

Survival and Chlamydospore Production of *Phytophthora ramorum* in California Bay Laurel Leaves¹

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Abstract

Sudden oak death manifests as non-lethal foliar lesions on bay laurel (*Umbellularia californica*), which support sporulation and survival of *Phytophthora ramorum* in forest ecosystems. The pathogen survives the dry summers in a proportion of attached bay leaves, but the propagules responsible for survival are unknown. This study focuses on summer pathogen survival associated with bay laurel in redwood-tanoak and mixed-evergreen forests with specific objectives including: i) detection of *P. ramorum* in leaf litter and soils throughout the annual disease cycle, ii) quantification of chlamydospores on and within attached symptomatic leaves, and in fresh and aged litter, iii) determination of chlamydospore germination, and, iv) assessment of pathogen survival within litter and canopy leaves, addressing the location of viable inoculum within foliar tissues. Ten trees were tagged for repetitive sampling in four redwood-tanoak and four mixed-evergreen forests. Sampling was conducted at four times between May 2006 and September 2007 to target different points during the disease cycle. To determine pathogen presence in leaf litter and soil, three soil samples and 20 symptomatic litter leaves were collected and independently bulked from each tree. Samples were then baited for *P. ramorum* with rhododendron leaves. Chlamydospore populations on surfaces of attached leaves, and fresh and aged litter were determined by scrubbing individual leaves with a moistened toothbrush and filtering the resulting suspension through 35µM nylon mesh. Chlamydospores were then counted under a dissecting microscope and a subsample of chlamydospores was placed on selective medium to observe germination potential. To evaluate chlamydospore production within tissue, leaves were cleared with KOH and then observed with light microscopy. Pathogen survival and colonization was determined by subdividing symptomatic tissue from each leaf for detection by PCR, culture, and microscopy. Chlamydospore populations on attached leaf surfaces were higher in redwood-tanoak than in mixed-evergreen forests, but chlamydospore germination was never observed. Pathogen recovery was highest in the late spring, but declined by the end of the summer survival period. Isolation recovery was higher in attached leaves than in freshly cast leaves, but was rare from aged leaf litter tissues. High pathogen detection frequency was achieved by PCR, even when recovery in culture was not possible. Pathogen detection by PCR and isolation did not correspond with the heightened chlamydospore production observed at some sites. Though bay laurel supports chlamydospore production, the quantity of chlamydospores produced per leaf varies between sites and the lack of germination of these survival propagules contributes to the mystery of their potential role in the epidemiology of sudden oak death.

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Introduction

Phytophthora ramorum, the cause of sudden oak death (SOD) in California (CA), is an exotic plant pathogen that has caused extensive mortality of susceptible oak (*Quercus* spp.) and tanoak (*Lithocarpus densiflorus*) in coastal California forests since the mid-1990s (Rizzo and others 2002, Rizzo and others 2005). The pathogen causes non-lethal infections on numerous hardwood and coniferous forest tree species, understory shrubs, and herbaceous plants (Davidson and others 2003, Garbelotto and others 2003). The non-lethal foliar lesions formed on California bay laurel (*Umbellularia californica*) by *P. ramorum* support prolific sporulation during rain events and summer survival of the pathogen in forest ecosystems (Davidson and others 2005b).

Though symptoms of *P. ramorum* on bay laurel are limited to foliar lesions, the sporulation and survival potential of the pathogen on this host elevate its epidemiological significance in the forest ecosystem. At an ecological level, presence of bay laurel correlates with occurrence of *P. ramorum* (Rizzo and others 2005), and on individual leaves the pathogen may produce as many as 5×10^3 zoospores during a single rain event (Davidson and others 2005a). Foliar infections of bay laurel generally precede the infection of adjacent oaks (Rizzo and Garbelotto, 2003) and isolate aggressiveness on bay laurel correlates directly with aggressiveness on oak (Hüberli and others 2005). The pathogen survives the hot, dry summer months in a proportion of symptomatic bay leaves (Davidson and others 2005a); but summer survival potential in bay leaves varies between sites. The phenomenon of site-influenced survival of *P. ramorum* within bay laurel has been documented in both a redwood-tanoak forest and a mixed-evergreen forest (dominated by coast live oak) at Jack London State Park (Glen Ellen, CA) and Fairfield Osborne Preserve (Pengrove, CA), respectively. Summer foliar survival in attached symptomatic leaves is higher in the redwood-tanoak forest (70 percent recovery) than in the mixed-evergreen forest (20 percent recovery), yet these two sites are located only 5km apart on opposite sides of Sonoma Mountain (J. Davidson, unpublished data). Furthermore, sporangia production is detected earlier and both disease spread and severity are higher in the redwood-tanoak forest than in the mixed-evergreen forest. The difference in the disease progress curve between the two sites suggests a disparity in amount of primary inoculum at the onset of the rainy season; however, the propagules responsible for pathogen survival are yet unknown.

Based on the hypothesis that primary inoculum is more abundant in redwood-tanoak forests than in mixed-evergreen forests at the onset of the fall rainy season, the overall focus of this work is to assess summer pathogen survival associated with bay laurel in the two forest types. Because infected bay laurel leaves are more likely to abscise than uninfected leaves, our investigations target both symptomatic attached leaves, and symptomatic leaves in the litter layer. Specific objectives include: i) detection of *P. ramorum* in leaf litter and soils throughout the annual disease cycle, ii) quantification of chlamydospores on attached symptomatic leaves, and in fresh and aged litter, iii) determination of chlamydospore germination, and, iv) assessment of pathogen survival within litter and canopy leaves, addressing the location of viable inoculum within foliar tissues.

Materials and Methods

Ten trees were tagged for repetitive sampling in four redwood-tanoak (Jack London State Park, Pfeiffer Big Sur State Park, Henry Cowell Redwoods State Park, and Samuel P. Taylor State Park) and four mixed-evergreen forests (Fairfield Osborne Preserve, Pacheco, Skyline Regional Park, and China Camp State Park). The redwood-tanoak and mixed-evergreen forest sites spanned latitudinal gradients of over 225 km, and 125 km, respectively. Sampling was conducted in May and August 2006 and in March and September 2007. At each sampling event, all samples were collected from the eight sights within two days. Three types of bay foliage were collected: symptomatic attached leaves, freshly cast litter leaves, and aged litter leaves. The September 2007 collection diverged from the normal sampling strategy because freshly cast leaf specimens were not available at all sites.

Baiting of Litter and Soil

To determine pathogen presence in leaf litter and soil, 20 leaves were collected and bulked from the forest floor and three soil samples were collected and bulked from under the canopy of each tree. Litter and soil samples were then baited with rhododendron cv. Colonel Coen leaves for *P. ramorum*. Lesion margins from symptomatic baits were placed on PARPH selective medium containing 0.05 g hymexazol/L for determination of presence of *P. ramorum*. A subsample of soil from each tree was passed through a 2 mm sieve and a 30-40 g sample was weighed and oven-dried for determination of soil moisture content.

Determination of Chlamydospore Populations

At each sample time, one symptomatic attached leaf, one symptomatic freshly cast leaf and one symptomatic aged litter leaf were collected for determination of chlamydospore presence on leaf surfaces of each of the 80 tagged trees. Additionally, one asymptomatic leaf was sampled from each of two trees at each site to assess for asymptomatic sporulation. Individual leaves were scrubbed on both sides with a moistened toothbrush and rinsed with deionized water before filtering the resulting suspension through a 35 μ M nylon mesh. Chlamydospores were then counted under the dissecting microscope and the total number of chlamydospores per leaf was recorded.

To look for chlamydospores within leaf tissue, another set of symptomatic attached-, freshly cast-, and aged litter leaves was gathered from each tagged tree and cleared in 1N KOH to render tissue translucent. After approximately three weeks in the KOH solution, leaves were clear enough to observe under the compound microscope.

Determination of Chlamydospore Germination Potential

At each sample time chlamydospore germination potential was determined by individually selecting 10 mature chlamydospores from the symptomatic attached-, freshly cast-, and aged litter leaves at each of the eight sites. For some sets of leaves, particularly those collected from mixed-evergreen forests, fewer than ten chlamydospores were available for germination studies. Individual chlamydospores were placed on PARPH selective medium, incubated in the dark at room temperature, and observed periodically over two weeks for chlamydospore germination.

Additionally, chlamydospores dislodged from symptomatic attached leaves in September 2007 were transferred to uninfected bay laurel and rhododendron leaves to

determine infection potential. Immediately after scrubbing leaves from Samuel P. Taylor State Park and Henry Cowell Redwoods State Park, chlamydospores were counted and 10 chlamydospores were placed on three detached, moistened bay laurel and rhododendron leaves. Leaves were then stored in moist chambers for approximately 2 weeks for observation of symptom development and subsequent isolation of symptomatic and asymptomatic tissue onto PARP.

Pathogen Survival and Colonization

To assess pathogen survival and colonization in leaf tissue, one symptomatic attached-, one freshly-cast-, and one aged litter- leaf was collected from each tagged tree. For each leaf, the area along the lesion margin was subdivided for detection by PCR, culture, and scanning electron microscopy (SEM). For detection in culture, tissue from attached symptomatic leaves was placed in PARP, whereas tissue sampled from the forest floor was placed in PARPH. Isolation plates were then stored in the dark for up to three weeks at room temperature for assessing recovery of *P. ramorum* and other *Phytophthora* spp. present. Only samples lacking recovery of *P. ramorum* in culture were assessed for pathogen presence using PCR. For visual observation of colonization, samples were fixed and preserved in formalin acetic acid (FAA) for future observation using scanning electron microscopy.

Results

Baiting of Litter and Soil

P. ramorum was baited from 60–90 percent of soil samples at all sites in May 2006 (fig. 1A), but was undetectable by August 2006. In May 2007, *P. ramorum* was baited from 0-70 percent of the soil samples (fig. 1B), with samples from redwood-tanoak forests exhibiting generally higher recovery than those from mixed-evergreen forests. In September 2007, the pathogen was only baited from one soil sample at Henry Cowell Redwoods State Park, a redwood-tanoak site. *Phytophthora ramorum* was only baited from the bulk leaf litter in March 2007, and only at sites experiencing a rain event concurrent with collection.

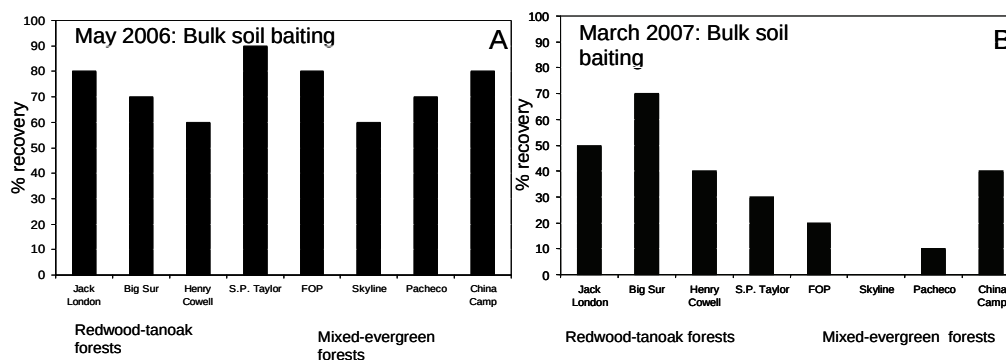


Figure 1δ Percent recovery from soil baits sampled at the end of the winter/spring rainy seasons. Graph A represents recovery in May 2006 and B from March 2007.

Determination of Chlamydospore Populations

Chlamydospore populations on attached leaf surfaces were higher in redwood-tanoak than in mixed-evergreen forests at all sample dates (fig 2). Chlamydospore populations were consistently higher at Henry Cowell Redwoods and Samuel P. Taylor State Park over all sample times. Chlamydospore levels were variable in fresh and aged litter.

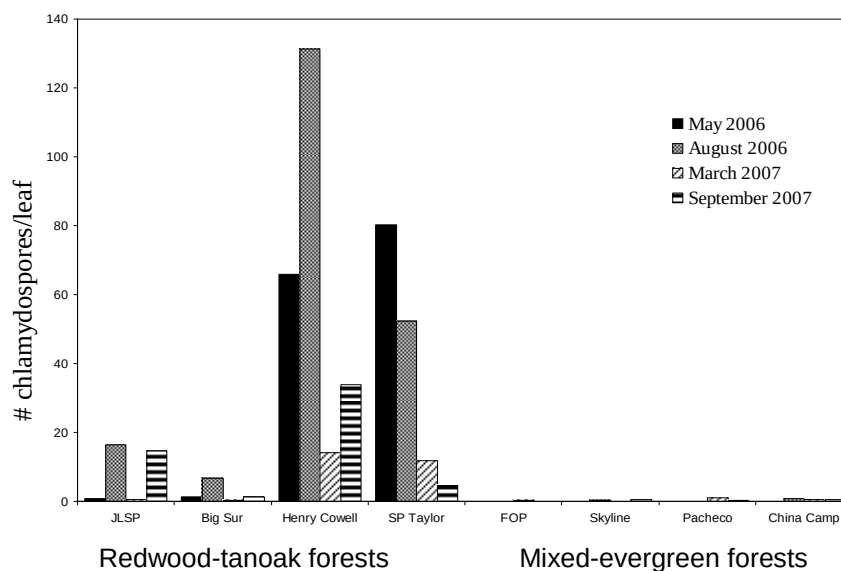


Figure 2d Chlamydospore populations on attached symptomatic bay laurel leaves. Values represent average number of chlamydospores per leaf at each of 8 sites and over four sample dates.

Chlamydospores were not observed in leaf tissues cleared in KOH, even in leaves from sites supporting high chlamydospore loads. To determine whether chlamydospores were sloughed off leaf surfaces while incubating in KOH, a drop of KOH was pipetted from the bottom of multiple test tubes and observed under the compound microscope. Chlamydospores were observed to accumulate at the bottom of test tubes containing leaves incubating in KOH.

Chlamydospore Germination

Chlamydospore germination was never observed on selective medium at any collection times. Similarly, chlamydospores placed on bay laurel and rhododendron leaves in September 2007 did not initiate infections of either leaf tissue.

Pathogen Survival and Colonization

Pathogen isolation from attached leaves ranged from 40-100 percent at each site in May 2006 and declined to a range of 0-40 percent in August 2006 (fig. 3). In August 2006 (fig 3B) and September 2007 (fig 3D), *P. ramorum* was not recovered from

attached leaves at four sites. The summer of 2006 was characterized by excessive heat, with numerous days having high temperatures over 38° C at most sites. Over the rainy season between August 2006 (fig 3B) and March 2007 (fig. 3D), recovery from attached leaves increased at five sites and remained static at three sites.

PCR resulted in higher detection of *P. ramorum* than isolation in culture (figs. 3, A and B).

Banksia attenuata

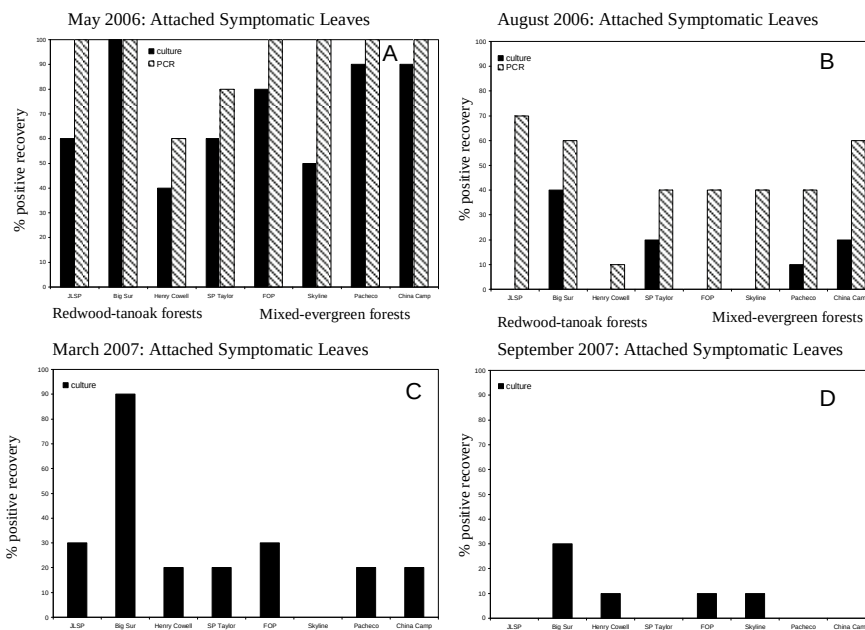


Figure 3D Recovery of *Phytophthora ramorum* from attached symptomatic bay laurel leaves. Graph A and B represent recovery by PCR and isolation in culture for samples collected in May and August 2006, respectively. C and D represent recovery in culture for samples collected in March and September 2007, respectively.

Fresh leaf litter was only collected at all eight sites in August 2006 (fig. 4A) and March 2007 (fig. 4B), whereas aged litter was collected from all eight sites in August 2006 (fig. 5A), March 2007 (fig. 5B), and September 2007. *Phytophthora ramorum* was recovered from freshly cast leaves with higher frequency in March 2007 than in August 2006 (fig. 4). Difference in isolation recovery of *P. ramorum* between attached leaves and freshly cast leaves was not more than 30 percent, with the exception of samples collected from Henry Cowell Redwoods in March of 2007. At this time, isolation recovery from fresh litter was 50 percent higher than recovery from attached leaves.

P. ramorum was isolated in culture from one aged leaf at each of two sites in March of 2007 (fig. 5B); however, the collection at these two sites was concurrent with a

heavy rain event. PCR detection of *P. ramorum* was possible in both fresh litter (fig. 4A) and aged litter (fig. 5A), even when the pathogen was not recovered in culture.

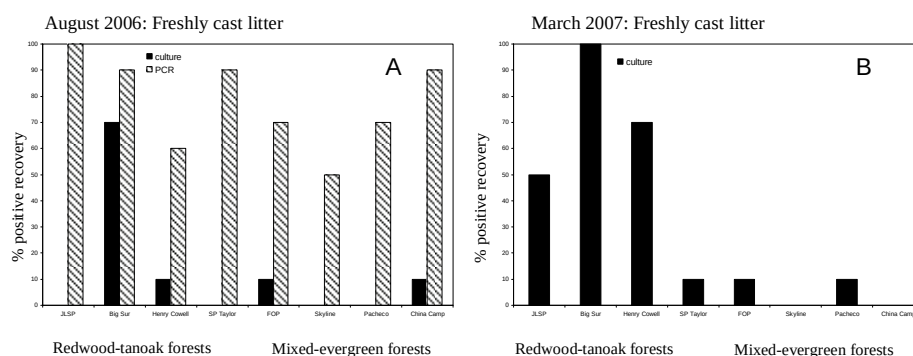


Figure 4d Recovery of *Phytophthora ramorum* in freshly cast leaf litter. A represents recovery by PCR and isolation in August 2006 and B represents isolation recovery in March 2007.

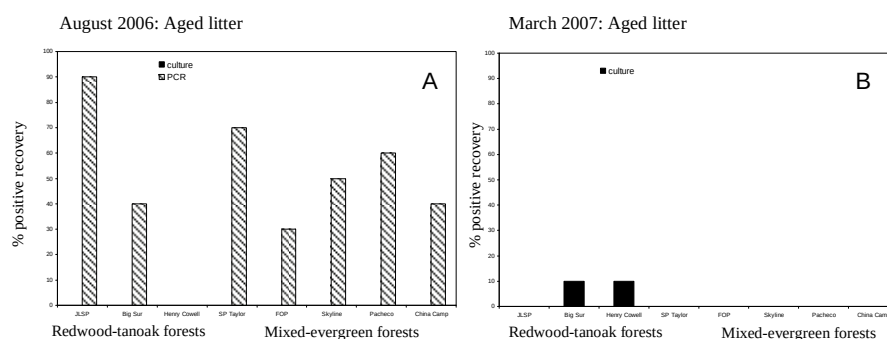


Figure 5d Recovery of *Phytophthora ramorum* in symptomatic, aged leaf litter. Pathogen detection by isolation in culture and PCR was completed for the sampling in August 2006 (A), and recovery by isolation was completed for samples collected in March 2007 (B).

Discussion

After four collection events spanning different times within the annual disease cycle of *P. ramorum*, our data suggest that bay laurel in redwood-tanoak forests support higher levels of foliar chlamydospore production than bay laurel in mixed-evergreen forests. Two sites in particular, Henry Cowell Redwoods and Samuel P. Taylor State Parks, exhibit higher chlamydospore loads on bay laurel than the other sites examined in this study. Presumably the heightened chlamydospore loads at these sites result in more primary inoculum at the onset of the disease cycle; however,

pathogen recovery at the end of the summer survival periods was not higher in sites exhibiting high chlamydospore loads. Furthermore, because chlamydospores produced on naturally-infected bay leaves did not germinate in vitro, one can only speculate on their viability, survival potential, and infectivity.

Infected bay laurel leaves are more likely to abscise than uninfected leaves, resulting in deposition of inoculum at the forest floor (Davidson and others 2005a). Pathogen recovery by isolation was higher in freshly cast litter than in aged litter, suggesting that survival declines rapidly after abscission. Detection of *P. ramorum* by PCR in fresh and aged litter remained high, even when the pathogen could not be recovered by isolation. PCR positives, however, may result from detection of non-viable pathogen tissue. Though PCR positives may result from presence of chlamydospores, the two sites exhibiting high chlamydospore loads did not have a higher frequency of PCR positives in aged or fresh litter than sites with low chlamydospore loads.

P. ramorum survives the hot, dry summers in a portion of infected bay laurel leaves, providing a source of primary inoculum at the onset of the fall rainy season. Chlamydospores are produced on infected leaves, however, their potential role in survival and infectivity is yet unknown. Chlamydospore production on bay laurel foliage varies between sites, but their potential role in shaping disease progress in epidemics in different forest types remains open for conjecture.

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