdot}08$ and $h^2 = 0{\cdot}22$.

Table 3 Nested analysis of variance of leaf lesion area within and among populations of *Umbellularia californica* in response to inoculation of detached leaves with *Phytophthora ramorum* zoospores. Population was modelled as a fixed effect, while Individual tree (population) was treated as a random effect

	SS	MS	d.f.	*F*	*P*
Trial 1					
Population	279·50	55·90	5, 1349	92·8	<0·0001
Individual tree (population)	150·07	1·79	84, 1349	4·5	<0·0001
Trial 2					
Population	8·48	1·70	5, 701	28·4	<0·0001
Individual tree (population)	16·18	0·22	72, 701	2·7	<0·0001
Trial 3					
Population	4·38	1·10	4, 649	10·0	0·03
Individual tree (population)	22·03	0·37	60, 649	2·2	0·005
Trial 4					
Population	9·90	2·48	4, 1049	7·4	0·03
Individual tree (population)	55·62	0·86	65, 1049	1·2	0·10
Trial 5					
Population	58·03	14·51	4, 909	20·9	<0·0001
Individual tree (population)	74·80	1·15	65, 909	2·7	<0·0001
Trial 6					
Population	7·07	2·36	3, 1019	17·2	0·002
Individual tree (population)	23·77	0·42	56, 1019	4·1	<0·0001

Actual and predicted disease severity

Of the seven populations where *P. ramorum* is not yet present, three (JF, SH and PC) had high disease severity risk as predicted by climate–host models (Meentemeyer *et al.*, 2004; Václavík *et al.*, 2010) and the susceptibility assays (this study, Table 1). Three populations (NF, MD and HH) had very low to low disease severity risk based on the climate–host models, but in susceptibility assays were found to have high to very high risks. Ten populations currently have been infested by *P. ramorum* (Table 1). For all these infested populations, with the notable exception of the three Oregon sites, both the climate–host models and our susceptibility assays predicted high to very high disease severity. For the three southern Oregon populations, the climate–host models predicted disease severity as high to very high, but the susceptibility assays suggested potentially very low to low disease severity.

Discussion

If outbreaks of *P. ramorum* are determined by climate and host susceptibility, then it could be predicted that (i) leaf infection should occur substantially within a limited range of environmental or climatic parameters, (ii) season

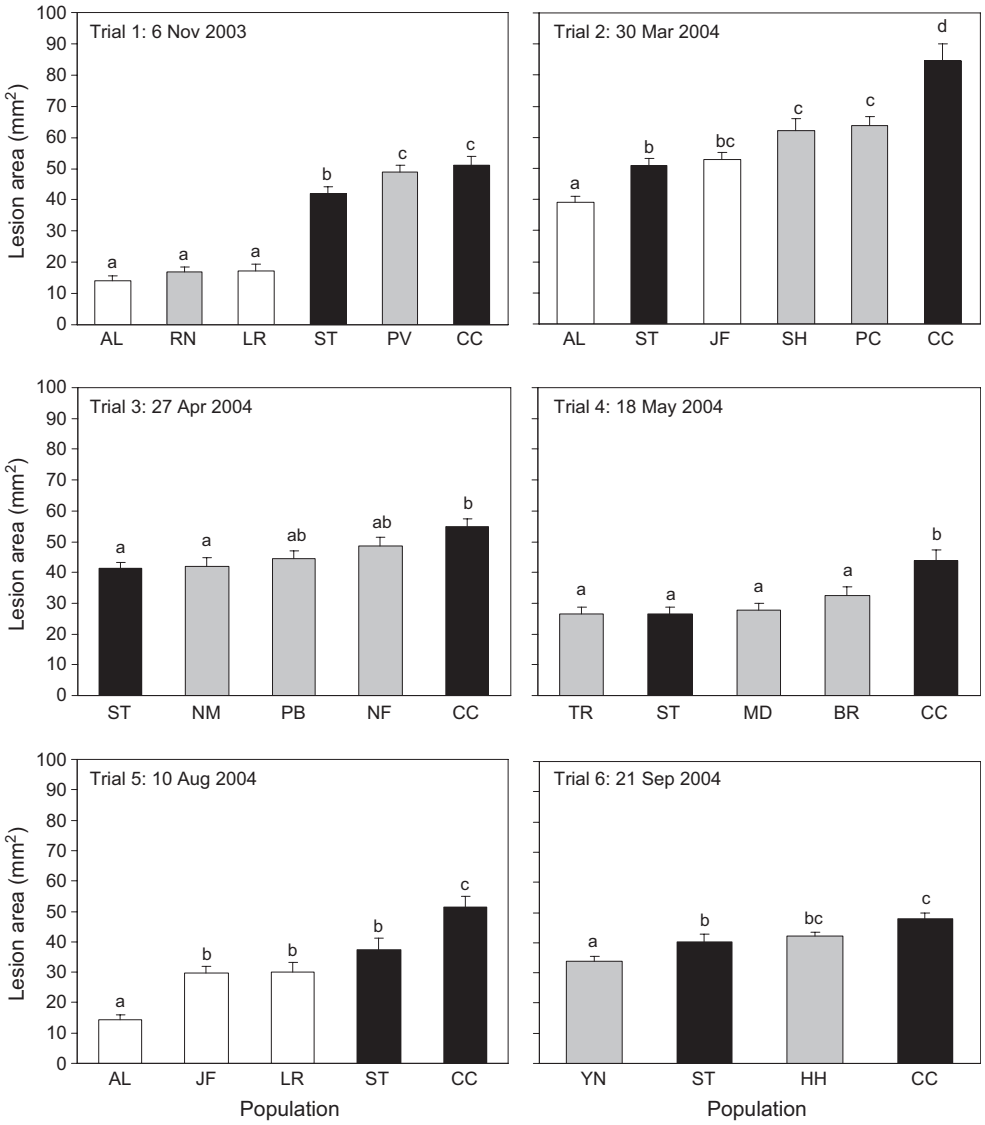

Figure 4 Mean leaf lesion area (±1 SE) per population produced after inoculation of detached *Umbellularia californica* leaves collected from 17 populations across California and Oregon with *Phytophthora ramorum* zoospores in trials 1–6. After deletion of samples to ensure a balanced design in each trial, $n = 15$ except trials 2 and 3 ($n = 13$) and trials 3 and 4 ($n = 14$). Reference populations (■), populations sampled more than once (□) and populations sampled once (▣). Populations with the same letter are not significantly different according to LSD ($P = 0.01$). See Figure 1 for location of populations.

should affect susceptibility of the host and susceptibility should be synchronous with the pathogen's capacity to infect and cause disease, and (iii) individual hosts should vary in susceptibility within and among populations. These predictions were met in this study. Additionally, the data suggest that high susceptibility of hosts may counterbalance and even outweigh the presence of climatic conditions that are not ideal for the pathogen.

Using parameters found to be optimal for disease to occur in detached leaves of *U. californica*, this study showed that season contributed to variation in susceptibility in two California populations. For both, susceptibility to *P. ramorum* in experimental inoculations was highest in concurrence with high successful isolation from naturally infected leaves. Temporal variation in susceptibility did not correlate linearly with mean climatic data, nor did experimental lesion size correlate to pathogen recovery rates from field-collected leaves with symptoms. Isolation of the pathogen was significantly higher in wetter months and peaked during wet-warm months. The parameters for optimal infection as determined through the controlled inoculations described here have not been formally published elsewhere but, in light of the threat represented by SOD, they were previously personally communicated to authors who used them when developing multifactor disease risk models (e.g. Meentemeyer *et al.*, 2004; Venette & Cohen, 2006; Magarey *et al.*, 2007; Václavík *et al.*, 2010).

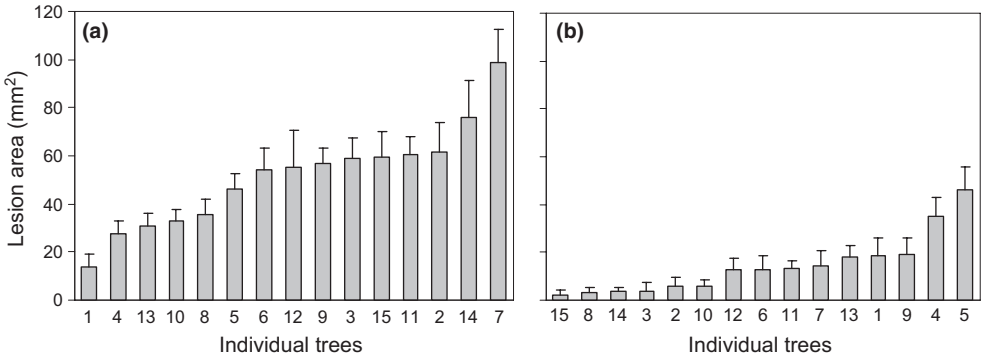

Figure 5 Mean lesion area (±1 SE) per tree produced on detached *Umbellularia californica* leaves collected from 15 trees growing at (a) site CC (California) or (b) site AL (Oregon) after inoculation with *Phytophthora ramorum* zoospores in trial 1 (n = 16 leaves).

Table 4 Nested analysis of variance of leaf lesion area within and among populations of *Umbellularia californica* seedlings collected from five parents from each of three populations (CC, ST and AL) in response to inoculation of detached leaves with *Phytophthora ramorum* zoospores. Each trial was analysed within its own model. Population was modelled as a fixed effect, while Parent (population) and Seedling (population, parent) were treated as random effects

	SS	MS	d.f.	*F*	*P*	Heritability[a] (h^2)
Trial 1						
Population	0·18	0·09	2, 225	0·57	0·58	0·03
Parent (population)	1·84	0·15	12, 225	1·12	0·36	
Seedling (population, parent)	8·27	0·14	60, 225	1·96	<0·001	
Trial 2						
Population	0·69	0·34	2, 222	0·56	0·58	0·22
Parent (population)	7·29	0·61	12, 222	1·74	0·08	
Seedling (population, parent)	20·62	0·35	59, 222	3·56	<0·001	

[a]Heritability was calculated from variance components as described in Materials and methods.

This study established that there is considerable variation in susceptibility to *P. ramorum* within and among 17 populations of *U. californica* from California and southern Oregon. The southern Oregon populations included in this study had lower susceptibility, and consistently so for site AL, than most California populations, independent of season (November 2003, and March and August 2004). Common garden inoculations of seedlings from two susceptible California and one relatively resistant Oregon population failed to identify strong differences in the 1- to 2-year-old seedlings. Disease tolerance may arise at a later developmental stage as reported for other pathosystems (Develey-Rivière & Galiana, 2007) or the differences

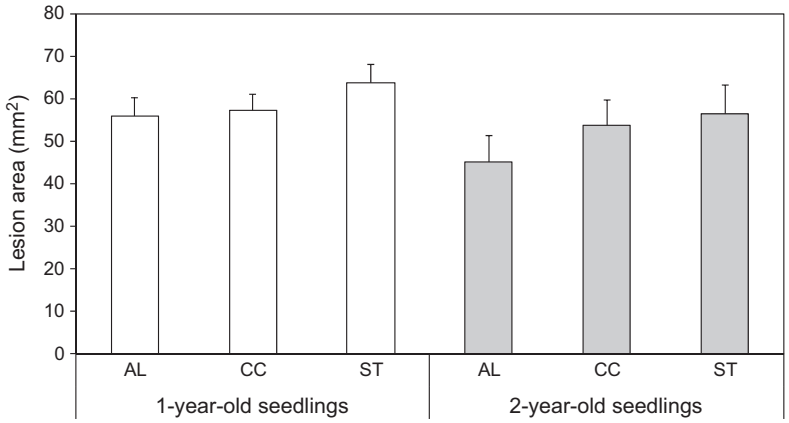

Figure 6 Mean leaf lesion area (±1 SE) per population produced after inoculation with *Phytophthora ramorum* zoospores of detached *Umbellularia californica* leaves collected from seedlings grown in the greenhouse for 1 (□) and 2 (▩) years. Seedlings were raised from drupes collected from two California (China Camp, CC; Samuel P. Taylor State Park, ST) and one Oregon (Alfred A. Loeb State Park, AL) population(s) beneath five mother plants per population (n = 5 per mother plant). See Figure 1 for location of populations.

observed in adults may be driven by environmental factors. These possibilities warrant further investigation.

Both temperature and moisture are known to influence sporulation and the infection cycle in the laboratory and field (Davidson *et al.*, 2005, 2008; Englander *et al.*, 2006). Although Tooley *et al.* (2009) showed some lesions can develop after 1 h of exposure to inoculum, their results indicated that the largest lesions develop at 20·5°C with an exposure period of 24–48 h in detached rhododendron leaves. Here, results indicated that optimal disease in detached *U. californica* leaf inoculations was produced at 19°C, with exposures to inoculum of at least 6–12 h and a zoospore concentration of approximately $2{\cdot}7 \times 10^4$ zoospores mL^{-1}. Up to approximately 2000 zoospores from 1 cm^2 lab-induced lesions (data not shown) were repeatedly obtained, indicating that concentrations of 10^4 zoospores mL^{-1} can easily be achieved in runoff from infected *U. californica* leaves. A strong dose response to zoospore concentration was demonstrated in the examined trees from California. In contrast, this relationship was not evident for *U. californica* from Oregon in tests by Hansen *et al.* (2005). Given that *U. californica* populations from the three sites in southern Oregon were less susceptible than the 13 California populations, it is reasonable to conclude that less susceptible hosts might have a limited response to inoculum concentration, simply because they are relatively tolerant to the disease.

In repeated testing of two populations, leaf susceptibility in detached inoculations and pathogen recovery rates from naturally infected leaves followed seasonal fluctuations. These fluctuations have also been reported for *Q. agrifolia* (Dodd *et al.*, 2005, 2008). More importantly, susceptibility of *U. californica* trees was found to be generally higher at times that the pathogen was recovered more frequently from naturally infected leaves. For canker disease to develop in *Q. agrifolia* there must be synchronism between colonization rate by the pathogen and host phenology, as active cambial tissue is required for infection (Dodd *et al.*, 2008). The data presented here suggests high susceptibility of the epidemiologically relevant *U. californica* is also synchronous with pathogen sporulation and infectivity and oak susceptibility, thus potentially explaining the reason for the high oak mortalities in California.

Although it was expected that recovery of the pathogen from naturally infected leaves would also be higher in warm-wet months than in cool-wet months, this expectation was correct only for the less susceptible ST site. In the highly susceptible site (CC), recovery rates were indistinguishable between wet-cool and wet-warm months. In the presence of highly susceptible individuals, disease can persist over a broader range of climatic and environmental parameters. Consequently, host susceptibility may counterweigh less than optimal climatic conditions (i.e. the wet cool period that is not ideal for the pathogen because of temperature limitations) and is likely to be an important, yet completely overlooked factor in predicting disease risk.

Considerable variability within most populations was found, as reported for *Q. agrifolia* (Dodd *et al.*, 2005) and for *U. californica* in Sonoma County (Anacker *et al.*, 2008). In the sites studied in southern Oregon, susceptible trees were few: five of 15 trees (trial 1), three of 13 trees (trial 2) and one of 14 trees (trial 5) from site AL were as susceptible as the most susceptible trees from site CC. Davidson *et al.* (2008) suggested that the lower density of *U. californica* in Oregon may be the limiting factor in epidemics in Oregon. A minor epidemiological role of *U. californica* in southern Oregon may also be due to the reduced susceptibility of *U. californica* trees. Trees from the studied sites in southern Oregon displayed morphological differences in leaf size and surfaces when compared to Californian trees; these differences, whether genetic or environmental in origin, warrant research for their role in susceptibility.

The lack of significant differences in the common garden inoculation trials performed in this study mirrors results of previous work (Anacker *et al.*, 2008). Factors other than genetics may cause most observed differences in host susceptibility. Nonetheless, in both trials the seedlings from the AL site always had lower susceptibility than those from the CC and ST sites. Further, our heritability estimate for susceptibility in 2-year-old seedlings was well within the range of quantitative traits used in tree breeding (Carson & Carson, 1989). Despite statistical uncertainty – the effect of shared parent had only an associated probability of $P = 0{\cdot}08$ – this trend implies a genetic contribution that should not be overlooked. The young age of the seedlings may have masked effects observed in adults, and a genetic contribution to susceptibility may only be detectable in certain environmental conditions. For example, the thicker cuticles anecdotally observed in leaves from Oregon populations may be caused by local climate or an interaction of genetics and local climate. Further work should assess genetic variation more definitively (e.g. the local study of *U. californica* by Anacker *et al.* (2008) and the range-wide study of *Q. agrifolia* (Dodd *et al.*, 2005)).

This study is the first to show the distinct difference in susceptibility of *U. californica* among populations sampled across a large native range (Table 1). The relative susceptibility of a population was found to be stabler than that of one tree, and could be determined with a single trial. In contrast, while within-tree susceptibility was significantly correlated among some trials, there was no absolute correlation for rank among trees tested at different times. The repeatability of assessment of relative susceptibility of an entire population makes this measure valuable for predicting the potential course of epidemics at different sites. Other studies on *P. ramorum* also concluded that geographic variation might play a direct or indirect (phenological) role in resistance and susceptibility of hosts including *U. californica* (Anacker *et al.*, 2008), *Q. agrifolia* (Dodd *et al.*, 2005, 2008) and *N. densiflorus* (Hayden *et al.*, 2011).

Based on the new data presented, the high susceptibility of *U. californica* individuals from CC may be the most

important factor in the determination of the highest SOD incidence in an oak forest in California, even if this site is not one of the oldest infestations in the state (Mascheretti *et al.*, 2008, 2009) and the climatic parameters are not as conducive as in other sites, as suggested by hotter than ideal maximum summer temperatures (http://www.cimis.water.ca.gov/cimis/data.jsp). The high susceptibility at CC occurs despite viability of the pathogen in the summer at CC having been found (by reverse transcription PCR data) to be only approximately 50% of that at ST, a site with ideal environmental conditions for *P. ramorum* (Chimento *et al.*, 2011). Conversely, populations from Oregon sites AL, LR and RN and from the Yosemite National Park site YN in the central Sierra Nevada (Table 1) had significantly reduced susceptibility. The overall risk in the YN site is low because climatic conditions are also not conducive to SOD outbreaks (Meentemeyer *et al.*, 2004; Magarey *et al.*, 2007). Conversely, the Oregon sites tested have a predicted high risk (Václavík *et al.*, 2010), but in this region the epidemic seems to be driven mostly by *N. densiflorus* (Hansen *et al.*, 2008) even where *U. californica* is present.

Nonetheless, evidence from California has shown that sympatry of *U. californica* and *N. densiflorus* can intensify disease severity (Cobb *et al.*, 2010). Hence, it could be predicted that in southern Oregon and central Sierra Nevada in California, disease should be less severe than in some *N. densiflorus* sites of California, either because of ideal climatic conditions (Sierra Nevada), or because *U. californica* populations are not as susceptible (Oregon) (Cobb *et al.*, 2010).

Uninfested populations (http://www.oakmapper.org, accessed 1 February 2010) that may be at high risk, based on the data in this study, include sites in Mendocino (SH), northern Humboldt (PC), Contra Costa (MD), Tuolumne (HH) and Santa Barbara (NF) counties (Table 1). It is assumed that all populations that were as susceptible as the highly susceptible CC (SH, PC, HH, NF) have the potential to face high inoculum loads of the pathogen even if environmental conditions are only moderately favourable. Sites including MD where *U. californica* populations were as susceptible as ST should witness high inoculum loads if environmental conditions are very favourable to the pathogen. Forests identified as at risk of witnessing high inoculum loads based on the combination of environmental and high susceptibility of sporulating hosts need to be managed appropriately now to ensure they remain free of the disease in the future (Meentemeyer *et al.*, 2004).

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