Detection, Distribution, Sporulation, and Survival of *Phytophthora ramorum* in a California Redwood-Tanoak Forest Soil

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ABSTRACT

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Recovery of *Phytophthora ramorum* from soils throughout sudden oak death-affected regions of California illustrates that soil may serve as an inoculum reservoir, but the role of soil inoculum in the disease cycle is unknown. This study addresses the efficacy of soil baiting, seasonal pathogen distribution under several epidemiologically important host species, summer survival and chlamydospore production in soil, and the impact of soil drying on pathogen survival. The efficacy of rhododendron leaves and pears as baits for detection of soilborne propagules were compared. Natural inoculum associated with bay laurel (*Umbellularia*

californica), tanoak (*Lithocarpus densiflorus*), and redwood (*Sequoia sempervirens*) were determined by monthly baiting. Summer survival and chlamydospore production were assessed in infected rhododendron leaf disks incubated under bay laurel, tanoak, and redwood at either the surface, the litter/soil interface, or in soil. Rhododendron leaf baits were superior to pear baits for sporangia detection, but neither bait detected chlamydospores. Most inoculum was associated with bay laurel and recovery was higher in soil than litter. Soil-incubated inoculum exhibited over 60% survival at the end of summer and also supported elevated chlamydospore production. *P. ramorum* survives and produces chlamydospores in forest soils over summer, providing a possible inoculum reservoir at the onset of the fall disease cycle.

Over the past decade, *Phytophthora ramorum* has incited extensive mortality of susceptible oak (*Quercus* spp.) and tanoak (*Lithocarpus densiflorus*) in discontinuous patches of coastal forests from Big Sur, California (CA) to southwestern Oregon (OR) (36,38). Though the pathogen has an extensive and expanding host range, the lethal cankers caused by *P. ramorum* on oak and tanoak prompted the term sudden oak death (SOD) for this disease on oaks (7,37). *P. ramorum* also causes Ramorum blight or Ramorum dieback, nonlethal diseases of numerous native hardwood and coniferous forest tree species, understory shrubs, and herbaceous plants (8,11). While *P. ramorum* has already impacted the stand structure of CA forests causing landscape level alterations, it continues to pose a threat to the integrity and biodiversity of native forests in North America (38).

P. ramorum was first associated with nursery plants in Europe (46) and was not associated with forest health until the elucidation of its role in SOD (37). Since its initial discovery in CA, *P. ramorum* has been found in southwest OR where the isolated disease incidence incited ongoing eradication efforts (12,13,23,38). Until recently, all European isolates of *P. ramorum* belonged to the A1 mating type, and all American isolates belonged to the A2 mating type, suggesting that the pathogen was introduced separately to the two continents (20). The alternate mating types have since been found in nursery settings in both continents (14,45), suggesting a role of the nursery industry in pathogen dispersal. Current strategies for prevention of long-distance spread focus on quarantine of nursery plants and plant products (1), while strategies for limiting spread within a landscape or region focus on early detection and inoculum management such as limiting movement of soils and water from infested to uninfested areas (38).

The epidemiology of *P. ramorum* in coastal forests is influenced by CA's Mediterranean climate with characteristic wet and dry seasons (9,38). Similar to the *P. palmivora* pathosystem on cacao (44), sporulation of *P. ramorum* is seasonal and correlated to rain events and warm temperatures (9,38). The disease manifests as nonlethal foliar lesions on bay laurel (*Umbellularia californica*) which support prolific sporangia production during rain events (9). Furthermore, chlamydospore production has been observed on naturally infected bay leaves (7), but the magnitude of chlamydospore production varies between sites (E. J. Fichtner, unpublished data). Conversely, no sporulation is observed on bleeding cankers on the main stems of oak and tanoak species (9); however, foliage and small twigs of tanoak do support sporulation (39). Emphasizing the role of bay laurel in the epidemiology of sudden oak death, presence of *P. ramorum* is correlated with presence of bay laurel, and foliar infections on bay laurel generally precede the infection of adjacent oaks (36). In addition to the heightened sporulation potential on bay laurel, *P. ramorum* also survives the hot, dry summer months in a proportion of symptomatic leaves (6).

Though many *Phytophthora* spp. are present or dispersed in soil (10), the role of soilborne inoculum in dissemination and survival of *P. ramorum* is largely unknown. *P. cactorum* and *P. palmivora* are generally considered above-ground pathogens of apple and cocoa, respectively, but both pathogens have been isolated from roots of asymptomatic trees (18,44). Unlike *P. quercina* which is associated exclusively with the roots of oaks (21), symptoms of *P. ramorum* have only been observed in above-ground plant parts with stem lesions ending at the soil line (11,37). Nevertheless, multiple lines of evidence suggest that *P. ramorum* may have a soil phase. In laboratory experiments, soilborne inoculum of *P. ramorum* has been shown to indirectly infect bay laurel seedlings via intermediate infection of leaf litter and subsequent simulated rain events (9). The pathogen has been isolated from tanoak roots (35), and dieback of tanoak sprouts has been observed following clear cut and burn treatments in the OR eradi-

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cation zone (13). In nursery systems, the pathogen has been isolated from rhododendron roots (2) and infects weeds of common container culture in experimental inoculations (39). *P. ramorum* can survive in potting media up to 12 months (27), and infested medium has been shown to incite root infections in rhododendron (26). The role of soil inoculum in the disease cycle within CA forests has not been directly addressed.

During the rainy season, *P. ramorum* has been pear-baited from soil under infected oaks in a CA mixed-evergreen forest, but recovery was low (20%) and undetectable a month after rains ceased (7,9). Lack of summer recovery by baiting suggests that the pathogen is either not viable, or viable but undetectable due to low population density or presence of dormant propagules. In general, *Phytophthora* spp. quickly lose viability upon drying conditions (32,41,42,44). Further studies are needed to determine the seasonal distribution of *P. ramorum* under host tree species and the relative efficacy of baiting techniques. For example, pears and apples are ineffective for baiting *P. quercina*, whereas oak-leaf baits consistently detect the pathogen in soil (21), and cocoa pod baits are more effective than direct isolation of *P. palmivora* from roots (44).

Because *Phytophthora* spp. may persist in soil for years in the absence of host tissue (44,47), the survival potential of *P. ramorum* in soil requires investigation. Long-term survival of *P. cinnamomi* is probably due to the formation of chlamydospores and oospores in roots and soil (32), yet in the *P. ramorum* system, production of chlamydospores in soil is unknown. In cocoa plantations, *P. palmivora* survival is inhibited by leaf litter mulch (25); however, preliminary data on *P. ramorum* illustrate higher recovery from the soil layer than the overlying litter layer (7). Given that some *Phytophthora* spp. may exhibit enhanced survival when buried in soil rather than incubated at or near the soil surface (18), the survival potential of *P. ramorum* should be investigated over a vertical distribution in forest soils.

P. ramorum produces abundant chlamydospores in vitro (37, 46), but the survival and role of these propagules in the SOD disease cycle are unknown. Chlamydospores are considered more resistant structures than zoospores and sporangia (15,16), but preliminary data on chlamydospores of *P. ramorum* suggest that they are killed by drying at 30% relative humidity at room temperature for 30 min (7). Chlamydospores of *P. ramorum* tend to germinate with a sporangiophore carrying a few sporangia, rather than with hyphae (46). While production of chlamydospores in soil varies between *Phytophthora* spp. (30), the production and persistence of *P. ramorum* chlamydospores in soils is unknown.

The overall focus of this work is to elucidate the role of forest soil in summer survival of *P. ramorum* and its ability to serve as a reservoir of inoculum at the onset of the fall rainy season. To achieve this goal, we address these specific objectives: (i) critically assess the efficacy of pear and rhododendron leaf baits at detecting populations of chlamydospores and sporangia in soil, (ii) investigate seasonal distribution of *P. ramorum* in soil and leaf litter under host tree species, (iii) assess the summer survival potential of *P. ramorum* in soils, (iv) determine the potential for chlamydospore production in soil, and (v) elucidate the role of soil drying on pathogen survival and chlamydospore production in mineral and organic forest soils.

MATERIALS AND METHODS

Study site. Field-based experiments and observations were conducted in a redwood-tanoak forest at Jack London State Park (JLSP) in Sonoma County, CA (122°W, 38°N). In November 2003, 15 trees each of bay laurel, tanoak, and redwood (*Sequoia sempervirens*) were tagged for repeated sampling over time. Tagged trees were selected within heavily infested areas, either within or adjacent to field plots established by P. Maloney (28). The total disease incidence within bay laurel, tanoak, and red-

wood was approximately 90, 55, and 10%, respectively, at JLSP in 2004 and 2005 (D. M. Rizzo, unpublished data). Individual trees were selected to maximize distance from dissimilar over-story species, thereby minimizing the influence of adjacent species on soil characteristics and input of natural inoculum from neighboring sources. The parent material of the soils is residuum weathered from metavolcanics and the dominant series are Spreckles and Goulding (4). Daily precipitation data were recorded by park staff.

Soil characteristics. Because soil characteristics vary greatly over short spatial distances, measurements of soil pH and organic matter content were assessed under the 45 tagged trees. Three soil samples were collected within a 1.5 m radius of each tree, bulked, and mixed thoroughly. The pH was determined for samples collected monthly from April 2004 through November 2004 in a 1:2, wt/vol, suspension in 0.01 M CaCl₂ solution (40). The pH values associated with the three host species over time were analyzed with a repeated measures analysis of variance (ANOVA). Soil samples collected in July 2004 were air dried, sieved, and analyzed for organic matter content using a modified Walkley-Black method (33) at the ANR Analytical Lab, UC Davis. The soil organic matter contents associated with the three host tree species were distinguished using an ANOVA followed by a Waller-Duncan *K* ratio test (*K* = 100) to separate means. All statistical analyses were conducted using SAS (SAS Institute, Cary, NC) statistical software.

Bait efficacy. The efficacy of pear fruits and rhododendron leaves to bait sporangia and chlamydospores from soil was determined in the lab using an isolate of *P. ramorum* recovered from soil at JLSP (D. M. Rizzo lab culture collection, UC Davis). To induce sporangia formation, agar plugs were excised from 1-week-old colonies of *P. ramorum* grown on 1/3 strength V8 agar and incubated for 48 h in soil water. Plates were shaken to dislodge sporangia, and the resulting spore suspension was quantified with a hemacytometer. Chlamydospores were harvested from 14-day-old colonies grown in V8 broth by blending 30 s in a Waring blender. Spores were then collected by passing the suspension through 100 and 50 µm nested sieves and resuspending in deionized water. The combined blending and sieving were essential to detach chlamydospores from hyphae and ensure that only chlamydospores were introduced to the system. Chlamydospore concentration was quantified in aliquots under a dissecting microscope.

Uninfested soil (Yolo silty clay loam) was collected from the Armstrong Field Station (Davis, CA), passed through a 2-mm sieve, mixed with coarse sand (1:1, vol/vol), and 0.5 liter of the soil/sand mixture was placed into each of 48 freezer bags. To reduce pesticide residue, D'Anjou pears were thoroughly washed prior to use and rhododendron cv. Colonel Coen were not sprayed with fungicides. The two propagule types were each embedded on a 4-cm² segment of fine filter paper by gentle application of negative pressure with an aspirator attached to a Büchner funnel. The application of approximately 30 spores to each segment was confirmed by transferring additional filter paper segments to a selective medium amended with 0.05 g of hymexazol per liter (PARPH) (10,29) and allowing 24 h for spore germination. After 24 h, filter papers were removed from agar and the number of propagules adhering to the agar was counted. The spore-embedded segments were either cut and broadcast in soil or placed in intimate contact with bait materials by adhering segments to leaves or fruit with flexible adhesive bandages. When affixing the filter paper segments to the baits, each segment was cut in half and adhered to the bait in two locations. For rhododendron leaf baits, the segments were placed on the abaxial and adaxial leaf surfaces, and for pears, segments were attached to opposing sides of the fruit. Uninfested filter paper segments were utilized as controls and four replicates were established for each treatment. Baits were then partially submerged in supersaturated soil. Baits

were incubated in soil for 2 days and then rinsed with deionized water. Rhododendron leaves were incubated in moist chambers for an additional 2 days prior to isolation, whereas lesions on pears were sampled immediately due to the onset of fruit rot while in saturated soils. Lesions were sampled from bait materials and submerged in PARPH. Isolation plates were stored 1 week in the dark before determination of percent *P. ramorum* recovery. The experiment was run three times to account for potential differences in pear quality throughout the year. All baiting and inoculum preparation in subsequent studies utilized rhododendron cv. Colonel Coen leaves and the same pathogen isolate.

Seasonal distribution. The distribution of inoculum under the 15 tagged bay laurel, tanoak, and redwood trees was monitored monthly from December 2003 through October 2005. Approximately 0.5-liter samples of both soil and leaf litter were collected and returned to the lab for baiting. Gravimetric soil moisture content was determined for 10 random samples from each host tree species with five subsamples (approximately 10 g each) per sample. Samples were weighed and then oven dried at 105°C for 48 h prior to determination of dry weight. *P. ramorum* was baited from soil and litter with rhododendron leaves according to the aforementioned procedure. Frequency of pathogen recovery and soil moisture content were recorded over time.

Summer pathogen survival. Ten tagged trees each of bay laurel, tanoak, and redwood were selected for assessment of survival in associated soils over the summer of 2004 and 2005. Approximately 4,000 rhododendron leaf disks were cut with a 1-cm-diameter cork borer, puncture wounded, and inoculated with agar slurry of *P. ramorum*. Inoculated leaf disks were incubated in moist chambers for 1 week. The agar was then rinsed off leaf disks, and 10 symptomatic leaf disks were placed in each of 360 mesh sachets. Sachets were stored in moist chambers until dispersal in the field the following day. To assess for initial recovery, 100 leaf disks were submerged in PARPH.

Mesh sachets were deployed to the field on 27 April 2004 and on 24 May 2005, near the end of the spring rainy season. Four points were selected at 1.5-m radii from each tree to allow for destructive sampling over time. Sachets were arranged vertically within partially submerged plastic cylindrical barriers (17 cm diameter) to prevent lateral pathogen movement. Soil was disturbed to approximately 8 cm below the surface. Sachets were arranged at three depths: 6 cm below the soil surface, the leaf litter/soil interface, and the leaf litter surface. One set of vertical sachets was retrieved at each of four time points: 1, 2, 8, and 25 weeks after deployment in 2004, and 1, 2, 8, and 22 weeks in 2005. To determine whether soil serves as an inoculum reservoir at the onset of winter, the 25 week sample time in 2004 was within 1 week after the first fall rain event, and the 22 week sample in 2005 was prior to the first fall rain event.

At each of the four time points, sachets were destructively sampled and leaf disks were then submerged in PARPH. After 3 weeks in selective medium, pathogen recovery was recorded. Based on the observations of Hüberli et al. (19) of enhanced recovery of *P. cinnamomi* after tissue hydration, leaf disks designated as *P. ramorum*-negative were then incubated for 3 weeks in deionized water before being returned to PARPH for subsequent reassessment of recovery. The influence of host tree association, and of vertical location on pathogen recovery over time were assessed using repeated measures ANOVA with between-subject factors and within subject interactions determined using a Wilks' Lambda test.

Soil samples were taken from each tree upon the introduction of inoculum to the field and at each sample time. Samples were taken both inside the cylindrical barrier and directly outside the cylindrical barrier in an effort to detect the potential transfer of the pathogen from the infested leaf tissue to the bulk soil. In the laboratory, gravimetric soil moisture content was assessed using the aforementioned technique and the average soil moisture con-

tent associated with each tree was recorded. Soil moisture content associated with tree species over time was assessed with repeated measures ANOVA. Soil samples were then baited for detection of *P. ramorum*. Frequency of bait recovery of the pathogen inside and outside the cylinders was determined for each sample time.

Chlamydospore production. All leaf disks ($n = 900$) from the last sample time were incubated in 1 M KOH for 3 weeks to render tissue translucent or clear enough to visualize chlamydospores under the light microscope. Five chlamydospore counts were taken under the $\times 10$ field of view (1.6 mm^2) and the average number of chlamydospores per field of view was recorded. Observations of chlamydospore germination events were also noted. Chlamydospore count data were recorded in correspondence with three possible pathogen recovery categories: prehydration positive, posthydration positive, or posthydration negative. It is important to note that all prehydration negative disks were hydrated. Utilizing the entire chlamydospore data set, a GLM procedure and Waller-Duncan K ratio test ($K = 100$) were employed to determine the influence of host soil association and vertical location on chlamydospore production. To determine whether differences in pathogen recovery upon initial isolation are related to varying levels of colonization of leaf disks, the chlamydospore populations in prehydration positive and prehydration negative disks were compared using a two-tailed t test. Lastly, sachets having contained both posthydration positive and posthydration negative leaf disks were selected for a paired, two-tailed t test to determine whether hydration-related recovery was linked to chlamydospore populations.

Effect of soil drying. A growth chamber experiment was designed to address the role of soil type and soil drying on survival and chlamydospore production of *P. ramorum*. In September 2005, approximately 1.1 liter of soil was collected from each of the 10 redwood and bay trees utilized in the field study. Soils associated with each host species were separately bulked and thoroughly mixed before distribution into 48 pots. Soils were then saturated with deionized water and drained to container capacity. Pots were placed into bins containing 2.5 cm of water, with the distance between the soil surface and the free water surface being 9 cm or 0.9 kPa matric potential (Ψ_m). After pots were allowed to equilibrate a few hours, infected leaf disk inoculum was introduced to each pot.

Leaf disk inoculum was produced by the aforementioned methodology and 15 leaf disks were placed into each of 48 sachets and then buried below the soil surface in each pot. Forty-five leaf disks were plated onto PARPH to determine initial percent pathogen recovery. Upon the introduction of inoculum, soil moisture content was measured in three randomly selected pots each of the redwood and bay laurel soils with five subsamples taken from each pot. Soil samples were specifically sampled from the soil surface to determine moisture content in the vicinity of incubating sachets. After inoculum introduction, pots were placed within a dark growth chamber maintained at 17°C and 64% relative humidity in a completely randomized design. Over the course of 24 weeks, half of the pots were allowed to dry, while the other half was maintained at 0.9 kPa Ψ_m .

The treatment design was a complete factorial with two soil types and two moisture regimes. Pathogen recovery was determined over time by destructively sampling three replicates of each treatment after 1, 4, 8, and 24 weeks. Soil moisture and pathogen recovery on PARPH were determined at every time point and chlamydospore production was assessed in disks recovered after 1 week. Effects of soil moisture regime and soil type on pathogen recovery over time were assessed with repeated measures ANOVA with between-subjects factors and differences within subjects assessed with a Wilks' Lambda test. The effects of soil moisture regime and soil type on chlamydospore production were assessed with PROC GLM and differences between

treatments were determined with a Waller-Duncan *K* ratio test (*N* = 100). The experiment was run twice.

RESULTS

Soil characteristics. Soil characteristics varied in association with different host tree species, with redwood-associated soils having higher organic matter content than those associated with either bay laurel or tanoak ($P \leq 0.01$) (Table 1). The average organic matter content of the organic redwood-associated soils was >20%, in comparison with the soils associated with bay laurel and tanoak. The pH range of soils was 5.5 to 6.1, with those associated with tanoak having a lower pH than those associated with redwood and bay laurel ($P \leq 0.01$) (Table 1). The pH values associated with specific host species did not change significantly over time.

Bait efficacy. Chlamydospores were not detected by use of either rhododendron leaves or pears. Rhododendron leaf baits were more sensitive than pears for detection of sporangia (Table 2). Pear baits were never effective at detection of broadcast sporangia and rarely facilitated detection of sporangia in intimate association with the fruit. Using rhododendron leaves, we detected sporangia between 75 and 100% of the time, whether broadcast or in intimate association with the leaf.

Seasonal distribution. *P. ramorum* was consistently recovered from soils and litter during the winter rainy season, and persisted in soil through July in 2004 and September in 2005 (Fig. 1A and B). Corresponding to pathogen recovery in the soil through the summer of 2005, spring rainfall events extended into June of 2005 (Fig. 1C), promoting late spring sporulation events (J. M. Davidson, unpublished data). *P. ramorum* recovery frequency from soil during the 2005 season was approximately three times higher than recovery in 2004 (Fig. 1A). Recovery frequency was greatest under bay laurel, followed by tanoak, with occasional recovery under redwood. Pathogen recovery in the leaf litter layer was generally less than in soil and persistence was limited to a shorter time period, with litter populations undetectable by late summer.

Summer pathogen survival. Soil moisture contents were different across the host-soil associations, with redwood soils having a higher soil moisture content than either bay laurel or tanoak soils in 2004 and 2005 ($P \leq 0.01$) (Fig. 2A and B). During both summer seasons, pathogen recovery was similar in the three host-associated soils; however, recovery varied across vertical locations of the leaf disks, with enhanced recovery observed in those incubated in soil ($P \leq 0.0001$) (Fig. 3A and B). In general, survival in all treatments was higher in 2005 than in 2004. Inoculum at the leaf litter surface exhibited <10% recovery 1 week after deployment (Fig. 3A and B), and in 2005 approximately 3% recovery was observed at the litter surface 1 day prior to the onset of the fall rains (Fig. 3B). Inoculum incubated at the interface of

litter and soil exhibited similar survival as that incubated in soils over the first 8 weeks, but interface recovery dropped drastically between the 8 week sample time and the onset of fall rains in October. Recovery was >60% in soil-incubated leaf disks at the close of the 2004 and 2005 summer seasons (Fig. 3A and B). Pathogen recovery was often observed in decomposing tissue, generally in residual vascular tissue.

Hydration of leaf disks enhanced pathogen recovery at every collection time (Table 3). Leaf disks incubated at the litter surface that were negative in the first assays were rarely rendered *P. ramorum*-positive after hydration. Because hydration-stimulated pathogen recovery was so dramatic at subsurface locations, the separation of means of percent recovery was impacted by post-hydration data; therefore, Figure 3 illustrates total (or post-hydration) pathogen recovery, and Table 3 depicts the change in recovery contributed by hydration. Chlamydospore germination was occasionally observed in post-hydration-positive leaf disks after tissue was cleared in KOH.

P. ramorum was baited from the bulk soil within cylindrical barriers throughout the entire summer season (Table 4). Conversely, recovery from outside the cylinders was minimal throughout the 2004 season, but >20% over the first 8 weeks of 2005 (Table 4).

Chlamydospore production. Chlamydospore production was observed in cleared leaf disk tissue from all treatments. In 2004, chlamydospore production was significantly higher in soil-incubated leaf disks than in those incubated at the interface or the surface ($P \leq 0.0001$) (Fig. 4A). In 2005, there was a significant interaction between host association of soil and vertical location of inoculum ($P \leq 0.001$), but the general trend was similar to the 2004 data with higher spore production in soils than at the interface or surface (Fig. 4B). In both seasons, no significant difference in chlamydospore production was observed in prehydration-positive versus prehydration negative leaf disks, suggesting that differences in initial recovery were not related to mere differences in extent of colonization by *P. ramorum*. When post-hydration negatives were paired with post-hydration positive leaf disks within a sachet, there were significantly more chlamydospores in disks that exhibited hydration-stimulated recovery than those nonresponsive to hydration ($t \leq 0.05$).

Effect of soil drying. Soil type had no effect on pathogen recovery over time, but did influence chlamydospore production.

TABLE 1. Soil organic matter content and pH associated with tagged bay laurel, tanoak, and redwood trees^v

Soil association ^w	% Organic matter ^x	Average pH ^y
Bay laurel	13.7 a ^z	5.93 a ^z
Tanoak	9.5 a	5.62 b
Redwood	20.1 b	5.88 a

^v Fifteen trees each of bay laurel, tanoak, and redwood were tagged for repetitive soil sampling over time.

^w Soil samples were collected within a 1.5 m radius of tagged trees.

^x Soils were collected in July 2004, air dried, passed through a 2 mm sieve, and organic matter content was determined with the Walkley-Black method (33). Values represent the average of 15 samples of each species.

^y pH was determined in a 0.01 M CaCl₂ solution. Samples were collected monthly from April 2004 through November 2004. Values represent average pH of soils associated with 15 tagged trees of each species over time.

^z Different letters within columns represent significant differences based on the Waller-Duncan *K* ratio test (*K* = 100).

TABLE 2. Percent recovery^v of *Phytophthora ramorum* from sporangia-infested^w soils using rhododendron leaves and pears as baits^x

Infestation treatment	Pear	Rhododendron
Run 1 ^y		
Uninfested ^z	0	0
Broadcast	0	100
Intimate	25	100
Run 2		
Uninfested	0	0
Broadcast	0	75
Intimate	0	75
Run 3		
Uninfested	0	0
Broadcast	0	75
Intimate	25	100

^v Percent recovery was based on number of positive isolations out of four replicate baits per treatment.

^w Sporangia were produced on 1/3 strength V8 agar plugs incubated for 48 h in soil water. Sporangia were enumerated with a hemacytometer and then embedded on moist filter paper strips before introduction to soil.

^x Baits were incubated in saturated soil for 2 days prior to isolation.

^y The experiment was run three times to account for potential differences in pear quality throughout the year.

^z Inoculum was introduced either in intimate association with the bait or broadcast in the soil. Spore-embedded filter paper strips were adhered to the abaxial and adaxial surfaces of rhododendron leaves and to opposing sides of pear fruits. Uninfested filter papers were utilized as controls.

Leaf disks incubated in redwood soils exhibited 40% fewer chlamydospores than those incubated in the bay laurel-associated soils ($P \leq 0.05$). The influence of soil drying on pathogen recovery was different in the two experimental runs due to overall

lower pathogen recovery in run 2 (Fig. 5). In comparison to inoculum maintained at 0.9 kPa Ψ_m , soil drying reduced pathogen recovery by 100 and 64% after 24 weeks of incubation in runs 1 and 2, respectively ($P \leq 0.05$) (Fig. 5). In these disturbed soils,

Fig. 1. The distribution of *Phytophthora ramorum* in soil and leaf litter in association with bay laurel, tanoak, and redwood was assessed monthly. **A** and **B** represent recovery from soil and leaf litter, respectively, with recovery frequency associated with each species ($n = 15$) stacked for a cumulative potential frequency of 45. Average soil moisture content ($n = 10$) corresponding with each collection time is included as a line graph in **A**. Error bars are inserted for points with a standard error ≥ 2.0 . **C** represents the daily rainfall recorded at Jack London State Park.

soil moisture content did not vary significantly between the redwood soil and the bay laurel soil; therefore, soil moisture was averaged between soil types. Differences in soil moisture content upon drying were evident at weeks 8 and 24, where dry treatments exhibited up to 75% less gravimetric water than moist treatments (Fig. 5).

DISCUSSION

The variation of soil characteristics and host distribution over short distances within a forest may impact the disease cycle of SOD in natural systems. For example, soils associated with redwoods tended to have high organic matter content, enhancing soil moisture retention. The redwood-associated soils may promote pathogen survival in exceptionally dry summers based on the general trend that most *Phytophthora* spp. lose viability upon the advent of drying conditions (42). Our investigation of the natural distribution of inoculum within a redwood-tanoak forest illu-

strates that *P. ramorum* was recovered more frequently under the bay laurel and tanoak hosts than under redwoods, suggesting that soil inoculum may originate from aerial sporulation on these hosts (9,38). Soil samples were collected at a 1.5 m radius from tagged trees based on the steep dispersal gradient observed in soil inoculum of *P. cactorum* under infected apple trees (18). However, inoculum of *P. ramorum* may be dispersed greater distances in windblown rain (9), resulting in pathogen presence in soils associated with nonhost species or nonsporulating hosts. In this study, trees were selected to maximize distance from dissimilar overstory species and thereby reduce the potential drift of inoculum from neighboring sources.

Soil populations of *P. ramorum* dropped after the conclusion of the rainy season. As illustrated by the seasonal distribution data, soil populations of *P. ramorum* were higher in 2005 than in 2004, presumably due to the latter year's late spring rains in May and June (Fig. 1C). In a comparable study in Nigeria, cocoa plantation sites exposed to seasons with low rainfall and severe soil drying

Fig. 2. Host species association influenced soil moisture content in **A**, 2004 and **B**, 2005. Soil moisture content was assessed upon the introduction of inoculum on 27 April 2004 and 24 May 2005, and also with each subsequent inoculum collection time until 20 October 2004 and 25 October 2005. Each point represents the average soil moisture content ($n = 10$). Error bars were inserted at each point with a standard error ≥ 4.0 .

Fig. 3. Summer survival of *Phytophthora ramorum* near the soil surface. Infected rhododendron leaf disks were incubated on the surface of the leaf litter, at the interface of the litter and soil, and below the soil surface. **A** and **B** represent pathogen recovery in 2004 and 2005, respectively. Inoculum remained in the field from 27 April through 20 October 2004, and from 24 May through 25 October 2005. Each point represents average percent pathogen recovery ($n = 300$). Error bars are present for standard error values ≥ 4.0 . Location of inoculum affected isolation recovery over time ($P \leq 0.05$).

exhibited low recovery of *P. palmivora* from soil (44). Davidson et al. (7,9) documented that *P. ramorum* was not detected in soils associated with oaks within approximately 1 month of the last spring rain event. In contrast, *P. ramorum* was recovered from

TABLE 3. Percent change in *Phytophthora ramorum* recovery from leaf disks after a 3-week incubation in deionized water

Location	1 week	2 weeks	8 weeks	22 weeks	24 weeks
2004 ^x					
Soil ^y	...	...	3.0 ^z	...	3.7
Interface	...	...	10.0	...	1.0
Surface	...	...	0.0	...	0.0
2005					
Soil	6.7	10.0	1.0	3.1	...
Interface	1.4	4.0	0.7	0.7	...
Surface	0.7	2.0	0.0	0.0	...

^x In 2004, post-hydration recovery data was recorded on inoculum incubated in the field for 8 and 24 weeks. In 2005, post-hydration recovery was assessed at every sample time.

^y Ten infected leaf disks were placed in each of 360 sachets and distributed under 30 trees at either of three vertical locations: below the soil surface, at the interface of the soil and litter, and at the litter surface. At each of four sample times, infected leaf disks ($n = 300$) were assessed from each vertical location to determine pathogen recovery.

^z The change in pathogen recovery contributed by hydration was determined by removing *P. ramorum*-negative leaf disk tissue from PARPH, incubating the leaf disks in deionized water for three weeks and then resubmerging the tissue in PARPH.

TABLE 4. Percent recovery of *Phytophthora ramorum* inside and outside cylinders^y

Sample location	1 week	2 weeks	8 weeks	22 weeks ^z	24 weeks ^z
2004					
Inside cylinder	33	16.6	20	...	20
Outside cylinder	3.3	0.0	0	...	0
2005					
Inside cylinder	63.3	86.6	46.6	23.3	...
Outside cylinder	56.6	46.6	20	0.0	...

^y Soil samples were collected both inside and outside each cylinder, and then baited with rhododendron leaves for determination of presence of *P. ramorum*. Values represent the percent of samples positive for the pathogen at each sample time ($n = 30$).

^z Soil samples were collected concurrent with leaf disk inoculum 1, 2, 8, and 24 weeks after inoculum deployment in 2004 and after 1, 2, 8, and 22 weeks in 2005.

soils at JLSP up to 3 months after the last rain event. Multiple factors may contribute to the difference in detection and persistence of soil inoculum in these two systems. Davidson et al. (7,9) monitored soil inoculum in a mixed-evergreen forest, whereas the present study was conducted in a redwood-tanoak forest. Though the two experimental sites are in close geographic proximity, the redwood-tanoak forest may have a higher inoculum load than the mixed-evergreen forest. Furthermore, the former study utilized pear baits, whereas rhododendron leaves may be more sensitive for detection of sporangia of *P. ramorum*.

Though rhododendron leaves were preferable to pears for baiting *P. ramorum* from soil, their application is limited due to the inability of the bait to detect chlamydospores. Consequently, we may presume that baits detected sporangia in soil for up to 3 months after the last spring rain event, but the potential for long-term chlamydospore survival is yet unknown. For routine baiting, however, rhododendron leaves are more cost effective and produce less bulk biohazard waste than pears. Additionally, enhanced pathogen colonization of rhododendron leaf baits may be achieved by incubating the tissue an additional 2 days in moist chambers, whereas pear baits were often rotting upon removal from saturated soil.

Given that some *Phytophthora* spp. may survive years in soil, even in the absence of a living host (43,47), the summer survival potential of *P. ramorum* in forest soil was addressed. *P. ramorum* was demonstrated to survive in soils over the course of the hot, dry summers typical of the Mediterranean climate of CA coastal forests. Enhanced survival was observed in leaf disks incubated in soil, with marginal survival observed at the interface of litter and soil, suggesting that natural pedoturbation processes resulting in inoculum burial may promote long-term pathogen survival. Similarly, *P. cactorum* exhibited better survival in soil than at the soil surface in New York State apple orchards (18). In the *P. ramorum* system, the litter layer tends to prolong survival of underlying inoculum, perhaps by reducing exposure to drying. In a jarrah forest soil, the incorporation of leaf litter has been shown to induce hyphal lysis of *P. cinnamomi* (34), and leaf litter was also antagonistic to hyphal survival of *P. palmivora* in vitro assays (25). Additionally, in a cocoa orchard, the litter layer supplies a natural barrier to rainsplashed inoculum originating from soil (24). Davidson et al. (9) demonstrated, however, that bay laurel leaf litter may serve as an intermediary between soil inoculum and aerial infections by *P. ramorum*, and the results of this

Fig. 4. Chlamydospore production in leaf disks. All leaf disks ($n = 900$) collected at the last sample time in both 2004 and 2005, were incubated in 1 M KOH until tissue was cleared of color. The average number of spores produced per square millimeter was determined for each disk by averaging five counts from under the 10 $\times$ field of view of the light microscope. A and B represent chlamydospore populations in 2004 and 2005, respectively. Different letters above bars designate significant differences based on the Waller-Duncan K ratio test ($K = 100$).

survival study suggest that the pathogen may survive below the litter layer and contribute to the SOD disease cycle.

Hydration of *P. ramorum*-negative leaf disks with deionized water enhanced recovery of the pathogen. This technique was employed because repetitive rinsing of *P. cinnamomi*-infected tissue promoted the detection of false negatives, often aiding in pathogen recovery from asymptomatic tissue (19). The potential detection of survival structures upon hydration is hypothesized in the *P. cinnamomi* system (19). Chlamydospores of *P. ramorum* were more populous in tissues responding to hydration than in those not stimulated by hydration, suggesting a potential role of chlamydospores in hydration-stimulated pathogen recovery. Additionally, the hydrated tissues (prehydration negative) had similar chlamydospore counts than the nonhydrated tissues (prehydration positive), suggesting that the difference in initial pathogen recovery was not due to disparity of tissue colonization by the pathogen. Chlamydospore survival was evident from the accounts of spore germination in the cleared leaf disks; however, it is impossible to elaborate or extrapolate on dormancy, survival, and germination of these spores from observation of individual germination events.

Chlamydospore production by *P. ramorum* was high in soil, but the potential role of these structures in pathogen survival and persistence is unknown. Chlamydospores play a role in long-term survival of *P. cinnamomi* in soils, persisting up to 6 years in the absence of a host (32,47). Conversely, *P. palmivora* and *P. megakarya* do not produce chlamydospores in cocoa orchard soils (44), but *P. palmivora* has been shown to produce chlamydospores under laboratory conditions (22,34). The role of chlamydospores in the SOD disease cycle is yet unknown.

The high frequency of recovery of *P. ramorum* from soil within cylinders suggests that inoculum is easily transferred to the bulk soil from infected tissue. Consequently, the introduction of infected leaf disks to the forest created numerous "hot spots" of infestation within this already heavily infested site. Soil populations of both *P. cactorum* and *P. palmivora* have been linked to incorporation of infected tissue from aerial plant parts into soil

(18,44). For example, the introduction of inoculated leaves to soil in microplots resulted in soil populations of *P. cactorum* 240% higher than bare soil control plots (18). In cocoa plantations, above-ground sanitation resulted in reduced populations of *P. palmivora* in soil (44). While sanitation is an effective disease management strategy in perennial crops, inoculum management strategies are limited within natural systems, relying mainly on reducing activities conducive to the movement of inoculum.

Similar to other *Phytophthora* spp. (42), *P. ramorum* loses viability upon soil drying. Because of the impracticality of maintaining a set of moist in situ field soils over the summer, a controlled experiment was designed by modifying a procedure designed for assessment of *P. cinnamomi* survival upon drying (5). After 6 months in drying soil, *P. ramorum* recovery drastically decreased regardless of the soil type. Though soil type did not influence survival, redwood soils were suppressive to chlamydospore production of *P. ramorum*, suggesting a limited production of survival structures in these soils. Suppression of *P. cinnamomi* has been observed in soils high in organic matter with complex and active microbial activity (17). Chlamydospores of *P. cinnamomi* were formed in soils both suppressive and conducive to avocado root rot, and disease suppressive soil had higher populations of bacteria and actinomycetes than conducive soils (3). The mechanism of suppression of *P. ramorum* chlamydospore production is unknown, but further studies will address this phenomenon under moist field conditions.

Multiple *Phytophthora* spp. cause root and crown rot of oaks in landscape and ornamental plantings (31), with the recently discovered *P. quercina* specifically associated with necrotic fine oak roots (21). Because the few reports of *P. ramorum* on roots involve isolation from asymptomatic tissue (2,26,35), it is possible that *P. ramorum* has a yet undetected persistence or presence in roots. Both *P. palmivora* and *P. cactorum* are considered aerial pathogens, but both are infrequently isolated from roots of cocoa and apple, respectively, even in asymptomatic plants (18,44). Consequently, determination of *P. ramorum* infection frequency and distribution in forest root tissues may pose a challenge due to

Fig. 5. Influence of soil drying on recovery of *Phytophthora ramorum*. **A** and **B** represent runs 1 and 2 of the experiment, respectively. Fifteen infected leaf disks were placed in each of 48 mesh sachets and then buried in soil maintained at container capacity. Half of the pots were maintained moist at 0.9 kPa matric potential, while the other half were allowed to dry over 24 weeks. Average percent soil moisture ($n = 15$) (solid lines) and percent pathogen recovery ($n = 45$) (dashed lines) are portrayed for drying (◆) and moist (▲) soil treatments over time. Error bars are inserted on points where the standard error is ≥ 5 .

the logistics of field sampling, potential for asymptomatic infections, and the identification of roots within heterogeneous plant populations. Infested soil or infected roots may provide hidden pathways for pathogen dispersal, evading detection and thwarting quarantine efforts.

Forest soils may act as inoculum reservoirs for *P. ramorum* within the SOD disease cycle, supporting both pathogen survival and chlamydospore production. The survival potential of *P. ramorum* in soil, combined with the recent reports of root infection (2,26,35,39) highlight the importance for enhanced understanding of the dynamic interaction between soil inoculum and the soil chemical, physical, and microbial environment. Further investigation is needed to specifically address the detection and survival of chlamydospores in forest soils, the infection potential of these spores, and to elucidate the frequency and distribution of root infections within infested forests. Furthermore, the potential for soil-mediated suppression of chlamydospore production is an intriguing phenomenon worthy of investigation under moist field conditions.

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