The Effects of Temperature and Inoculum Concentration on Susceptibility to Infection by *Phytophthora ramorum* in Tanoak and California Bay Laurel

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Abstract Despite widespread concern about risk factors associated with Sudden Oak Death, little is known about the conditions that limit infection. This study examines the effect of temperature and zoospore concentration, two factors hypothesized to limit infection rates and the extent of disease caused by *Phytophthora ramorum* in tanoak (*Lithocarpus densiflora*) and intermediately resistant California bay laurel (*Umbellularia californica*). Three trees of each species were sampled in the fall of 2004 in Marin County, CA. Leaves were inoculated at concentrations 3.3x10, $3.3x10^2$, $3.3x10^3$ and $3.3x10^4$ spores/ml, then incubated at ideal growth conditions (19 °C). In a separate experiment leaves were inoculated with $3.3x10^4$ spores/ml at temperatures 5 °C, 12 °C, 19 °C, 26 °C and 33 °C. To determine how lesion size varied within these conditions, lesion area was measured. Both experiments yielded leaf infection at all conditions, including the lowest concentration levels. Increasing zoospore concentration led to (a) statistically significant increases in infection rates and (b) increasing mean lesion sizes for both species. Infection rates in bay leaves were greatest at intermediate temperatures, although unexpectedly, relatively high infection rates were found at the highest and lowest temperatures. Significant differences in lesion size in bay were found between intermediate temperature groups and those at the extremes. Infection rates and lesion size in tanoak suggested an inhibitory effect of heat, but not of cold. These experiments demonstrate a remarkable ability of *P. ramorum* zoospores to infect leaves, even at suboptimal conditions.

Introduction

Sudden oak death (SOD) is a recently discovered epidemic that occurs throughout the coastal regions of California and Oregon, from the counties of Monterey to Mendocino in California and is isolated in Curry County in southwest Oregon (McPherson et al. 2003). Over the past ten years SOD has had a devastating effect on oak and tanoak species in forests along 185 miles of the Central Coast in California (Garbelotto et al. 2003) and could dramatically impact ecosystem structure and function in the future (Kelly and Meentemeyer 2002). Currently, SOD is a threat to native oak forests and the ecosystems supported by these forests. In addition, it is a disease that has serious implications for land management policy. (M. Garbelotto, per. comm. April, 2004) It is especially important to study this disease as little is known about how it spreads across the stand and the ecosystem level. Given that tanoak, coast live oak, and black oak trees are distributed along 1,500 miles of the coast of California and Oregon (Garbelotto et al. 2001), the potential for huge negative ecosystem impact is high.

Sudden oak death is caused by the pathogen *Phytophthora ramorum*, a new species of *Phytophthora* in the phylum Oomycota (Werres et al. 2001, Garbelotto et al. 2003) identified via DNA-based phylogenetic analysis in 2002 (Garbelotto et al. 2002a, Rizzo et al. 2002a). Although *P. ramorum* looks and behaves like a fungus it is actually a water mold, phylum Oomycota (Garbelotto et al. 2001), that causes two types of diseases in hosts, one that lethally infects branches and stems, and the other that non-lethally infects foliage and twigs (Rizzo and Garbelotto 2003, Garbelotto et al. 2003). This study examines two possible conditions that affect infection rates and lesion size of disease caused by *P. ramorum* in tanoak (*Lithocarpus densiflora*), a tree species that is fatally affected by the pathogen and intermediately resistant California bay laurel (*Umbellularia californica)*, a non-lethally damaged tree host for the pathogen.

The lethal form of the disease is an infection of the trunk of oaks and tanoaks that creates black bordered sap-seeping cankers (Rizzo et al. 2002a). *P. ramorum* kills tanoak, coast live oak (*Quercus agrifolia*), California black oak (*Quercus kellogii*), and Shreve's oak (*Quercus parvula var shervei*) in the greatest numbers (Rizzo et al. 2002). The disease girdles the infected tree, effectively cutting off its' vascular transport mechanism for water supply (Rizzo et al. 2002a). *P. ramorum* non-lethally colonizes the foliage of an ever increasing number of overstory and understory hosts. These foliar hosts, which are affected by pathogen infection in leaves only, are

thought to be the main source of disease spread of *P. ramorum*, which is then spread by wind and rain to susceptible trees and subsequently leads to infection and eventual mortality (Kelly and Meentemeyer 2002). Foliar hosts have been hypothesized to play a key role in the spread of *P. ramorum*, and could be solely responsible for triggering the current forest epidemics (Garbelotto et al. 2003).

Over the last few years there have been increasing discoveries of foliar host species associated with the spread of *P. ramorum*; a good example is the California bay laurel. In a 2002 report of stem water potential as a risk factor for *P. ramorum* infection, Swiecki and Bernhardt (2002) found that there was a positive association between the density of California bay trees and SOD. They hypothesized that this connection was due to the creation of favorable microclimates for pathogen infection (Swiecki and Bernhardt 2002); later, it was found that the California bay is a foliar host (Rizzo et al. 2002b).

Currently, the known geographic *P. ramorum* spread consists of forest types that have cool and moist climates (Rizzo et al. 2002a). Studies done on inoculum control in *P. ramorum* have shown that the pathogen is sensitive to high temperatures (higher than 35 °C) (Garbelotto et al. 2001), and is therefore thought to favor wet and cool environments (Swiecki and Bernhardt 2002, Garbelotto et al. 2002a, Garbelotto et al. 2003). Because foliar hosts have been hypothesized to be important in spreading the pathogen to oaks, forests that have a high diversity of plant hosts may be more at risk for invasion by *P. ramorum* (Rizzo and Garbelotto 2003). In contrast, a lower density of favored hosts may result in a more gradual spread of the disease, and allow forest managers to identify and protect stands before they are fully destroyed. In this light, studies need to be conducted that examine the conditions that affect the rate of infection of *P. ramorum* in both types of hosts.

Given the relatively small amount of time that the pathogen *P. ramorum* has been studied, and the limited understanding its effect on forests and ecosystems at the landscape scale, it is crucial that more research be done in this area. This study focuses on the environmental conditions that limit infection rates and lesion size of disease caused by the pathogen *P. ramorum* among bay of intermediate resistance and tanoak. I was specifically interested in looking at how infection rates and lesion size, the measurable size of the infection, varied within a range of temperature and inoculum levels. This information could shape future forestry management strategies.

Methods

Zoospores of *P. ramorum* (with which sample leaves were infected) were grown by infecting three bay laurel and three tanoak leaves collected on the UC Berkeley campus, Berkeley, CA, with an isolate of *P. ramorum*, Pr-52 (Rizzo 2000). To grow each isolate, 2 mm^2 plugs of the isolate were placed on each leaf of each species and grown at room temperature for 10 days (bay leaves were infected with Pr-52 at the leaf tip and tanoak leaves were infected with the isolate at the petiole, methodology explained below). I took a portion of the lesion resulting from the inoculation from each leaf and plated it on PARP agar, a medium that allows the pathogen to grow selectively. After 10 days of growth in a 20 °C incubator, 2 mm^2 plugs of hyphae were taken from the growing edge of the mycelium and were transferred to a V8 plate (a more selective medium), and placed in a 20 °C growth chamber. Next, 2 mm^2 plugs of hyphae were transferred from the growing edge of the original V8 plates to new V8 plates. These plates were allowed to grow at 20 °C in an incubator for 10 days. Sporangia growth was induced three days before the experiments began by cutting out the circular hyphae growth from each V8 plate, transferring them to sterile 10 cm diameter Petri dishes, and slicing them into 20 small cubes. The cubes were equally spaced on the Petri dish and flooded with sterile water. The dishes were placed in the dark at room temperature from three days. This step allows the release of sporangia into the sterile water, producing a spore suspension with which sample leaves are infected.

Sample leaves were collected from three tanoak individuals and three bay individuals in Samuel Taylor State Park in Marin County. I randomly selected three bay trees of intermediate resistance from a previously randomly selected set of 10 trees using unpublished data by Tami Harnik and Daniel Hüberli, ESPM, UC Berkeley, and a map provided by the Garbelotto Lab, UC Berkeley. Because no information exists on genetic variability within the tanoak species at Samuel Taylor State Park, three tanoak trees were chosen in a redwood grove about a mile away from the selected bays and each tree was tagged for future reference.

After an initial visual sweep of each tree and branches with at least 200 leaves on a majority of their branches were selected. Every effort was made to avoid sampling bias by choosing branches at a similar height on all trees. After cutting off the chosen branches using a saw-pole apparatus I clipped limbs off the branches to create three bundles of 10 limbs per tree. Limbs were chosen based upon the visual appearance of each leaf. If a limb was thought to be infected by *P. ramorum* (detected visually by the appearance of lesions) or was otherwise infected or

contaminated it was not chosen. The bundles were then placed in buckets of water, draped with wet towels, placed in a cooler with ice and brought to the lab.

An hour before leaves were inoculated I induced zoospore growth by chilling the sporangia/ water solution in beakers on ice, causing the sporangia to release their zoospores (UC Berkeley protocol, unpublished). To prepare the zoospore solution, the zoospores were first quantified by using a haemocytometer. Three sample counts were taken for each zoospore isolate (bay and tanoak) and from these sample counts an average concentration was calculated.

I used a detached leaf assay inoculation method. Since bay is a foliar host and tanoak is stem host, each species becomes infected differently; bay is infected through their leaves and tanoak is infected through their woody structures. Therefore, to inoculate the leaves, the tip of a bay leaf and the petiole of a tanoak leaf are placed in a 50 ml conical Falcon tube and 300 microliters of the zoospore solution is pipetted into the tube and left for 24 hours at the experimental temperature/ inoculum condition (K. Hayden 2004, pers. comm.).

In the temperature experiment, the zoospore concentration was held constant at $3.3x10^4$ spores/ml for each trial, which is currently the standard concentration used in most inoculation experiments in the Garbelotto laboratory (K. Hayden, pers. comm.). There were five different temperature conditions, 5 °C, 12 °C, 19 °C, 26 °C, 33 °C. Twenty leaves from each bay and twenty leaves from each tanoak were incubated after inoculation at each temperature for 24 hours.

In the inoculum experiment, the temperature was held constant at 19 °C, ideal growth conditions (K. Hayden 2004, pers. comm.). The variable in this experiment was the zoospore concentration. Four different concentrations of inoculum were made, 3.3x10, $3.3x10^2$, $3.3x10^3$ and $3.3x10^4$ spores/ml by first determining the initial zoospore concentration as previously described, then diluting the solution as necessary with sterile water. Twenty bay leaves from each bay tree (60 total) and twenty tanoak leaves from each tanoak tree (60 total) were inoculated at each zoospore concentration for one day. A negative control consisting of a single leaf inoculated in sterile water was included for each tree at each condition. Dodd, et al. (2004) found that only one negative control was necessary for their experiment involving *P. ramourm* and coast live oak. Additionally, Schmale and Gordon (2003) found that no negative control was needed in their inoculation experiments. The protocol in the Garbelotto Laboratory for experiments using *P. ramorum* is to use only one negative control (K. Hayden, per. comm.).

After the 24-hour inoculation period all leaves were removed from suspension, the excess solution was blotted off, and the leaves were laid on wet paper towels and incubated in moist chambers at 19 °C for 10 days.

The inoculated leaves were placed on paper and digitally scanned. The computer program ASSESS (American Phytopathology Society, St. Paul, Minnesota) was used to measure lesion area, using a protocol developed by D. Hüberli and T. Harnik, UC Berkeley. All data were gathered on two separate factors, based on visual information. The first was if the pathogen caused infection of the leaf. The second was the size of the lesion on infected leaves. Lesions were determined visually; bay lesions less than 0.5 mm in diameter were not counted as official lesions for statistical analysis (K. Hayden 2004, pers. comm.). Statistical analyses were performed in JMP 5.0 (SAS Institute, Cary NC). Average lesion size and infection rates for all treatments were compared within and between hosts using analysis of variance (ANOVA).

Results

Leaf infection occurred at all experimental zoospore concentrations for both tanoak and bay leaves. There were no infections on control leaves and therefore control results are not shown. For both species zoospore concentration was positively related to infection rate (Fig. 1a,b; bay: Pearson chi square= 136, $p<0.0001$; tanoak: Pearson chi square= 165, $p<0.0001$). Post-hoc comparison between treatments of both bay leaves and tanoak leaves revealed that infection rates fell into two groups: 3.3x10 and $3.3x10^2$ spores/ml and $3.3x10^3$ and $3.3x10^4$ spores/ml (bay: df=1, Pearson chi square=134, $p<0.0001$; tanoak: df=1, Pearson chi square= 164, $p<0.0001$).

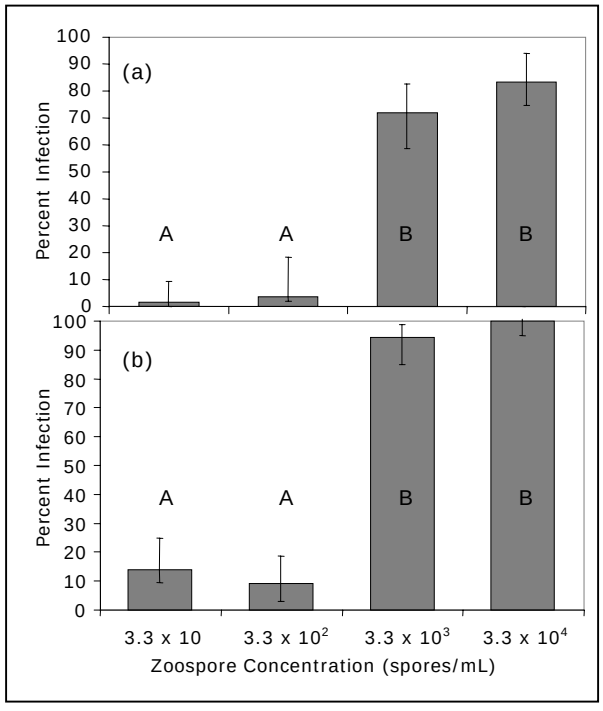

Figure 1a,b: Infection rates in (a) bay leaves and (b) tanoak leaves at experimental zoospore concentrations. Treatments with the same letter are not significantly different. Error bars represent one standard error of the mean.

There was a significant effect of zoospore concentration on bay lesion size (ANOVA, F=10, $p<0.0001$). Lesion size in bay leaves fell into three significantly different concentration groups, $3.3x10^4$, $3.3x10^3$ and $3.3x10^2$ spores/ml (Fig. 2a; Tukey-Kramer comparisons). The concentration 3.3x10 spores/ml was not statistically different from any of the other groups. Comparisons between the treatments of tanoak leaves revealed mean lesion sizes to fall into two significantly different groups: 3.3x10 spores/ml and $3.3x10^2$, $3.3x10^3$, $3.3x10^4$ spores/ml (Fig 2b; Tukey-Kramer comparisons).

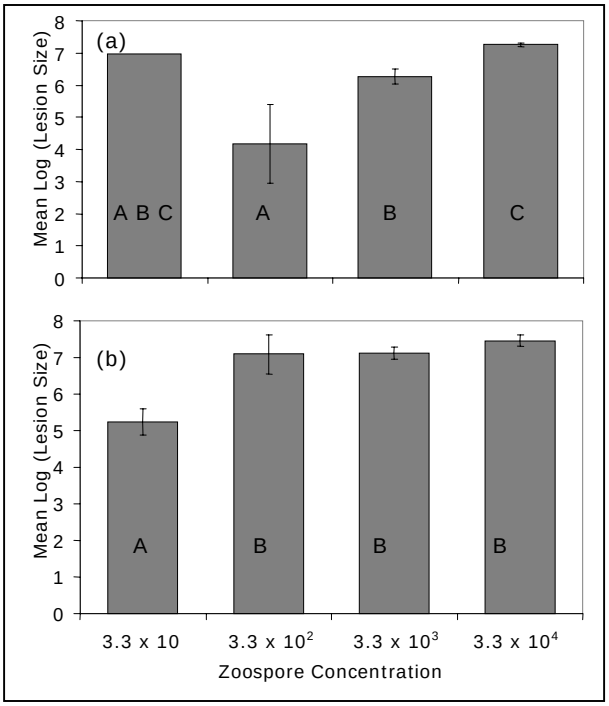

Figure 2a,b: Mean lesion size in (a) bay leaves and (b) tanoak leaves at experimental zoospore concentrations. Treatments with the same letter are not significantly different. Error bars represent one standard error of the mean.

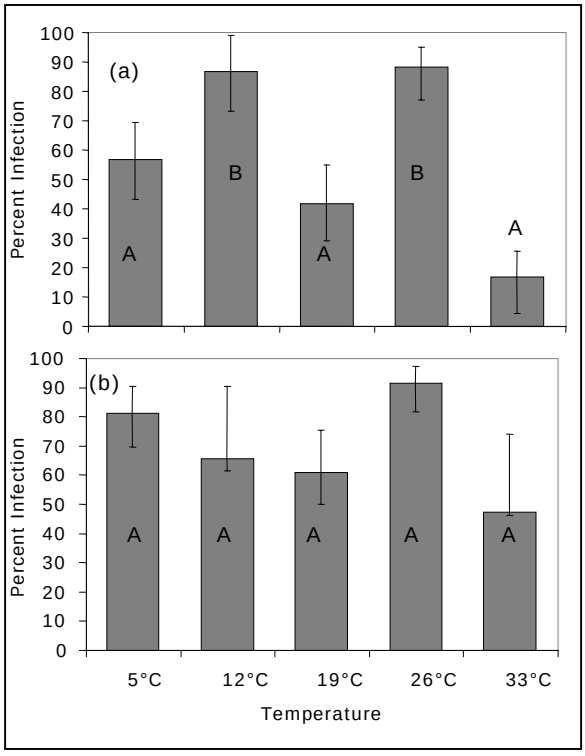

Figure 3a,b: Infection rates in (a) bay leaves and (b) tanoak leaves at experimental temperatures. Treatments with the same letter are not significantly different. Error bars represent one standard error of the mean.

In both tanoak and bay leaves temperature had an erratic effect on infection rates. Post-hoc comparison of temperature treatments revealed that infection rates of bay leaves fell into two groups that were significantly different from each other: 5 ºC, 19 ºC, 33 ºC and12 ºC, 26 ºC (Fig. 3a; ANOVA, df=1, Pearson chi square= 65, $p<0.0001$). There were no significant differences between infection rates in tanoak leaves among the five experimental temperatures (Fig. 3b; ANOVA, Pearson chi square= 32.15, $p<0.0001$).

Comparison between treatments showed that mean lesion size in bay leaves fell into two statistically different temperature groups (Fig. 4a; Tukey-Kramer comparisons). These groups were 5ºC and 12ºC, 19ºC, and 26ºC. Mean lesion sizes in bay leaves at 33ºC were not statistically different from other temperatures. Significant differences in mean lesion size in tanoak leaves over the five experimental temperatures fell into two groups: 26ºC and 33ºC (Fig. 4b; Tukey-Kramer comparisons). Mean lesion sizes at 5ºC, 12ºC and 19ºC were not significantly different from the other temperatures.

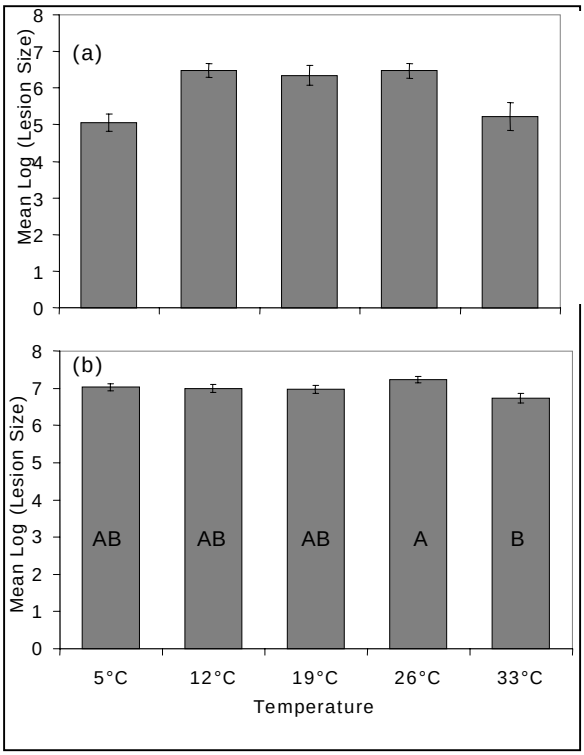

Figure 4 a,b: Lesion size in (a) bay leaves and (b) tanoak leaves at experimental temperatures. Treatments with the same letter are not significantly different. Error bars represent one standard error of the mean

Discussion

Zoospore concentration had an unambiguous effect on infection rates in both bay as well as tanoak leaves. As leaf infection was not expected to occur at the lowest concentration levels, it was remarkable that infection took place at these low levels. At 3.3x10 spores/ml, one bay leaf (1.7% of total) and 8 tanoak leaves (14.3%) were infected. Although infection did occur at these conditions, there was a significant decrease in infection rates for both species at $3.3x10^2$ and 3.3x10 spores/ml (Figs. 1a,b). These results indicate that there was a clear zoospore threshold in both tree species between zoospore concentrations $3.3x10^2$ and $3.3x10^3$ spores/ml. Above this threshold infection rates increased dramatically and below this point they were very small. Clearly, decreasing zoospore concentration decreases infection rates, but may not completely eliminate infection, even at very low spore loads. Hansen et al. in 2005 found that a similar significant dose response relationship for tanoak, but not for bay. In particular, the 2005 study showed similar threshold results for tanoak; in a detached leaf study they found a large jump in infection rates in tanoak, on the same order of magnitude as this study, between zoospore concentrations on the order of 10^2 spores/ml and 10^3 spores/ml (Hansen et al. 2005). However, in

the Hansen study bay leaves were not shown to have a dose response relationship, as infection rates at all zoospore concentrations were very close to zero. The difference between these two studies could arise from the fact that the Hansen study used a sample size of five leaves per treatment, where this study used a sample size of 60 leaves per treatment.

For both species results are strongly suggestive of a correlation between zoospore concentration and lesion size. Increasing zoospore concentration in bay leaf inoculations led to an increase in mean lesion size (Fig. 2a). Interestingly, the one bay leaf that was infected at the lowest concentration showed a remarkably large lesion size, not following this trend. However, because this infected sample size was so small compared to an infected sample size of 50-60 leaves in the higher concentrations, there was little power to detect differences. Therefore this lesion size was noted, but not considered to be an indicator of the trend. Although there was no significant trend in the mean lesion size of tanoak leaves, the ordering of the means follows the trend of increasing mean lesion size with increasing zoospore concentration (Fig. 2b).

Effects of temperature on infection rates and lesion size of bay and tanoak leaves were erratic. Surprisingly, for both tree species there were relatively high infection rates at the extreme temperatures (5 ºC and 33 ºC, Figs. 3a,b). In bay leaves there was a statistically significant difference in infection rates at the extremes and those in the mid-temperatures (12 ºC and 26 ºC). A clear picture of how temperature affects bay leaves was not developed because infection rates at the optimum temperature (19 ºC), where highest infection rates were expected to be found, were not statistically different from those at the extremes (Fig. 3a).

There were no significant differences between infection rates in tanoak leaves at experimental temperatures. However, both bay and tanoak leaves showed higher infection rates at the coolest temperature compared to the warmest temperature. Despite the lack of statistically significant results, the overall trend indicates an inhibitory effect of heat but not cold. This trend support previous research that suggests that *P. ramorum* is sensitive to high temperatures and favors cooler ones (Swiecki and Bernhardt 2002, Garbelotto et al. 2002a, Garbelotto et al. 2003).

Mean lesion sizes in bay leaves inoculated at experimental temperatures showed a hump-shaped response to temperature (Fig.4a). A similar but much less pronounced effect of temperature was reflected in the lesion size of tanoak leaves (Fig. 4b). At the highest temperature mean lesion sizes were significantly smaller than mean lesion sizes at 26ºC. This suggests that by

increasing temperatures in tanoak leaves from the intermediate temperature range lesion sizes will decrease.

Overall, a highly statistically significant trend was found in mean lesion size on bay and tanoak leaves: increasing zoospore concentration lead to increased infection rates. The temperature experiment resulted in less obvious outcomes. Trends suggested infection rates for bay leaves were highest in intermediate temperatures. Mean lesion sizes for both tanoak and bay leaves pointed to larger lesion sizes at cooler temperature when compared only to warmer temperatures. The anomalous results of the temperature portion of this study were perhaps due to an incubator idiosyncrasy. Because of time constraints, incubators were not checked daily to ensure temperature accuracy. The growth chambers used in this experiment were old and notoriously faulty. Additionally, peculiar outcomes could possibly be attributed to experimental methods as all inoculations were performed at room temperature and therefore zoospores and leaves were heated or chilled after inoculation was initiated.

Several remarkable and important results that have serious implications for tree stands on the west coast were found through this study. Infection occurred in both bay and tanoak at very small zoospore concentrations. This finding has huge ramifications for both tree stands and ecosystems. Tanoak species are the most susceptible species to infection by *P. ramorum* (Rizzo and Garbelotto 2003, Garbelotto et al. 2002b). Although tanoak is highly resistant to temperature changes and has been historically very adaptable to other types of environmental conditions, such as volcano erruption and drought (McDonald 1987), *P. ramorum* is seriously impacting tanoak stands (Hayden, per. comm. April 2004) throughout their extensive coastal range (Garbelotto et al. 2001).

The vulnerability to the pathogen that tanoak possesses puts whole ecosystems at risk because tanoak systems provide an ecologically important niche for animals and plants (McDonald 1987). In a study of Douglas-fir forests in northwestern California it was found that wildlife populations are positively correlated to the occurrence of tanoak stands (Raphael 1987). The pathogen has a large host range and it is able to spread through air (Garbelotto et al. 2003), and therefore has an enormous potential to produce drastic changes in the foodweb and the ecosystem.

Over the course of history aggressive oomycete pathogens have caused a great deal of destruction. A case similar to *P. ramorum* was *P. cinnammomi*, a tree pathogen which caused

devastation in western Australia's forests. Since *P. cinnammomi* was introduced, it has eradicated almost every tree species in the *Eucalyptus marginata* forests, resulting in the transformation of these ecosystems into grasslands (Weste and Marks 1987). The results of these experiments demonstrate that even though infection in both bay and tanoak species drops off significantly at small zoospore concentrations, the potential for infection still exists at very small spore loads. This potential could have drastic effects on tanoak ecosystems throughout the range of *P. ramorum*.

It is obvious that more work must be done on this topic in particular. Future research directions to yield more unambiguous results could involve altering zoospore temperatures before inoculation, include temperatures beyond the range tested, and perhaps an expanded, smaller interval of temperatures. Additionally, it would be interesting to take a daily zoospore count on random incubating leaves to determine the ability of temperature to decrease zoospore loads during the incubation period.

Despite some anonmolus results, it is unequivocally clear that *P. ramorum* zoospores have a remarkable ability to infect leaves even at suboptimal conditions.

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