Phytophthora ramorum is a generalist plant pathogen with differences in virulence between isolates from infectious and dead-end hosts

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Summary
Variation in virulence was examined among isolates of *Phytophthora ramorum* from epidemiologically important or infectious (non-oak) and transmissive dead-end (oak) hosts from North America. Twelve isolates representative of the genetic, geographic and host range of *P. ramorum* in the western United States were inoculated on leaves of *Umbellularia californica* (bay laurel or bay) and stems of *Quercus agrifolia* (coast live oak). In spite of extreme genetic similarity among the isolates employed, and even within the same genotype, significant differences in lesion size were measured, suggesting virulence in this pathogen is also controlled by epigenetic factors. A strong positive correlation between lesion size on bay laurel and coast live oak provides experimental evidence *P. ramorum* is a generalist pathogen that lacks host specificity. Isolates from non-transmissive oaks were significantly less pathogenic both on oaks and bays than isolates from infectious hosts. These results are essential to further our understanding of the epidemiology and evolutionary potential of this pathogen. A quantitative differential in virulence of isolates from hosts with different epidemiological roles has been described for many animal diseases, but is a novel report for a plant disease.

1 Introduction
Phytophthora ramorum is a newly described plant pathogen that causes a forest disease known as sudden oak death (SOD) (Rizzo *et al*. 2002). This organism is unique among *Phytophthora* species of temperate forest ecosystems because of the significant aerial component of its life cycle (Davidson *et al*. 2005, 2008). The known distribution of the pathogen includes nurseries of ornamental plants in Europe and North America, as well as in wild woodlands of the western United States (Werres *et al*. 2001; Rizzo *et al*. 2002; Davidson *et al*. 2003). Recently, isolated outbreaks have also been reported in forest settings in Europe, including a significant infestation of planted larch in the UK (Brasier *et al*. 2004; Brasier and Webber 2010). Based on its limited geographic distribution in the wild, the severity of symptoms it can cause on some hosts and the limited genetic diversity of its populations, *P. ramorum* is regarded as an exotic pathogen of unknown origin to both Europe and the United States (Rizzo and Garbelotto 2003; Ivors *et al*. 2004, 2006). It has been recently shown that the infestation in the wild is linked to multiple escapes of the pathogen from nursery plants and that the pathogen is reproducing only asexually in California (Ivors *et al*. 2006; Mascheretti *et al*. 2008, 2009). Genetic evidence has further indicated the pathogen has been transferred among commercial nurseries both within North America and between North America and Europe (Goss *et al*. 2011). Several countries, including the United States, have imposed strict quarantines and regulations aimed at preventing the further spread of this microbe through infected ornamental plants (Anonymous 2006, 2008). Regulatory action of this scope
and intensity is historically unprecedented for a generalist plant pathogen.

In nursery settings, *P. ramorum* infects many different plant species (Werres *et al*. 2001; Rizzo *et al*. 2002; Davidson *et al*. 2003). Symptoms include foliar necrosis, branch die-back, and, at times, lethal stem infection. The most common nursery hosts include several species and varieties within the genera Rhododendron, Camellia and Viburnum (arrowood). The wild-land infestation of *P. ramorum* ranges from central California to southern Oregon and seriously affects populations of coastal oaks in the section Lobatae (commonly referred to as red oaks) and the related *Notholithocarpus densiflorus* (synonym *Lithocarpus densiflorus*, tanoak) (Rizzo *et al*. 2002; Rizzo and Garbelotto 2003). On the US west coast, oak and tanoak stem cankers are generally lethal, while the pathogen is known to cause less serious diseases on a wide range of native hosts including coniferous and broad-leaved trees, shrubs, herbaceous plants and even ferns (Davidson *et al*. 2003; Rizzo and Garbelotto 2003).

While it is useful to partition hosts on the basis of severity of disease symptoms, a far more meaningful grouping is that based on the role played by each host in the epidemiology of the disease. In Californian forests, sporulation (production of deciduous sporangia) by *P. ramorum* during favourable conditions is always greatest on bay laurel trees, with less abundant sporulation observed on other hosts such as tanoak twigs and redwood needles. Conversely, sporulation on oaks has not yet been observed, and consequently, it is believed that oak-to-oak infection may be insignificant and that bay laurels may effectively represent the most important reservoir of the pathogen in oak woodlands. (Davidson *et al*. 2005). Further evidence that supports the role of plants such as bay laurel in the epidemiology of the disease is the fact that presence of infected bay leaves is strongly correlated with girdling stem cankers on *Quercus agrifolia* (coast live oak) (Kelly and Meentemeyer 2002; Rizzo and Garbelotto 2003), and, in fact, foliar infections of bay laurel generally precede the infection of oaks (Rizzo and Garbelotto 2003). An analogous situation has been observed in nursery settings or in European wild infestations associated with plantings of ornamental plants where rhododendrons and camellias are reported as hosts of major epidemiological importance (Brasier *et al*. 2004).

Epidemiological models describing hosts acting as an infectious reservoir for a pathogen, differing from hosts representing transmissive dead-ends for the pathogen, are common among zoonotic and vector-borne diseases (Morens *et al*. 2004). Pathogen infectiousness and disease severity are normally extremely different between the two types of hosts in these diseases (Woolhouse *et al*. 2001). SOD in the evergreen coastal forests of California is one of the first examples of a plant disease in which lethally affected hosts (i.e. oaks) are transmissive dead-ends for the pathogen, while hosts spreading the disease (i.e. bay laurels, redwoods, and rhododendrons) are not necessarily lethally affected by the disease (Garbelotto *et al*. 2003).

Although the bay-oak and rhododendron-oak relationships have been described in the field, and a generalist nature of *P. ramorum* has been postulated, the presence or absence of host specificity has not been rigorously tested. The presence of undetected host specificity, a common trait among individuals in many pathogen species, may change our current hypothesis on how this disease spreads. Furthermore, although sporulation has not been observed on oaks, it may occur undetected, potentially challenging our current definition of oaks as dead-end hosts.

In this paper, we test the hypothesis that *P. ramorum* may be a true generalist by inoculating both coast live oak seedlings and bay laurel leaves with a selection of 12 isolates representative of the genetic, geographic and host range of *P. ramorum* in the western United States. A strong correlation of disease severity caused by the same isolate on both hosts would support the generalist hypothesis and negate the presence of host specificity, at least with regards to bay laurels and coast live oaks. We further test the hypothesis that isolates from coast live oak (putative dead-end hosts) may differ in virulence level when compared to isolates obtained from epidemiologically important or infectious hosts such as bay laurels, redwoods, tanoaks and rhododendrons. Reduced virulence of isolates from oaks may indicate they are of reduced fitness and may provide additional evidence in support of a substantial ecological difference between pathogen populations from infectious and dead-end hosts.

2 Materials and methods

2.1 Isolates

All 12 isolates of *P. ramorum* were collected from diseased native (10 isolates) and ornamental (two isolates) plants from California and Oregon (Table 1). To include samples representative of the entire North American population, isolate selection was performed taking into account the genotypic diversity described previously using amplified fragment length polymorphism (AFLP) analysis (Ivors *et al*. 2004). Five isolates were chosen from the dominant AFLP genotype I, which represents 75% of 67 isolates screened from California and Oregon (Ivors *et al*. 2004). The remaining seven isolates were selected randomly from 13 rare AFLP genotypes. Recent genotypic analysis using seven polymorphic SSR markers (based on Mascheretti et al. 2008) on five of the 12 genotypes selected has confirmed these genotypes are either identical or extremely closely related. Six isolates were from coast live oak (*Q. agrifolia*) and five were from a range of hosts on which sporulation of *P. ramorum* has been reported or observed in nature. One isolate (Pr-70) was from huckleberry (*Vaccinium ovatum*), a host for which *P. ramorum* sporulation is uncertain. This isolate was not used in the final analysis comparing isolates from oaks vs. isolates from putatively epidemiologically important hosts.

2.2 Capacity to form lesions on bay laurel leaves

Branches of about 10 cm in length with asymptomatic leaves were collected from a single bay laurel (*Umbellularia californica*) tree located at the University of California, Berkeley. Leaves from this tree were found to be of intermediate susceptibility compared to other trees previously tested (Meshriy *et al*. 2006). All leaves, except the first mature leaf, were carefully removed from the branch and discarded. To maintain turgidity of the attached leaf, each stem was placed into an individual flask containing sterile deionized water.

Each isolate was used to inoculate ten leaf tips with 300 ll of zoospores (1 x 10^4 spores/ml) and incubated for 14 days in humid chambers as described previously (Hüberli *et al*. 2003). The experiment was replicated once. Isolates Pr-27 and Pr-159 produced no (trial 1) or very low (trial 2) numbers of zoospores. The zoospore concentration for trial 2 for Pr-27 was 20 spores/ml and for Pr-159 was 1000 spores/ml. The controls were ten leaf tips inoculated with sterile deionized water. The average minimum and maximum temperatures were 18 and 24°C for trial 1 and 19 and 23°C for trial 2.

At harvest, lesions were traced onto film and the lesion area was calculated by counting the number of 1 mm squares within the tracing. To confirm the presence of *P. ramorum*, two small leaf pieces from the lesion margin were plated onto corn meal agar amended with pimaricin–ampicillin–rifampicin–PCNB (P_{10}ARP) containing 25 mg PCNB, a selective medium for *Phytophthora* spp. (Erwin and Ribeiro 1996). For leaves that had no lesions, the leaf tips exposed to zoospores or sterile water were plated onto P_{10}ARP.

2.3 Capacity to form lesions in stems of coast live oak seedlings

Two-year-old coast live oak (*Q. agrifolia*) seedlings (North Coast Native Nursery, Petaluma, CA, USA) in circular pots (6.5 x 25 cm) were used in two separate shade-house inoculation trials. In each trial, there were 10 replicate seedlings per isolate and control. The average minimum and maximum temperatures for the duration of the experiment were 8 and 14°C for trial 1 and 8 and 18°C for trial 2. All lateral shoots were pruned 6 weeks prior to inoculation.

A sterile scalpel was used to cut a bark-flap, about 6 mm long and 4 mm wide, upwards at approximately 15 cm above the potting medium surface on each stem. A 5-mm-diameter agar disc, cut from the margin of a 14-day-old culture growing on V8 juice agar (V8A; 100 ml non-clarified V8 juice and 17.5 g of agar/l), was inserted mycelium-side-down under the flap, the flap closed and the wound sealed with Parafilm and silver Nashua® tape (Tyco Adhesives, Franklin, MA, USA). Controls were inoculated with sterile V8A discs. The average stem diameter at the site of inoculation for both trials was 5.5 mm (range 3.5–9.5 mm).

Thirty-five days after inoculation, the stems were excised from the seedlings and the outer bark was carefully scraped back with a sterile scalpel blade at the site of inoculation to expose the maximum extent of any lesion that may have developed. The acropetal, basipetal and circumferential lengths of the lesion present in the phloem were measured. A small piece of phloem tissue cut from the margins of the longitudinal lesion and the site of inoculation was plated onto P_{10}ARP to confirm the presence of *P. ramorum*.

2.4 Data analysis

Data for parametric tests were screened for assumptions of homoscedasticity, presence of outliers, normality and noncorrelations of means and variances (Tabachnick and Fidell 1996). Significant main effects were compared using Fisher's least significant difference (LSD) test ($p = 0.05$) using STATISTICA (StatSoft Inc., Tulsa, OK, USA). For the coast live oak seedling inoculation experiment, stem diameter was included as a covariate and lesion lengths for both experiments were analysed together, because the data for both correlated significantly ($r = 0.48$, $p < 0.001$) using the Spearman rank *r*. Lesion areas in the bay laurel inoculation experiments were square-root transformed and were also analysed together, because data for both correlated significantly ($r = 0.44$, $p < 0.001$). The experiment-by-isolate interaction was analysed for the bay laurel and coast live oak experiments. Rank correlations were investigated among bay laurel and coast live oak lesions using both the Spearman rank *r* and linear regression.

3 Results

3.1 Capacity to form lesions on bay laurel leaves

Most isolates formed lesions on bay laurel leaves and these typically developed from water-soaked lesions after 1–2 days, to tan or brown lesions after 14 days. All isolates, except Pr-1, caused lesions that were not significantly different between experiments. Because isolate Pr-1 was consistently in the most aggressive group, the combined

data are presented. A significant difference ($p < 0.0001$) in mean lesion area was found among isolates, with a range of 0–31 mm^2 (Fig. 1). A group of three isolates, Pr-1, Pr-52 and Pr-106, consistently ($p < 0.006$) formed much larger lesions than the other isolates. Mean lesion sizes of isolates from AFLP genotype I were significantly different ($p < 0.001$) and ranged from 0 to 13 mm^2. Five isolates (Pr-27, Pr-36, Pr-75, Pr-159, Pr-345) consistently ($p < 0.02$) formed no or very small lesions on leaves (Fig. 1). Recovery of *P. ramorum* from leaves without lesions or with only very small lesions was unsuccessful with the exception of isolate Pr-345, which was recovered from up to 30% of the small lesions. Recovery from all other inoculated leaves was successful, while no recoveries were obtained from controls.

3.2 Capacity to form lesions in stems of coast live oak seedlings

All isolates formed water-soaked to brown lesions in the exposed phloem and longitudinal lesions were significantly ($p < 0.0001$) larger than the wound in the controls (Fig. 1). Lesions were generally not evident through the bark. Circumferential lesions or girdling were not a reliable measure of aggressiveness because stem circumferences were small and the size of inoculation wounds was too variable. They were therefore excluded from the analysis. Significant differences were found among isolates ($p < 0.0001$) and within AFLP genotype I ($p < 0.0001$), with mean lesion lengths ranging from 19 to 52 mm and 19 to 46 mm, respectively (Fig. 1). Three virulence groups were identified; isolates Pr-27 and Pr-159 produced the smallest lesions ($p < 0.05$), isolates Pr-1, Pr-52 and Pr-106 produced the largest ($p < 0.02$) lesions, while the remaining were intermediate in virulence. Isolates Pr-57, Pr-71 and Pr-345 caused a significant main effect for experiment ($p < 0.001$) and the interaction, experiment-by-isolate ($p < 0.001$). However, because these three isolates were consistently of intermediate aggressiveness in both experiments and changed their rankings only within this category, the combined data are presented (Fig. 1).

3.3 Comparative data analysis

Lesion length in coast live oak stems was significantly correlated with lesion area on bay laurel leaves ($r = 0.89$, $p = 0.0001$) (Fig. 2). Isolates from oaks were significantly less pathogenic than isolates from other hosts both on bay laurel leaves ($p = 0.003$) and coast live oak seedlings ($p = 0.04$) (Fig. 1).

4 Discussion

Results from this study suggest a strong positive correlation between virulence of all isolates on bay laurel and coast live oak. Virulence with regards to these two hosts is thus a generalist trait, i.e. isolates display equal virulence levels on both hosts. The lack of host specificity is further supported by the fact that the group of isolates from oaks was not more aggressive on their original host than the group of isolates collected from other hosts. Although we cannot exclude different outcomes from tests involving other isolates and/or plant hosts, the data from the experiments outlined here support that virulence in *P. ramorum* is a general trait. This analysis is particularly compelling in the light of the good genotypic, host and geographic representation of the selected isolates.

While isolates from oak and non-oak hosts were represented in all three virulence groups (low, intermediate and high) (Fig. 2), isolates from oaks were less aggressive on both hosts (Fig. 1). If the aggressive isolate Pr-70 was to be included in the comparative analysis, based on laboratory evidence that sporulation is possible on Vaccinum (Tooley *et al*. 2004), the difference between the two groups would even be larger (data not shown). This result supports a differential ecological role played by isolates from the two groups of hosts, with isolates obtained from epidemiologically relevant hosts being more pathogenic than those obtained from the dead-end oak hosts. We hypothesize that different virulence levels may be correlated with the high selection pressure on isolates from epidemiologically important hosts vs. a low selection pressure on dead-end hosts (Parker and Gilbert 2004).

A differential selection pressure for isolates from the two types of host is plausible based on our current knowledge of transmission and sporulation of *P. ramorum*. Bay laurel leaf infection, colonization and sporulation are crucial epidemiological steps for the spread of the disease (Rizzo and Garbelotto 2003; Davidson *et al*. 2005). Infected bay laurel leaves and tanoak twigs are the most important sources of inoculum in California. Infection and sporulation on foliar hosts are fast processes, often requiring just a few days to be completed in favourable weather conditions (Davidson *et al*. 2005). Furthermore, we have determined (data not shown) that lesion size and amount of sporulation in inoculated bay laurel leaves in vitro are strongly correlated. This correlation implies that the most pathogenic isolates will also be the ones most likely to sporulate successfully and infect new hosts. Intense competition among isolates for leaf infection and colonization may provide the necessary selection pressure needed to maintain high virulence levels among isolates in bay leaves and other epidemiologically important hosts (Bull 1994).

Conversely, oak infection is a comparatively rare event and usually occurs with the association of infected bay laurel (Kelly and Meentemeyer 2002; Swiecki and Bernhardt 2002; Davidson *et al*. 2005). The infection process is followed by a much slower process of host colonization, lasting between 6 months to several years (Rizzo and Garbelotto 2003). Isolates that have successfully infected oaks are not likely to encounter conspecific competitors; this lack of competition and the lack of opportunity/ability to spread to other hosts may lead to an ageing of the isolates and result in reduced virulence. This reduction in virulence may be a similar process that occurs in isolates that are maintained on agar media in the laboratory for extended periods (Erwin and Ribeiro 1996; Linde *et al*. 1999). In fact, we have observed that many oak isolates eventually die in culture compared to isolates from other hosts (unpublished data). Whether the reduced virulence of oak isolates may be attributed to an ageing effect, to a lack of competition among isolates, to a direct effect of the host (e.g. by producing chemicals inhibiting overall virulence) or to any combination of these factors, needs to be investigated further.

Although the study presented here provides valuable information on the epidemiology of SOD by analysing the virulence of a group of isolates representative of the overall diversity of *P. ramorum* in North America, it does not allow further determination of differences among epidemiologically important hosts, or among the distinct geographic regions in which this pathogen can be found. Multiple isolates per host and region of provenance need to be employed to answer such detailed questions. Nonetheless, this is the first study to show the range of virulence variation occurring within a molecular genotype (AFLP genotype I). SSR results have confirmed AFLP genotypes within each lineage of *P. ramorum* are closely related and generated by the fixation of mutations or mitotic recombination events (Ivors *et al*. 2006; Mascheretti *et al*. 2008, 2009; Vercauteren *et al*. 2010). Results of significant phenotypic variation within the same or very closely related genotypes are analogous to those found by previous studies on the related pathogen *Phytophthora cinnamomi* (Dudzinski *et al*. 1993; Hüberli *et al*. 2001) and indicate that phenotypic expressions including virulence are complex (Vercelli 2004), governed by the plastic nature of the phenotypes (Brasier 1991) and/or by epigenetic differences.

Overall, results from this study support the hypothesis that the epidemiology of SOD in oak-bay woodlands of North America may be consistent with the disease models developed for zoonotic and vector-borne diseases rather than other plant diseases. This may be different from the epidemiology of the disease in redwood-tanoak stands in the United States (Maloney *et al*. 2005; Davidson *et al*. 2008) or in larch plantations in the UK (Brasier and Webber 2010), where lethally affected hosts also appear to spread the disease. However, the distinct virulence of isolates from hosts characterized by different epidemiological importance here described may be present in analogous situations in Europe where rhododendrons are reported to be the infectious hosts, while oaks and beeches may in fact represent lethally affected dead-end hosts (Brasier *et al*. 2004).

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Table 1. North American isolates of *Phytophthora ramorum* used in this study.

Isolate numbers[1]	**Source**	**State, county**	**Year**	**AFLP genotype**[2]
Pr-1, CBS110534	*Quercus agrifolia*	California, Marin	2000	VII
Pr-27	*Q. agrifolia*	California, Marin	2000	I
Pr-36, CBS110953	*Q. agrifolia*	California, Sonoma	2000	III
Pr-52, CBS110537, ATCC MYA-2436	*Rhododendron* sp.	California, Santa Cruz	2000	V
Pr-57	Notholithocarpus *densiflorus*	California, Santa Clara	2001	I
Pr-70, CBS110539	*Vaccinium ovatum*	California, Marin	2001	I
Pr-71, CBS110539	*Q. agrifolia*	California, Sonoma	2001	II
Pr-75	*Q. agrifolia*	California, Monterey	2001	I
Pr-102, ATCC MYA-2949	*Q. agrifolia*	California, Marin	2001	I
Pr-106, CBS110956	*Umbellularia californica*	California, Sonoma	2001	VIII
Pr-159, CBS110543	*Lithocarpus densiflora*	Oregon, Brookings	2001	IV
Pr-345, CBS110544	*Sequoia sempervirens*	California, Sonoma	2002	VI

[1]All isolates with Pr numbers are from a culture collection maintained at University of California, Davis; some isolates were submitted to ATCC (American Type Culture Collection, Manassas, VA) and CBS (Centraal Bureau voor Schimmelcultures, Baarn, the Netherlands).
[2]Amplified fragment length polymorphism (AFLP) genotypes described previously (Ivors *et al*. 2004).

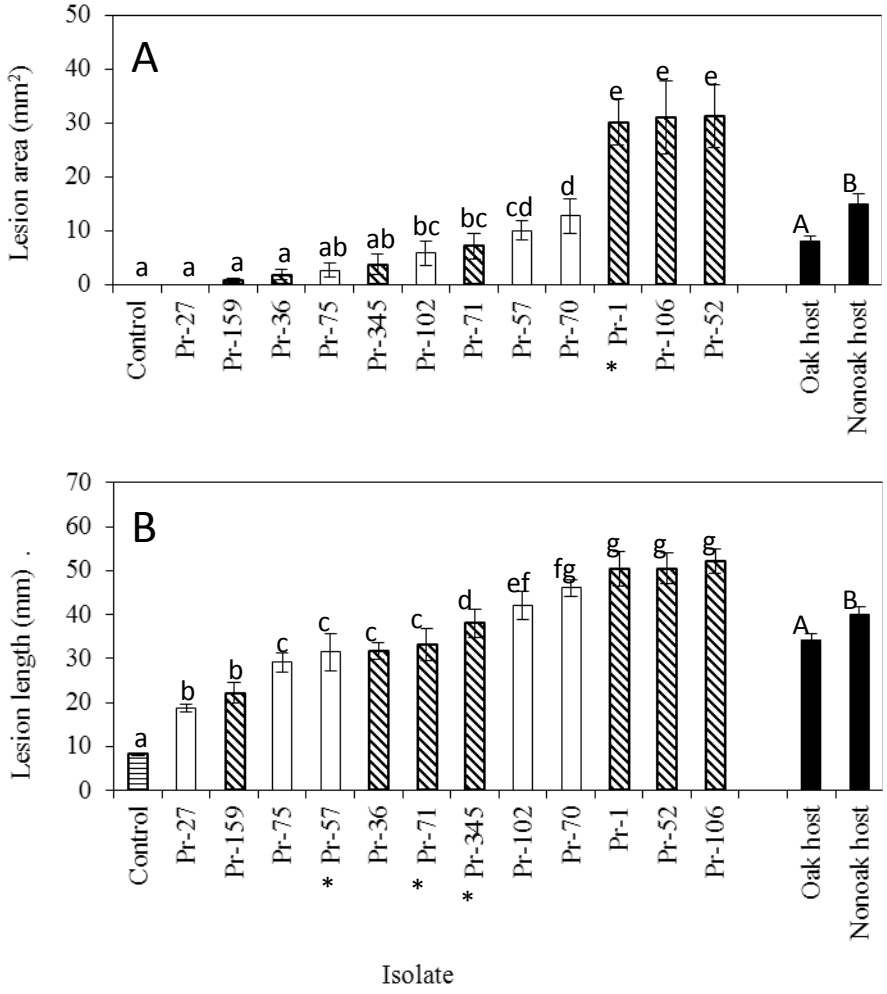

Fig. 1. Mean lesion area on leaves (n = 20) of *Umbellularia californica* (A) branches and mean lesion length in stems (n = 20) of *Quercus agrifolia* (B) seedlings 14 and 35 days after inoculation, respectively, with 12 North American isolates of *Phytophthora ramorum*. Error bars are standard errors of the mean for two pooled experiments. White bars are isolates from the AFLP clone, while striped bars are from the rare AFLP genotypes; those with the same letter are not significantly different (P = 0·05). Black bars are the mean data for host origin; those with the same capital letter are not significantly different (P = 0.05). *Lesions produced by these isolates were significantly different (P < 0.05) in the two experiments.

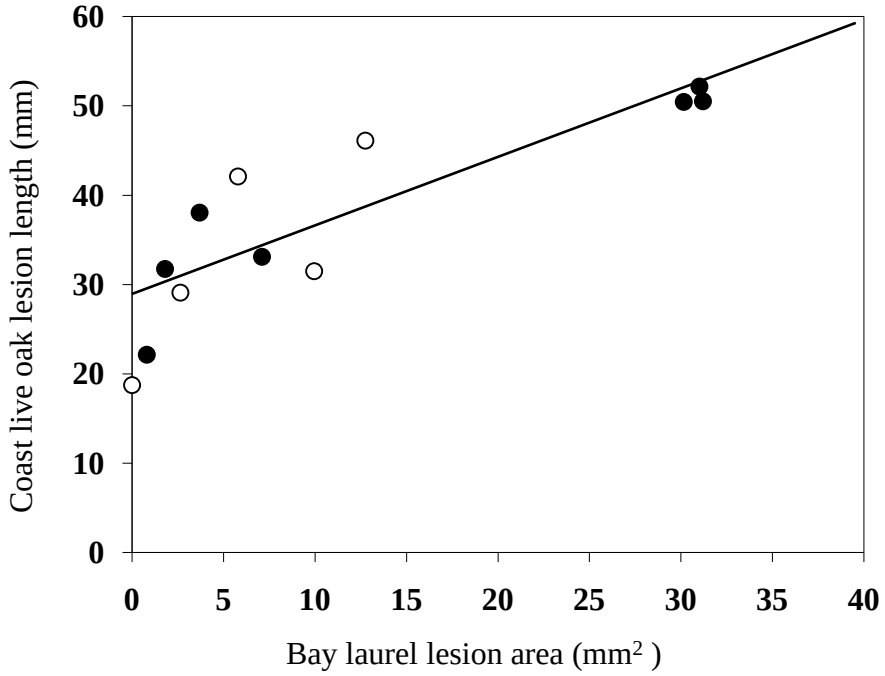

Fig. 2. Correlation between mean lesion area on leaves of *Umbellularia californica* (bay laurel) branches and mean lesion length in stems of *Quercus agrifolia* (coast live oak) seedlings 14 and 35 days after inoculation, respectively, with 12 North American isolates of *Phytophthora ramorum*. Non-filled points are isolates from AFLP clone, while filled points are from the rare AFLP genotypes. Spearman's rank correlation showed there was a significant positive correlation ($r = 0.89$, $p < 0.001$).