Disease Risk of Potting Media Infested with *Phytophthora ramorum* Under Nursery Conditions

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ABSTRACT

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Phytophthora ramorum has been found in potting media of containerized plants; however, the role of infested media on disease development under nursery conditions is unknown. This study assesses pathogen survival, sporulation, and infectivity to rhododendron plants in nursery pots with infected leaf litter that were maintained under greenhouse and field conditions. The influence of environmental conditions and irrigation method on disease incidence was also assessed. Infected leaf disks were buried below the soil surface of potted rhododendrons and retrieved at approximately 10-week intervals for up to 66 weeks. Pathogen survival was assessed by either isolation or induction of sporulation in water over three experimental periods. *P. ramorum* was recovered from infected leaf disks incubated in planted pots for longer than 1 year. Chlamydospores and sporangia formed on hydrated leaf disks but relative production of each spore type varied with the duration of incubation in soil. Root infections were detected after 40 weeks in infested soil. Foliar infections developed on lower leaves but only after spring rain events. Sprinkler irrigation promoted the development of foliar infections; no disease incidence was observed in drip-irrigated plants unless foliage was in direct contact with infested soil. Management implications are discussed.

Phytophthora ramorum, the causal agent of sudden oak death (SOD), produces trunk cankers and widespread mortality on tanoak (*Lithocarpus densiflorus*) and oak (*Quercus* spp.) (17) and leafspots and blights on numerous other native hosts in California and Oregon woodlands (1,3). The pathogen was described as a new *Phytophthora* spp. in 2001 but it was observed as early as 1993 to cause leaf blights and mortality on rhododendron and viburnum in nurseries and public gardens in Germany and The Netherlands (24). With the association of *P. ramorum* with SOD, intensive nursery stock and public garden inspections ensued, resulting in documentation of the pathogen in several European countries. In December 2000, *P. ramorum* was first discovered infecting rhododendron nursery stock in California (10). By 2003, agricultural inspectors found the pathogen infecting nursery stock in California, Oregon, Washington, and British Columbia, Canada. In 2004, the disease became a national concern when a large wholesale nursery in California shipped camellia plants infected with *P. ramorum* to nurseries and other customers in 40 states. Presently, Animal and Plant Health and Inspection Service Plant Protection and Quarantine lists 110 plant taxa as proven or associated hosts (1). State, federal, and international quarantine restrictions have been placed on the movement of plants or plant parts of these listed hosts.

In addition to causing aboveground cankers and leaf blights, *P. ramorum* can survive in soil and can serve as an inoculum reservoir for both root and shoot infections. *P. ramorum* survived in infected camellia leaf tissue buried for almost 2 years and in root pieces up to 11 months in laboratory and greenhouse conditions, respectively (19). Furthermore, the pathogen survived in various potting media or soil for up to 6 months as sporangia or 12 months as chlamydospores (13). Infected rhododendron leaf disks buried in California forest soil retained 60% pathogen survival over the summer (7) and up to 80% survival after a year-long incubation initiated during the winter rainy season (E. Fichtner, *unpublished data*). This demonstrated survival potential of *P. ramorum* in both forest soils and in various potting media suggests their role as an inoculum reservoir in both natural ecosystems and in nurseries.

P. ramorum in infested soil has been shown to serve as primary inoculum for root and foliar infections. Under laboratory conditions, foliar infections of bay laurel were induced by splash dispersal of inoculum from infested soil covered with bay laurel green leaf litter (4). Similarly, in situ studies in a California forest demonstrated that splash dispersal of infested soil after rain events can infect tanoak and bay laurel foliage up to 30 cm above the forest floor in both the presence and absence of a leaf litter layer (E. Fichtner, *unpublished data*). *P. ramorum* has been shown to cause asymptomatic infections of rhododendron roots in infested U.K. woodlands (8) and of potted rhododendrons grown in artificially infested potting medium (16). Additional laboratory studies demonstrated that *P. ramorum* may infect roots of weeds found in ornamental container culture (20) and roots of numerous nursery ornamental plants (19). In commercial nurseries, *P. ramorum* has been detected both beneath and within containers of asymptomatic nursery stock, demonstrating the potential for undetected pathogen movement in the nursery stock trade (2).

Long-term survival of *P. ramorum* in container and field soil may at least partially explain the challenge of eradication efforts, but the influence of nursery practices and environmental conditions on pathogen longevity, transmission, and infectivity in infested containers under nursery conditions are unknown.

The epidemiological importance of infected leaf litter in potting soil in transmission and survival of *P. ramorum* in container-grown rhododendrons was assessed under outdoor-nursery and greenhouse conditions. The objectives of this study were to (i) determine in situ survival potential of *P. ramorum* in potting media; (ii) investigate the relative production of sporangia and chlamydospores on buried, infected leaf litter over time; (iii) assess potential for pathogen transmission from infected leaf litter to aboveground plant parts and roots; and (iv) investigate the influence of rain events and irrigation practices on foliar disease incidence in pots containing infected leaf litter. The results from these experiments will help assess disease risk associated with soilborne inoculum and provide information for the development of improved nursery practices for disease management.

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MATERIALS AND METHODS

Plant material and container soil. Nursery stock consisted of established 3.8-liter *Rhododendron* 'Cunningham's White' obtained from a commercial nursery at the beginning of each of the three experimental seasons. Plants were maintained without the use of fungicides under 40% shade cloth, fertilized with slow release fertilizer (21-5-6 Apex; J. R. Simplot, Boise, ID) at 6 g/pot every 4 months. The potting media consisted of equal portions of Douglas fir bark, peat moss, and perlite.

Experimental sites. Field experiments were conducted in Felton, CA, an area characterized by a Mediterranean climate, with cool wet winters and hot, dry summers. Rainfall was supplemented with drip or overhead-sprinkler irrigation in the experiment comparing irrigation practices. Greenhouse experiments were maintained in a research greenhouse (Salinas, CA; maximum day temperature of 23°C and minimum night temperature of 11°C) and plants were irrigated every second day with drip irrigation.

Production of leaf disk inoculum. The *P. ramorum* isolate (Pr-52) used in all experiments originated from an infected *Rhododendron* sp. from a commercial nursery in Santa Cruz County, CA (12,17,23,24). Seven 6-mm-diameter V8 agar plugs were placed into sterile 10-cm-diameter petri plates containing 7 ml of sterile deionized water, incubated at 20°C for 3 days in the dark to induce sporangia production, chilled at 4°C for 20 min, and returned to room temperature for 1 h to induce zoospore release. One hundred 5-mm-diameter rhododendron leaf disks were evenly dispersed over the water surface of each plate and incubated for 24 h at 20°C. Leaf disks were removed, covered with moist paper towels, and then incubated for another 48 h at 20°C in the dark, at which point darkened lesions completely covered most leaf disks. These symptomatic leaf disks were arranged in groups of 100 for processing in the experiments described below.

Evaluation of leaf disk decay, pathogen recovery, and sporulation potential. Symptomatic rhododendron leaf disks were placed in potted rhododendron plants on 9 February 2004 (season 1), 25 February 2005 (season 2), and 17 February 2006 (season 3). February was chosen to infest the soil because this is when the highest concentration of inoculum would be expected in California nursery conditions. Each pot contained 100 symptomatic leaf disks placed in a 36-cm^2 nylon mesh sachet (7 openings/cm) and buried horizontally 1 cm below the soil surface. Each season, up to 48 potted rhododendrons were infested and randomly selected for incubation under field or greenhouse conditions. At approximately 10-week intervals, the sachets were removed from three potted rhododendrons that were randomly selected at each location. Seasons 1, 2, and 3 lasted a total of 66, 50, and 60 weeks, respectively.

On most retrieval dates, leaf disks were evaluated for decay rate and pathogen recovery. Infested rhododendron leaf disks were removed from the sachets and washed with sterile deionized water (SDW) to remove most of the adhering soil. Leaf disk decay was quantified by counting the number of nondecayed leaf disks (usually less than 50% decomposed) and subtracting this number from the total number of originally buried leaf disks. These disks were again washed with SDW and transferred to PARP semiselective medium (6) for pathogen detection. Pathogen recovery in the leaf disks was quantified by counting the number of disks that produced *P. ramorum* colonies in culture after 10 to 14 days. In season 2, the leaf disks incubated in the greenhouse after 30 weeks were not plated and used instead to evaluate sporulation potential.

In seasons 2 and 3, a portion of inoculated leaf disks was periodically removed from the soil, rinsed with SDW, flooded with SDW in the laboratory, and evaluated for sporulation potential. Six leaf disks were randomly removed from each of three sachets incubated in field and greenhouse conditions and placed in a circular pattern in a sterile 10-cm-diameter petri plate. The disks were covered with a 9.5-cm-diameter transparent nylon mesh to hold the disks in place and facilitate spore counting. Plates were then flooded with SDW to just cover the top of the disks without dislodging them. After incubation in the dark for 7 days at 20°C, the number of sporangia and chlamydospores that developed around the edge of each disk were counted.

Evaluation of root infections. In season 3, roots were evaluated for infection by *P. ramorum* at 20, 30, 40, and 60 weeks after incubation. After leaf disks were retrieved from three pots each of greenhouse- and field- incubated rhododendrons, roots were excavated for destructive sampling. The roots of each plant were thoroughly washed to remove all visible soil and a sample of clean roots from each plant was selected to fill the volume of a 140-by-15-mm petri plate. Washed roots were cut into 3-cm-long fragments with a razor blade, then covered with SDW. Fifty rhododendron ('Colonel Coen') leaf disks (5 mm in diameter) were floated above each set of roots for 4 days at room conditions, and then were placed on PARP containing hymexazole at 50 mg/liter. *P. ramorum* and *P. cinnamomi* were identified morphologically. Three isolates of *P. cinnamomi* were subcultured for identity confirmation by sequencing the internal transcribed spacer 1 and 2 region of ribosomal DNA and comparing the sequence to those in GenBank.

Evaluation of foliar disease risk associated with sprinkler or drip irrigation. In a separate experiment, incidence of foliar disease in containerized rhododendrons growing in infested potting medium was assessed under sprinkler and drip-irrigation systems. Experiments in all three seasons were established at approximately the same time (February) and in the same field as the experiments described above. The experiments received natural rainfall and were irrigated when necessary with domestic water. Treatments were (i) sprinkler irrigation with soil inoculum, (ii) sprinkler irrigation without soil inoculum, (iii) drip irrigation with soil inoculum, and (iv) drip irrigation without soil inoculum. The experiment was established annually in a randomized complete block with six blocks, and each block containing treatment plots with three rhododendrons surrounding a central rhododendron with plants spaced approximately 5 cm apart. In infested treatment plots, 100 leaf disks prepared as described previously were uniformly mixed into the top 1 cm of soil in each pot. Each plot grouping was arranged in the center of a wooden pallet, 95 by 82 by 16 cm (height); pallets were spaced 1 m apart. This eliminated any pot contact with the soil and rain splash onto foliage from the field.

Each sprinkler irrigation plot utilized brass nozzle sprinkler heads (SF-9, 140 kPa [20 psi], 6 liters/h; Champion-Arrowhead, Los Angeles) on inverted "J"-shaped risers that allowed the sprinkler heads to be installed upside down and directed sprinkler output primarily to the tops of the experimental plants. This system, along with minimal sprinkler pressure and relatively small spray droplet size, provided an environment that did not allow water splash at the soil surface but did completely wet the leaf canopy at each irrigation. Drip-irrigation plots utilized pressure-compensated drippers (no. 12503117, 1.9 liters/h/plant; Pepco Irrigation, Fresno, CA); water was always applied directly to the soil and, therefore, never covered the foliage. Irrigations were made when approximately half the available soil water had been used by the plant as measured by gravimetric methods. Water was applied until container capacity was reached and an additional 20% leaching fraction was added. This took approximately 45 min for sprinkler and 1 h for drip irrigation.

Every 2 weeks, plants were monitored for symptoms of *P. ramorum* infection. If characteristic leaf lesions were observed, these leaves were removed, pieces of the lesions plated on PARP, and the number of confirmed infected leaves recorded.

Environmental monitoring. Rainfall and temperature were recorded at the field experimental site with a portable digital weather station (Onset Computer Co., Pocasset, MA). The greenhouse was kept at a maximum day temperature of

23°C and minimum night temperature of 10°C.

Statistical analyses. Leaf disk decay was described with the following Logit model:

$$\mathrm{Log}\left(\frac{\mathrm{Decay}_{ij}}{1-\mathrm{Decay}_{ij}}\right) = a_i + loc_i * \mathrm{weeks}_j + error_{ij}$$

where a_i is the intercept, loc_i is the slope for each location (greenhouse or field location), respectively, weeks_i is time (in week units; j = 1,2,…) since sachets were buried and $error_{ij}$ is the over-dispersion error. The response, Decay_{ij} (the number of remaining disks from 100 at loc *i* for week *j*) was assumed to be an overdispersed binomial random variable. The intercepts and slopes were estimated using the maximum likelihood estimation (MLE) technique (15) correcting for overdispersion with the SAS (v.9.1.3) GENMOD procedure (18). The leaf disk decay was assessed by comparing the slopes of the models for greenhouse and field locations for each of the three experiments.

Leaf disk recovery was described with the following Logit model:

$$\mathrm{Log}\left(\frac{\mathrm{Recovery}_{ij}}{1-\mathrm{Recovery}_{ij}}\right) = a_i + loc_i * \mathrm{weeks}_j + error_{ij}$$

where a_i, loc_i, weeks_i, and $error_{ij}$ were defined as above. The response, $\mathrm{Recovery}_{ij}$ (the number of positive disks from the number of remaining disks 100 at loc *i* for week *j*) was assumed to be an overdispersed binomial random distribution. Again, the intercepts and slopes were estimated using MLE correcting for overdispersion with the SAS (v.9.1.3) GENMOD procedure. The leaf disk recovery was assessed by comparing the slopes of the models for greenhouse and field locations for each experiment.

Sporulation potential was described with the following Poisson regression model:

$$\mathrm{Expected}\left[\mathrm{Log}\left(\mathrm{count}_{ijk} \mid Year_k\right)\right] = a_i + s_{1i}\mathrm{week}_j + s_{2i}\mathrm{week}_j^2 + Year_k$$

where a_i (i = 1 for chlamydospores and i = 2 for sporangia) are the intercepts; s_{1i} are the coefficients for weeks_i and s_{2i} are the coefficients for weeks_i squared for chlamydospores and sporangia, respectively; weeks_i = time in weeks since leaf disk sachets were buried; and *year* is random effect due to year (the overdispersion error assumed to be normally distributed). The response, count (chlamydospores or sporangia), was assumed to be an overdispersed Poisson random variable with expected mean (15). The intercepts a_i and coefficients s_{1i} and s_{2i} were estimated by MLE correcting for over-dispersion with the SAS (v.9.1.3) GLIMMIX procedure. The greenhouse and field data were analyzed separately with a similar Poisson model.

RESULTS

Leaf disk decay. Leaf disks decayed slowly, leaving a skeleton of leaf veins in the general shape of a leaf disk before becoming indiscernible fragments. In season 1, leaf disks decayed more quickly in the greenhouse than in the field (P = 0.008) based on comparisons of estimated

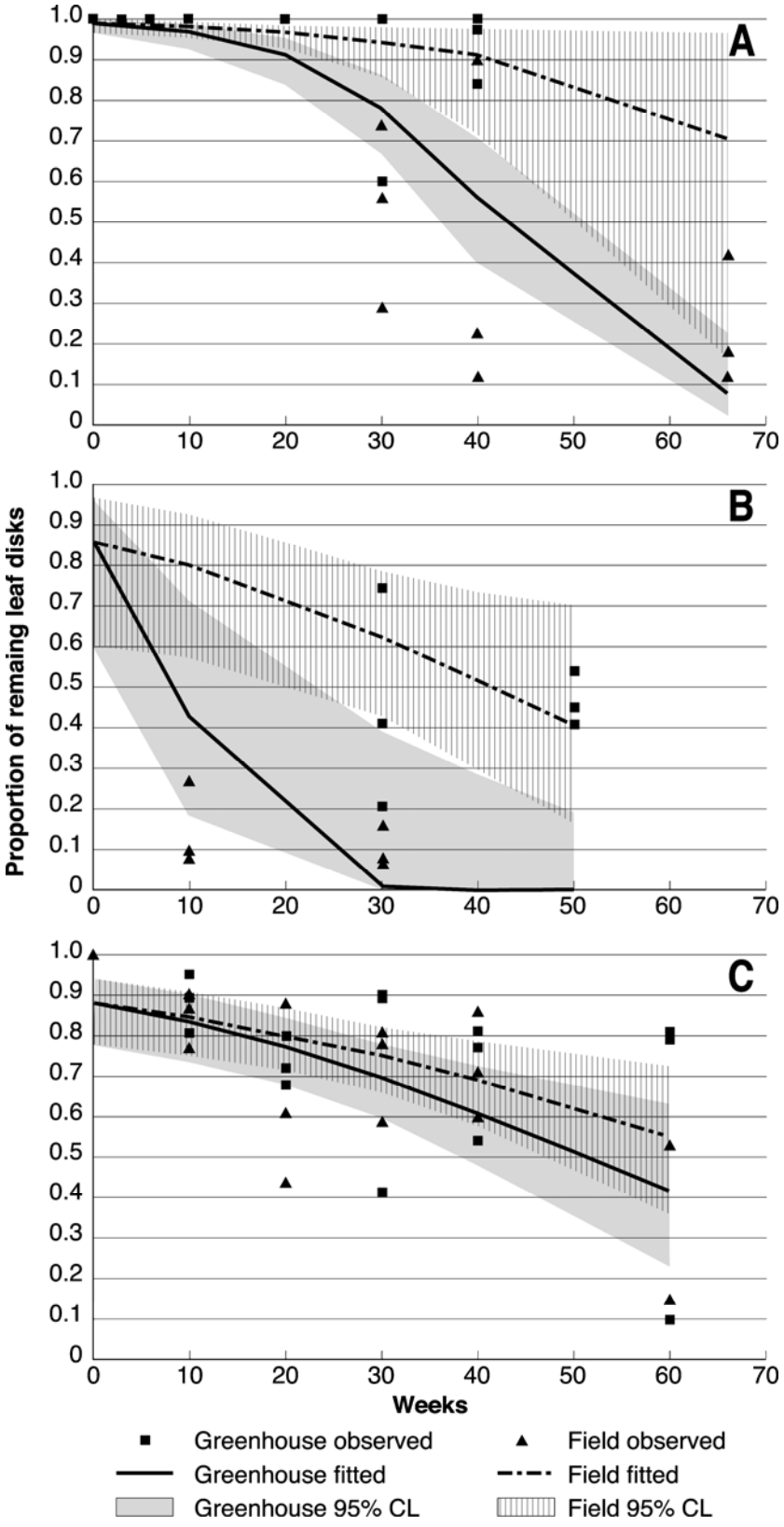

Fig. 1. Effect of incubation time after inoculation with *Phytophthora ramorum* and burial in planted potting medium on the proportion of remaining leaf disks in greenhouse or field conditions. The observed proportion of remaining leaf disks and estimated proportion from the fitted Logit model with their respective 95% confidence limits (CL) are shown for season **A,** 1; **B,** 2; and **C,** 3 (inoculation and burial dates in February 2004, 2005, and 2006, respectively).

slopes for greenhouse (–0.105) and field (–0.054) from their respective fitted Logit models. There were no significant differences between decay rate in greenhouse- and field-incubated disks in seasons 2 and 3 (P = 0.840), although the estimated slopes for this experiment also indicated that greenhouse-incubated disks decayed slightly faster than field-incubated disks (Fig. 1).

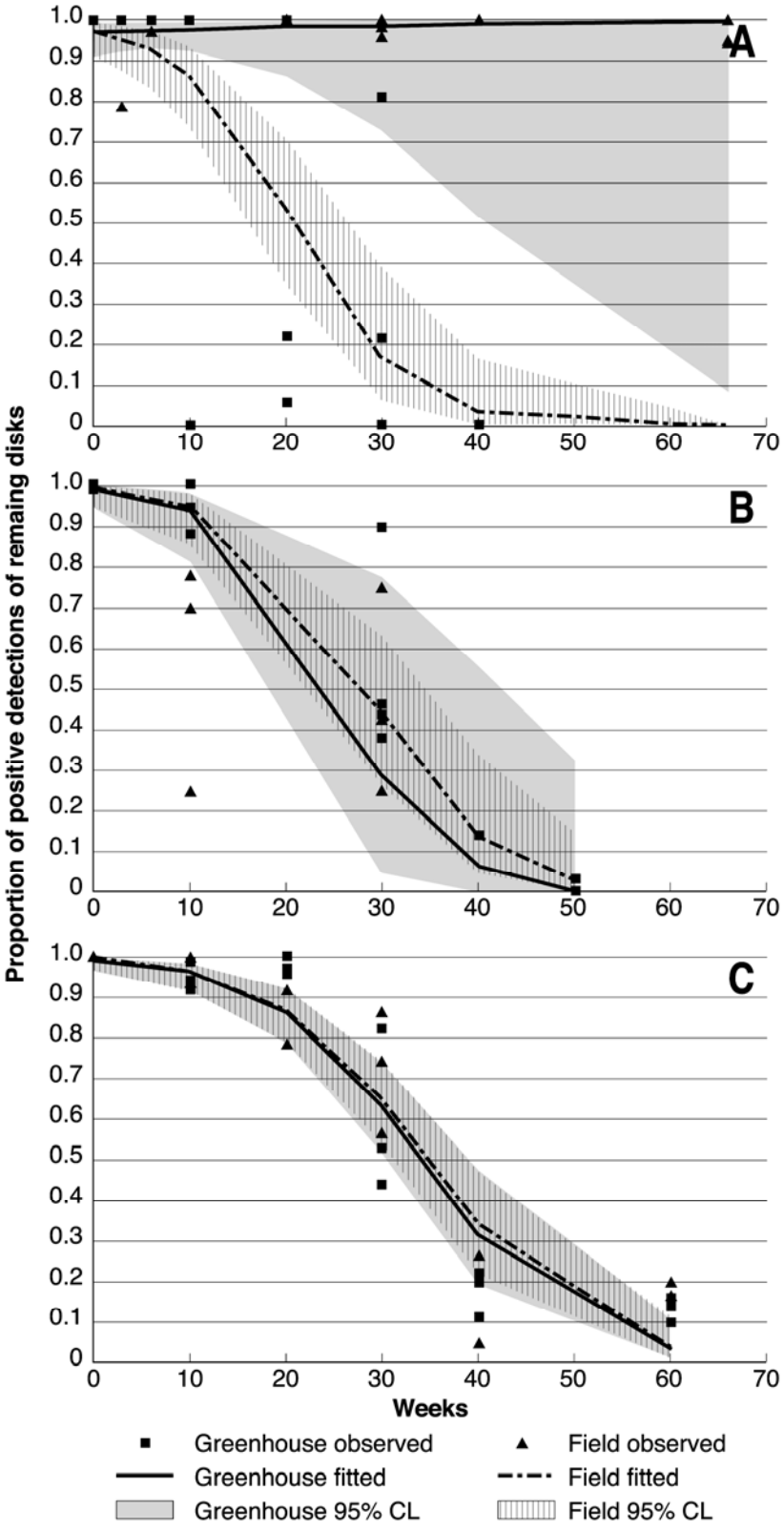

Fig. 2. Effect of incubation time after inoculation and burial in planted potting media on the proportion of disks where *Phytophthora ramorum* was detected in the remaining inoculated leaf disks. The observed positive detections and estimated positive detections from the fitted Logit models for the field or greenhouse and their respective 95% confidence limits (CL) are shown for season **A,** 1; **B,** 2; and **C,** 3 (inoculation and burial dates in February 2004, 2005, and 2006, respectively).

Pathogen recovery from leaf disks. In general, pathogen recovery from leaf disks declined over time. In season 1, the pathogen was recovered significantly less frequently in the field-incubated disks than in the greenhouse-incubated disks (P = 0.0008) based on comparisons of estimated slopes for field (–0.17) and greenhouse (0.02) recovery from fitted Logit models. In season 1, even though the greenhouse-incubated leaf disks retrieved at 66 weeks were badly decayed, the pathogen could be recovered from 96.5% of discernible leaf disks. This high recovery and slightly positive slope of the fitted Logit model, however, was exceptional compared with all other evaluations, where recovery declined with time. In seasons 2 and 3, the estimated slopes from the fitted Logit recovery model for field- and greenhouse-incubated disks were negative and not significantly different between the two locations (P = 0.925 and 0.840, respectively; Fig. 2).

Sporulation potential on leaf disks. After flooding in water, sporulation was observed on perimeters of leaf disks that were incubated in both greenhouse- and field-planted pots for up to 50 and 60 weeks in seasons 2 and 3, respectively (Fig. 3). Chlamydospores were also seen within the leaf disk tissue when examined after flooding. The fitted Poisson model showed that significantly more chlamydospores than sporangia were produced soon after inoculation (0 and 10 weeks; P < 0.0001); however, after 10 weeks, the leaf disks produced increasingly more sporangia than chlamydospores (P = 0.0001). This temporal asynchronous relationship between the formation of sporangia or chlamydospores was similar in greenhouse- and field-incubated leaf disks.

Root infection occurrence. *P. ramorum* was not recovered from roots in pots that had been infested with inoculated leaf disks for 20 or 30 weeks. *P. ramorum* was baited from roots of two infested pots that were incubated under field conditions for 40 weeks and from one pot incubated in the greenhouse for 60 weeks. *P. cinnamomi* was also recovered from roots in both greenhouse- and field-incubated plants and, occasionally, both *P. cinnamomi* and *P. ramorum* were recovered from roots of the same plant.

Foliar disease incidence under drip and sprinkler irrigation. In season 1, two diseased plants were detected before irrigation treatments were initiated on 24 March 2004. One leaf tip on each infected plant was inadvertently partially buried in infested soil. In season 2, disease occurred on the lower, shaded portions of sprinkler-irrigated plants on 22 April 2005 in three separate treatment plots. Disease only occurred on the centrally located, infested plants; no foliar infections occurred on

plants adjacent to the infested pots. In season 3, no foliar infections occurred on any of the irrigated plots.

DISCUSSION

Although leaf disks slowly decayed for over a year and, eventually, only a skeleton of leaf veins was discernible, the remains supported recovery of *P. ramorum* in PARP medium and sporulation in flooded conditions for the duration of all the experiments. Leaf veins consist of highly lignified vascular tissue and, therefore, are more resistant than cellulosic leaf blades to microbial decomposition in the soil (9,14). The generally slower decay rate of vascular tissue may protect *P. ramorum* from adverse environmental and moisture fluctuations.

In season 1 (2004), the infected leaf disks supported significantly lower pathogen viability in the field than in the greenhouse even though the decay rate of the leaf disks in the field was slower. This could be explained by the environmental conditions in the spring period following infestation of pots. In 2004, the spring period (March, April, and May) immediately following the leaf disk placement in the field was unusually dry (38.6 mm mean rainfall) and warm (24.3°C mean maximum), whereas the equivalent periods in the other two seasons for 2005 and 2006 were notably cooler (20.7 and 17.9°C mean maximum, respectively) and wetter (158.6 and 607.3 mm mean rainfall, respectively). Leaf disks were placed just under the soil surface where they would be particularly vulnerable to drying and high temperatures under field conditions. In contrast, the regular irrigation and moderate environment in the greenhouse would be less subject to these extremes and could account for the significantly better pathogen recovery. In addition, the environmental extremes in 2004 may have adversely affected populations of decay organisms in field-incubated pots, thereby reducing leaf disk decay rate.

There are two possible explanations for the asynchronous production of chlamydospores and sporangia on flooded leaf disks. First, chlamydospores could support later sporangia production via germination of chlamydospores to form sporangiophores (19,24). Perhaps a reservoir of dormant chlamydospores forms early within leaf tissue and, over weeks, conditioning in soil may induce the gradual breaking of dormancy of a portion of the chlamydospore reservoir. When these leaf disks are placed in flooded conditions, mycelia grow into the water and form sporangia relative to the number of chlamydospores that germinate. Although observations were not made intentionally to detect chlamydospores within leaf disks, chlamydospores were observed only occasionally in moderate numbers within leaf disks during the normal spore-counting procedure in this study. Second, the propensity for later sporangia production could be related to the gradual decline of nutrient reserves in colonized tissue. Soilborne *Phytophthora* spp. usually produce sporangia optimally after colonies are transferred from nutrient-rich media to nutrient-poor conditions by frequent rinsing of water or salt solutions (5,11). In this case, perhaps the nutrient depletion associated with tissue decay and the constant leaching contributed by irrigation created conditions conducive for sporangia production.

Root infections were detected only after pots were infested for at least 40 weeks. Though chlamydospores placed in the rhizosphere have been observed to germinate and infect roots in vitro (19), the infected leaf disks utilized in this study had minimal root contact. Detection of root infections corresponded with periods of heightened sporangia production potential on leaf disk inoculum, suggesting that a threshold level of sporangia was necessary for induction of root infections. Additionally, roots were generally precolonized with *P. cinnamomi* which may have put *P. ramorum* at a competitive disadvantage for infecting roots.

Foliar disease on leaves supported above the soil occurred only in the spring, when both significant rainfall occurred and the plants were supplemented with sprinkler irrigation. *P. ramorum* blight on rhododendron was also found during the same period in another unrelated simultaneous experiment at the same field site (22). In that experiment, long periods of leaf wet-

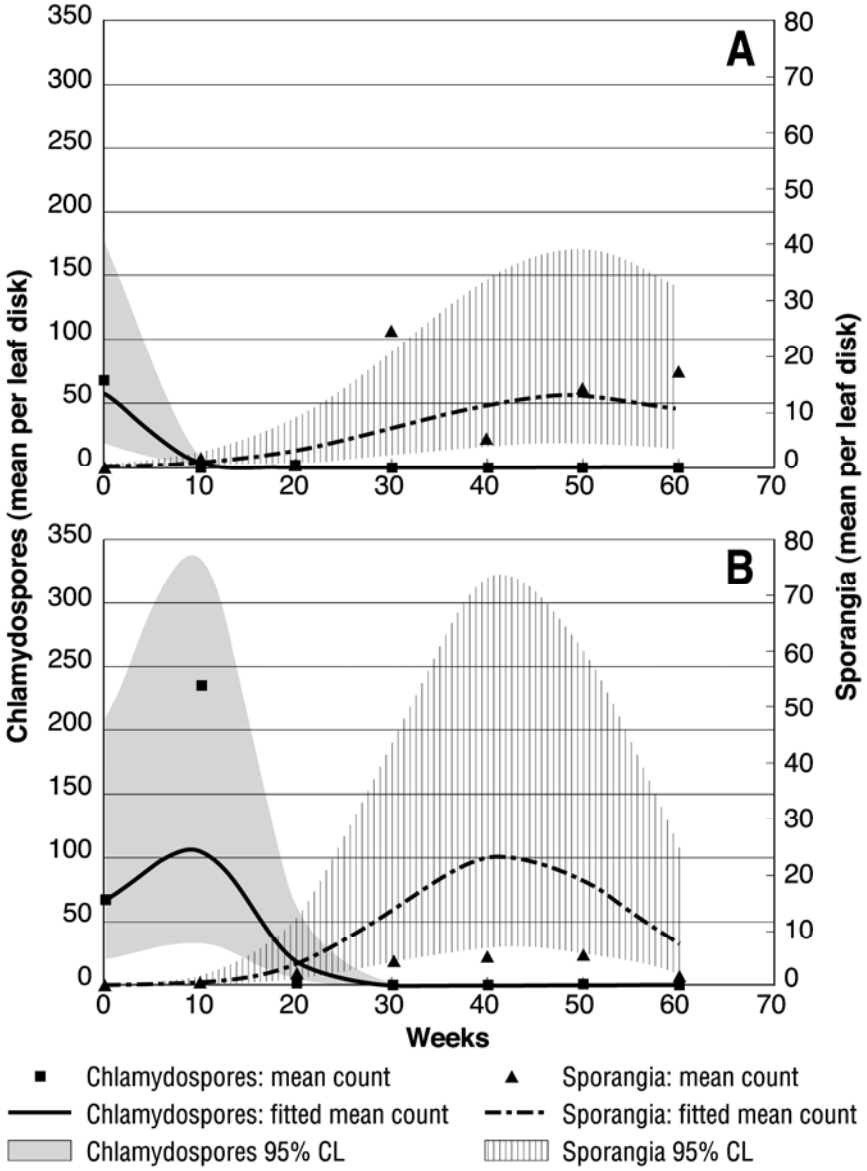

Fig. 3. Effect of incubation time after inoculation and burial in planted potting medium on the production of *Phytophthora ramorum* sporangia and chlamydospores from flooded leaf disks. Sporulation was measured after flooding with sterile deionized water in petri plates for 1 week in the dark at 20°C. The observed spore production and estimated mean spore counts for the fitted Poisson models and associated 95% confidence limits (CL) in field and greenhouse conditions (data combined) are shown for seasons **A,** 2 and **B,** 3 (inoculation and burial dates in February 2005 and 2006, respectively).

ness and cool temperatures preceding infection were found to be conducive to disease. In this experiment, the transmission of inoculum from infested soil to the leaf surfaces was likely associated with vigorous rain events causing splash dispersal to leaves supported above infested soil.

Considering the findings in this study that infected leaf disks could remain viable for lengthy periods in greenhouse and field conditions and then produce infectious spores, one must consider infected leaf litter at or just under the soil surface as an important inoculum reservoir for *P. ramorum* in nursery production. This inoculum source may arise when infected leaves are blown in the wind, drop into nursery stock containers or field soil underlying the infected plant, and gradually work their way into the soil or litter. Infected leaves are a likely source of soil inoculum because they tend to abscise when lesions enlarge significantly (21). Additionally, populations of *P. ramorum* in infested pots may increase due to colonization of roots.

Potting media may support long-term pathogen survival and serve as a source of primary inoculum for foliar infections. Infected leaves may also be a source of inoculum for root infections of nursery stock. As an inoculum management strategy, fallen or intact leaves with lesions should be removed from nursery stock and destroyed. Nurseries should inspect incoming stock not only for infected intact leaves but also for fallen leaves in the containers or in the bottom of trucks bringing in shipments to the nursery. Rainfall events and spring conditions are most conducive to splash dispersal and lower leaf infection, and lower leaves touching infested soil may become infected. Irrigation timing should allow for the drying of the soil surface and root ball, if possible, to decrease the wet conditions suitable for propagule production and root and leaf infection. Sprinkler irrigation methods that minimize splash at the soil surface, or drip irrigation, could help reduce or eliminate splash dispersal and transmission to lower leaves.

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