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Effects of Inoculum Density and Wounding on Stem Infection of Three Eastern US Forest Species by *Phytophthora ramorum*

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Abstract

Seedlings of three Eastern US forest species *Quercus rubra* (northern red oak), *Quercus prinus* (chestnut oak) and *Acer rubrum* (red maple) were inoculated by applying *Phytophthora ramorum* sporangia to stems at different inoculum densities with and without wounding. Disease occurred in all treatments involving wounds, and no disease was observed in unwounded treatments. Younger seedlings (2–3 years old) did not differ significantly from older seedlings (5–6 years old) in disease incidence, but older seedlings sustained smaller lesions compared with younger seedlings. For both old and young seedlings, disease on wounded stems was observed down to the lowest sporangia concentration utilized (500 sporangia/ml for old seedlings and 100 sporangia/ml for young seedlings). The results show that in the presence of wounding, even very low sporangia concentrations can result in disease, and further suggest that wounding caused by insects and other factors may play an important role in *P. ramorum* epidemiology in forest environments.

Introduction

Knowledge of inoculum density relationships is important for understanding the epidemiology of plant pathogens (Van der Plank 1975). Knowledge of the minimum number of spores required for infection can help with predictive disease models and pest risk assessments. In some pathosystems, the relationship between inoculum density and disease is directly proportional, while in others, there exists a minimum inoculum density above which disease occurs. Knowledge of inoculum density relationships, combined with knowledge of levels of inoculum present in natural ecosystems in which the disease occurs, can allow predictions to be made regarding the likelihood of epidemic development. It has also been shown that high inoculum levels may allow a given pathogen to overcome host resistance that may have been expressed at low inoculum levels. Such knowledge is important to apply during screening studies so that potentially valuable forms of resistance are not overlooked through the use of excessive inoculum levels. In screening studies, it is desirable to use inoculum levels that increase the likelihood that resistance will be identified, and these levels need to be determined experimentally.

Phytophthora ramorum is a destructive pathogen which causes sudden oak death and ramorum blight, (Rizzo et al. 2002, 2005; Grunwald et al. 2008, 2012) as well as sudden larch death in the UK (Brasier and Webber 2010; Webber et al. 2010). With *P. ramorum*, inoculum density studies have been performed on foliage of selected hosts (Tooley et al. 2004, 2013; Hansen et al. 2005; Hüberli et al. 2008), but not on host stems. In nature, *P. ramorum* has been found trapped in rain water (Davidson et al. 2005, 2008) indicating that *P. ramorum* sporangia are likely to be present on stems and trunks of young and old trees, respectively, during rain events. It has also been shown that *P. ramorum* is able to infect host species directly through tree bark (Brasier et al. 2003), and to colonize sapwood from inoculum placed in the bark (Parke et al. 2007). Knowledge of inoculum density relationships on tree stems in addition to foliage would thus be helpful in understanding forest epidemics.

Phytophthora ramorum has been found in several states in the Eastern US, the result of movement of infected plants within the nursery trade (Stokstad 2004). Monitoring efforts are underway in many Eastern forests to determine whether the pathogen is present and attacking oaks and other species. Three species that play important roles in Eastern US forest ecosystems include *Quercus rubra* (northern red oak), *Quercus prinus* (chestnut oak), and *Acer rubrum* (red maple; Burns and Honkala 1990). These species were chosen for these studies because all three have been found to be susceptible to *P. ramorum* in greenhouse studies (Tooley and Kyde 2007; Tooley and Browning 2009) and would likely have a large impact on epidemic severity should *P. ramorum* become established in the Eastern US

It is known that wounding can increase susceptibility of certain host species to infection by *Phytophthora* species (Bostock and Doster 1985; O'Gara et al. 1996; Adorada et al. 2000; Thomidis 2003; Granke and Hausbeck 2010; Alao et al. 2012). However, the role of wounding in infection of host species by *P. ramorum* has not been investigated. The presence of wounding might allow fewer spores to be required to infect a given host species compared with the number of spores required to infect unwounded tissue. In these studies, we examined the effects of *P. ramorum* sporangial inoculum density and wounding on infection of stems of three forest species with the potential to play an important role in epidemiology of sudden oak death.

Materials and Methods

Seedlings of *Q. prinus* (chestnut oak), *Q. rubra* (northern red oak) and *A. rubrum* (red maple) were obtained from the John S. Ayton State Tree Nursery, Preston, MD, and were transported to the USDA-ARS Foreign Disease-Weed Science Research Unit (FDWSRU) Biosafety Level 3 (BSL-3) plant pathogen containment facility at Ft. Detrick, MD (Melching et al. 1983), where experiments were performed. The six *P. ramorum* isolates chosen for these studies (Table 1) varied in host of origin and clonal lineage (Grunwald et al. 2009), and sporangia of the six isolates were combined for inoculation. To produce sporangia, number 3 cork-borer discs were cut from the margins of *P. ramorum* colonies growing on 20% V8 juice agar at 20°C in darkness. Discs (15–20) were incubated in 1% soil extract as described previously (Tooley et al. 2004). Two series of experiments were conducted with 2- to 3-year-old seedlings of *Q. prinus*, *Q. rubra* and *A. rubrum*. Eight inoculation sites were marked on stems at a spacing of 10 cm, starting at the base of the plant. Treatments consisted of 0, 500, 1000 and 3000 sporangia/ml applied to wounded and unwounded stem tissue in a randomized pattern. Bark was abraded with 220 grit sandpaper to expose the vascular tissue. The sporangia suspension was applied to the stem with a small paintbrush. An acetate template was employed to standardize the treatment area to 0.5 × 1.0 cm. Immediately following inoculation, stems were wrapped with a wad of moistened cheesecloth and sealed with parafilm. Six plants per species were treated in each of three experiments. In the second series, three plants per species were treated with 0, 100, 250 and 3000 sporangia/ml in each of three experiments. We also conducted three experiments with 5- to 6-year-old seedlings of *Q. prinus* and *Q. rubra.* Six trees per species were treated with 0, 500, 1000 and 3000 sporangia/ml as described above. Inoculated trees in all experiments were maintained in a climate-controlled, Biosafety Level 3 greenhouse at 20°C for 2 months, after which stems were evaluated for *P. ramorum* lesion formation. The stem diameter at each inoculation site was recorded, and the bark was scraped off to expose the vascular tissue. Using a black marker, the perimeters of stem lesions were traced onto a single layer of no. 50 cheesecloth held firmly in place over the stem section. Lesion perimeters were traced onto paper from the cheesecloth and scanned. Stem lesion areas were determined using the ASSESS software program (Lamari 2002). Sections of the discoloured stem tissue were plated on PARP selective agar medium (Jeffers and Martin 1986) with 4% buffered and clarified V8 juice added, to confirm the presence of *P. ramorum*. Data are reported as mean percentage infection and adjusted lesion area (measured lesion area/stem diameter).

In an attempt to determine the number of spores actually deposited on stems during treatment, spore suspensions were painted onto Durapore ® polyvinylidene fluoride (PVDF) membrane filters (EMD Millipore, Billerica, MA, USA) in the same manner as

Table 1 Isolates of *Phytophthora ramorum* used in these studies

Isolate	Host	Location	Lineage[a]
Pr-6	Coast live oak	Marin Co., CA	NA1
Pr-52	Rhododendron	Felton, CA	NA1
CAM 5C	Camellia	CA	NA1
WSDA 1838	Rhododendron	WA	NA1
WSDA 1772	Viburnum	OR	NA1
BBA 9/95	Rhododendron	Germany	EU1

[a]As described by Grunwald et al. (2009).

stems, utilizing the acetate template which delineated an area of 0.5 cm^2. Filters were then turned over onto the surface of 1.25% water agar plates. The number of spores deposited on the water agar was counted in replicates of 12 at each sporangium concentration (0, 100, 250, 500, 1000, 2000 and 3000 sporangia/ml), and four experiments were completed.

Data were analysed by analysis of variance and regression analysis using the SAS statistical package (SAS Institute Inc. 2008). Simple linear regression models were fit to the lesion data, and calibration estimates were made of the threshold at which point any amount of lesion area was observed. With y used to represent adjusted lesion area and x representing the sporangia concentration, we employed the linear model $E(y) = \beta \log(x + 0.1) - \delta)$, where δ is the sporangia threshold parameter at which the expected adjusted lesion area becomes positive (O'Brien 2010). The SAS/OR procedure NLP was used to estimate profile likelihood confidence intervals for these threshold estimates. Threshold estimates and 95% confidence limits were exponentiated (exp (δ to obtain threshold estimates which were on the original scale of sporangia/ml. We also estimated the number of sporangia/ml required to attain the median-adjusted lesion area.

Results

Wounding was required for infection of stems by *P. ramorum*, as confirmed by isolations from wounded and not unwounded stem tissue on PARP-V8 selective medium. For both old and young seedlings, disease on wounded stems was observed down to the lowest sporangia concentration utilized, 500 sporangia/ml for old seedlings and 100 sporangia/ml for young seedlings (Fig. 1). For young seedlings at 100 sporangia/ml, 33% infection was observed on 2- to 3-year-old seedlings of *A. rubrum*, while 67 and 78% infections were observed for *Q. prinus* and *Q. rubra*, respectively (Fig. 1). Stem diameters averaged 6.35, 6.88 and

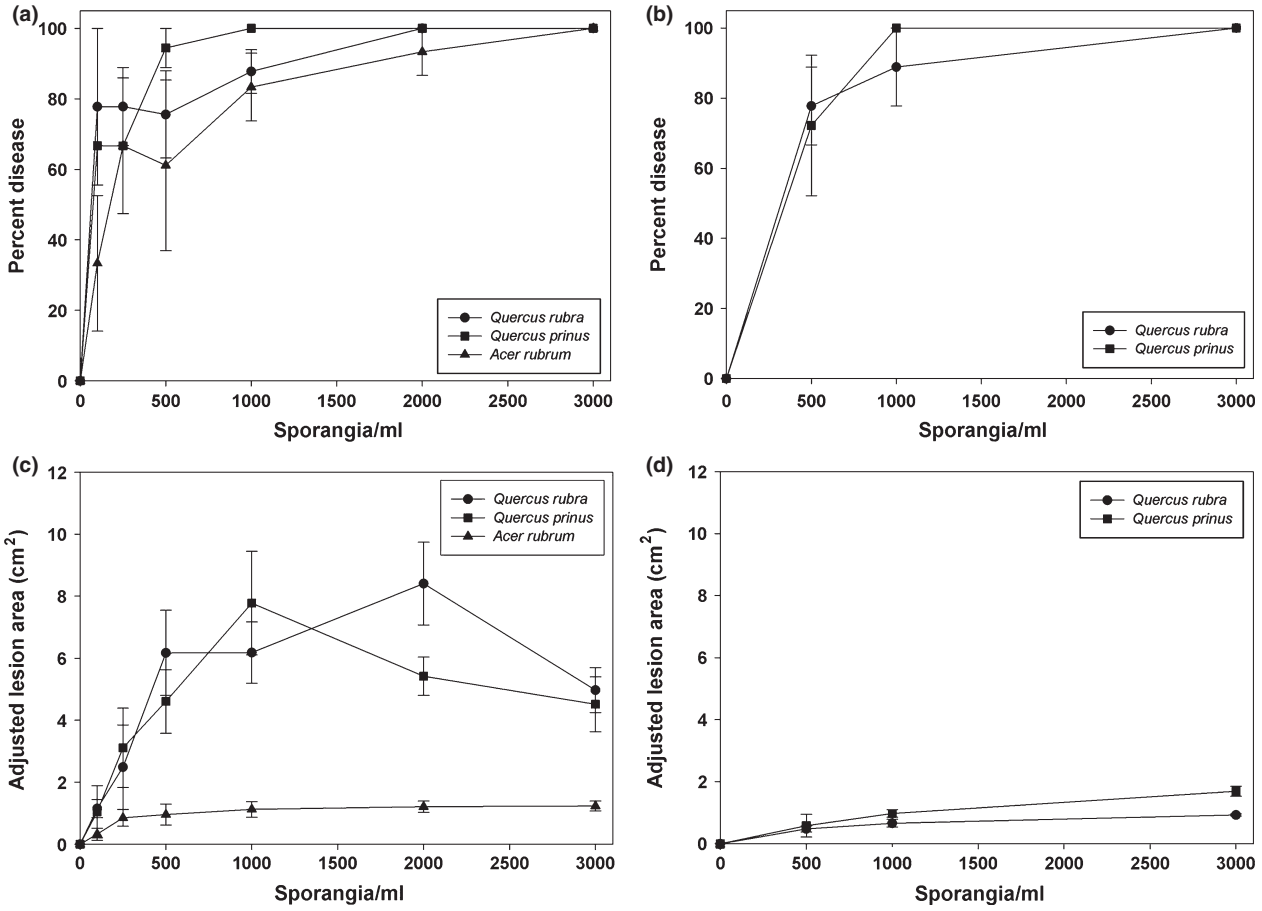

Fig. 1 Percentage disease (a, b) and adjusted lesion area (c, d) plotted against *Phytophthora ramorum* inoculum density (sporangia/ml) for three Eastern US forest species; 2- to 3-year-old seedlings (a and c) and 5- to 6-year-old seedlings (b and d). Bars represent standard errors.

7.06 mm for 2- to 3-year-old seedlings of *Q. prinus*, *Q. rubra* and *A. rubrum*, respectively. In 5- to 6-year-old seedlings, mean stem diameters were 9.19 mm for *Q. prinus* and 9.61 for *Q. rubra*. For young seedlings, lesion areas on infected stems were marginally smaller with *A. rubrum* than with the two oak species ($Pr > F = 0.0853$). When the adjusted lesion area on old and young seedlings were compared at the sporangia concentrations that were common to both sets of experiments, lesions were significantly smaller for older seedlings than for younger seedlings at inoculum concentrations of 500, 1000 and 3000 sporangia/ml. However, disease incidence (percentage infected stems) did not differ significantly ($P = 0.05$) between old and young seedlings.

Linear regression models were used to estimate calibration thresholds representing the minimum disease levels observed (Table 2) and describe the relationship between adjusted lesion area and the log of the spore concentration (Table 3). We did not observe a statistically significant difference in thresholds among species, and threshold estimates for older seedlings were similar to those observed for younger seedlings (Table 2). When the slopes of the linear regressions for the three species were compared, we observed a difference in the rates of increase in mean-adjusted lesion area with increased spores across the species (Table 3). *Q. rubra* and *Q. prinus* showed significantly higher rates of increase in infected lesion area ($P < 0.0001$ and $P = 0.0011$, respectively) compared with *A. rubrum*.

We also estimated the number of sporangia/ml required to attain the median-adjusted lesion area (Table 4). This value is roughly equivalent to the LD50 values calculated in studies of foliar disease levels (Tooley et al. 2013), but in the present analyses, percentage disease was not assessed and so the calculation was based on median lesion areas. Among the younger trees, the value observed for *A. rubrum* was significantly higher than those obtained for *Q. rubra* or *Q. prinus* (Table 4). In older trees, values for *Q. rubra* and *Q. prinus* were significantly higher than those

Table 2 Threshold estimates of number of sporangia/ml required for the lowest observable amount of disease, and 95% confidence intervals (CI) for three forest species stem-inoculated with *Phytophthora ramorum*

Species	Estimate	Lower 95% CI	Upper 95% CI
2- to 3-year-old seedlings			
Quercus rubra	0.0790	−0.0969	1.3394
Quercus prinus	0.0089	−0.0987	0.8963
Acer rubrum	0.0476	−0.0854	0.5726
5- to 6-year-old seedlings			
Q. rubra	0.0470	−0.0802	0.4917
Quercus prinus	0.0608	−0.0946	1.0231

Table 4 Estimates of number of sporangia/ml required for attaining the median-adjusted lesion area of disease, and 95% confidence intervals (CI) for three forest species stem-inoculated with *Phytophthora ramorum*[a]

Species	Estimate	Lower 95% CI	Upper 95% CI
2- to 3-year-old seedlings			
Quercus rubra	0.6631	−0.0718	4.5583
Quercus prinus	0.5084	−0.0812	3.8398
Acer rubrum	692.40	251.29	2518.36
5- to 6-year-old seedlings			
Q. rubra	373.43	157.61	989.88
Quercus prinus	15.38	2.59	57.65

[a]Transformation log(spores+0.1) and the median-adjusted area of all trees 1.00909 for young seedlings and 0.6125 for old seedlings.

Table 3 Regression estimates for three forest species (2- to 3-year-old seedlings) of intercepts and slopes that measure the rate of change in mean log lesion area with increased *Phytophthora ramorum* sporangia concentration

Parameter	Species	Estimate	Standard error	*t* value[a]	Pr > |t|
Intercept	*Quercus prinus*	1.3005	0.5904	2.20	0.0284
Intercept	*Acer rubrum*	0.2284	0.5901	0.39	0.6990
Intercept	*Quercus rubra*	1.1973	0.5905	2.03	0.0435
Slope	*Quercus prinus*	0.5864	0.1002	5.85	<0.0001
Slope	*A. rubrum*	0.1194	0.1001	1.19	0.2338
Slope	*Q. rubra*	0.6960	0.1012	6.88	<0.0001

Pairwise slope comparisons	Estimate	Standard error	*t* value	Pr > |t|
Quercus rubra minus *Quercus prinus*	0.1096	0.1424	0.77	0.4422
Q. rubra minus *Acer rubrum*	0.5766	0.1423	4.05	<0.0001
Quercus prinus minus *A. rubrum*	0.4670	0.1416	3.30	0.0011

[a]There were 310 degrees of freedom for all comparisons.

for the younger trees, and *Q. rubra* showed a value significantly higher than that obtained for *Q. prinus.*

The mean number of spores per 0.5 cm^2 following deposition onto membrane filters utilizing the acetate template was 0, 1.6, 4.3, 9.7, 18.4, 28.8 and 54.3 for 0, 100, 250, 500, 1000, 2000 and 3000 sporangia/ml, respectively [minimum significant different by Tukey's studentized range (HSD) test = 5.53].

Discussion

In this study, we quantified the relationship between inoculum density and infection of stems of three Eastern US forest species by *P. ramorum*. Infection was only observed to occur on stems that were wounded with sandpaper prior to inoculation with *P. ramorum*. We also estimated the number of sporangia/ml required to attain the median-adjusted lesion area. This value is roughly equivalent to the LD50 values calculated in earlier studies based on foliar inoculation (Tooley et al. 2013), but in the present analyses, percentage disease on a per-plant basis was not assessed, and thus, the calculation was based on lesion areas. Threshold estimates were comparable to those previously observed for whole plants and detached leaves of these same host species (Tooley et al. 2013, Table 2). We found that threshold estimates for the median amount of observed disease in *Q. rubra* and *Q. prinus* were substantially higher for 5- to 6-year-old seedlings compared with 2- to 3-year-old seedlings. It is thus possible that resistance to *P. ramorum* is more pronounced in older seedlings and may increase further with increased tree age.

In terms of the number of sporangia/ml required to attain the median-adjusted lesion area, we found that for younger seedlings, the value observed for *A. rubrum* was significantly higher than those obtained for *Q. rubra* or *Q. prinus,* which indicates a higher level of resistance to *P. ramorum* within *A. rubrum*. This observation is consistent with ratings of *A. rubrum* for resistance in earlier studies (Tooley and Browning 2009). In older trees, values for *Q. rubra* and *Q. prinus* were significantly higher than those for the younger trees, and *Q. rubra* showed a value significantly higher than that obtained for *Q. prinus*, indicating that perhaps resistance increases with age for *Q. rubra*.

Estimates of sporangia numbers actually applied to the 0.5 cm^2 of stem area at each inoculum concentration based on applying spores to a similar area of polyvinylidene fluoride membrane ranged from ca. 2 to 54 at initial concentrations of 100 to 3000 sporangia/ml. At 100 sporangia/ml, percentage disease ranged from 33 to 78% for the three species. As some sporangia painted onto the membranes could have failed to transfer to the agar prior to counting, the accuracy of such counts in representing the true number of sporangia deposited on the 0.5 cm^2 of stem tissue is unknown. Nonetheless, the results indicate that very few sporangia may be required to produce substantial amounts of disease on wounded stem tissue of these three forest species.

Hansen et al. (2005) used stem inoculation to assess reactions of Oregon forest trees and shrubs to *P. ramorum*. Inoculations were performed on potted plants with a cut made through to the cambium and colonized agar placed in the wound for 3 weeks. These workers found that stem-wound inoculation correlated with field symptoms for several hosts and that significantly larger lesions were noted on dieback and SOD hosts. Many additional studies demonstrate the effects of wounding of various host species on infection by *Phytophthora* species. Bostock and Doster (1985) found that *P. syringae* cankers on almond trees were associated with pruning wounds. Wounding also increased the susceptibility of pickling cucumbers to *P. capsici* (Granke and Hausbeck 2010), and *Eucalyptus marginata* to *P. cinnamomi* (O'Gara et al. 1996). The effect of injury of trees was also noted for *P. cactorum* and *P. citrophthora* on peach trees by Thomidis (2003). Root trimming enhanced infection of pepper roots by *P. capsici* (Adorada et al. 2000; Alao et al. 2012). Wound repair was also observed to occur, in that plants on which wounds were aged prior to inoculation showed less disease from *P. capsici* compared with freshly wound-inoculated plants (Adorada et al. 2000). Our results show that injury was required to obtain infection of seedling stems by *P. ramorum*. This suggests that in nature, injured host plants may require extra protection against *P. ramorum*. Trees that have sustained some type of injury, such as weather related, animal or insect damage, would be more likely to become infected by *P. ramorum*. The relationship between certain types of beetles and *P. ramorum* infection in California forests has been explored (McPherson et al. 2008), and the role of insects in forest ecology may be an important factor in terms of numbers of trees that will become infected by *P. ramorum*. In addition, our results suggest that homeowners who have susceptible species on their property should treat observable injuries with creosote or other means to prevent entry by *P. ramorum*.

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