

Responses of swamp bay, *Persea palustris*, and avocado, *Persea americana*, to various concentrations of the laurel wilt pathogen, *Raffaelea lauricola*

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Summary

Laurel wilt, caused by the fungus *Raffaelea lauricola* and transmitted by the exotic ambrosia beetle *Xyleborus glabratus*, has killed members of the Lauraceae plant family throughout the southeast United States. A series of experiments were conducted to examine the effects of inoculum concentration on the development of laurel wilt in swamp bay, *Persea palustris*, and avocado, *Persea americana*. In each experiment, host plants were inoculated with aqueous suspensions of 10^2 , 10^3 , 10^4 or 10^5 conidia of *R. lauricola*, and plants were rated periodically for external symptom development (wilting and foliar dieback). At the end of experiments, plants were rated for internal symptoms of the disease (discoloration of sapwood) and assayed for *R. lauricola* on a semi-selective medium. Symptom severity in swamp bay was significantly lower for the 10^2 treatment than at higher (10^3 – 10^5) concentrations, whereas 10^2 and 10^3 conidia caused less disease than 10^4 and 10^5 conidia in avocado. At the lowest inoculum concentration, 67% of the swamp bay plants and 20% of the avocados died by the time the respective experiments were terminated. The pathogen was recovered from a high proportion of the symptomatic sapwood of swamp bay (100%) and avocado (94%), and sapwood discoloration and recovery of *R. lauricola* from inoculated stems of swamp bay were highly correlated with recovery of the pathogen and symptom development in roots. Clearly, swamp bay and avocado are very sensitive to *R. lauricola*. The ability of only 100 conidia of this pathogen to kill these hosts suggests that few individuals of *X. glabratus* or other ambrosia beetles that carry low levels of the pathogen would be sufficient to transmit conidia that infect and lead to disease development. The results are also relevant to the development of disease-tolerant host selections, as they indicate levels of the pathogen appropriate for use in screening plants for disease resistance.

1 Introduction

Laurel wilt, caused by the asexual fungal symbiont (*Raffaelea lauricola* T.C. Harr, Fraedrich & Aghayeva) of the exotic red-bay ambrosia beetle (*Xyleborus glabratus* Eichhoff), has ravaged members of the Lauraceae in the south-eastern United States (Fraedrich et al. 2008; Harrington et al. 2008). *Raffaelea lauricola* is presumed to have accompanied *X. glabratus* when the vector arrived in the USA in 2002 (Ploetz et al. 2013). Fraedrich et al. (2008) reported that *X. glabratus* visited, but did not establish a brood in healthy trees in the USA. However, during these initial boring attempts by adult *X. glabratus* females, in which spores are released from the beetles' mandibular mycangia (spore-bearing structure), trees are likely inoculated with *R. lauricola*. Susceptible species then develop laurel wilt, decline and became more attractive to *X. glabratus* and other ambrosia beetles which then propagate in these trees.

Ambrosia beetles typically reproduce in stressed and dead trees, and they are usually not considered to be tree-damaging insects (Batra 1967). Their preference for weakened or dying trees has led to a general assumption that ambrosia beetles do not interact with healthy trees; consequently, the interaction of *X. glabratus* with healthy trees in the USA has been viewed as atypical behaviour (Hulcr and Dunn 2011). The extent to which the interaction of *X. glabratus* with healthy trees is unusual or a function of new associations of an exotic vector and pathogen with naive host trees requires further study (Ploetz et al. 2013; Carrillo et al. 2014).

Recently, eight ambrosia beetle species in the USA other than *X. glabratus* were shown to carry *R. lauricola*, albeit at lower levels than in *X. glabratus* (ca. 1/100th to 1/1000th the amounts detected in *X. glabratus*) (A. Campbell, unpublished data, Carrillo et al. 2014). As the other species were present in the USA prior to the introduction of *X. glabratus*, their associations with *R. lauricola* appear to be recent developments (after 2002) (Ploetz et al. 2013). Inoculations with *R. lauricola* via no-choice tests with *X. glabratus* have shown that mortality of avocado can occur with as few as five females of the species, with a single female necessary on redbay (Fraedrich et al. 2008; Mayfield et al. 2008a). For other ambrosia beetle species that carry lower levels of *R. lauricola*, higher numbers may be needed to induce laurel wilt (40 beetles were used per plant in Carrillo et al. 2014).

Six species of *Raffaelea* have been isolated from mycangia of *X. glabratus*, and as many as four species have been found in an individual of this species (Harrington et al. 2010). However, in several different examinations of *X. glabratus* collected from the south-eastern USA, Japan and Taiwan, *R. lauricola* has been the most common fungus in this beetle's mycangia (A. Campbell, personal communication, Harrington and Fraedrich 2010; Harrington et al. 2011; Carrillo et al. 2014). Although *R. lauricola* was not recovered from a small subset of the individuals of *X. glabratus* that were assayed from the USA and Asia, most contained hundreds to thousands of colony-forming units (CFUs) of

the pathogen (A. Campbell, personal communication, Harrington and Fraedrich 2010; Harrington et al. 2011; Carrillo et al. 2014).

Prior work indicated that single-point inoculations of susceptible tree species with either pathogen mycelia or conidia led to laurel wilt development (Fraedrich et al. 2008; Mayfield et al. 2008b; Ploetz et al. 2012; Hughes et al. 2013). Other aspects of the pathogen \times vector \times host tree interactions are poorly understood. For example, the amount of inoculum that *X. glabratus* delivers into trees is not known, as is the dosage of inoculum that is needed to incite wilt. As some individuals of *X. glabratus* carry no, or low levels of *R. lauricola*, it would also be useful to know whether trees that are infested with relatively few propagules of the pathogen could survive and/or recover from infection. Finally, research programmes aimed at identifying laurel wilt tolerance would benefit from information on how inoculum concentration affects the extent and timing of disease development (Hughes 2013).

These studies were conducted to determine the effects of inoculum concentration on the development of laurel wilt. Specifically, the objectives were to (1) assess if laurel wilt symptom development (internal and external) is dependent on conidia concentration in swamp bay and avocado, (2) determine the lower thresholds of *R. lauricola* needed to incite disease in these hosts, and (3) determine whether *R. lauricola* can move into the root system of swamp bay.

2 Materials and methods

2.1 Plant material

Plants of swamp bay were purchased from a commercial nursery in northern Florida and of avocado cultivar 'Simmonds' from a nursery in south Florida. Swamp bay plants were grown from seed in 12.5-l containers, had a single dominant stem 1.5 cm in diameter at 10 cm above the soil line and were 1.5 m in height. Avocado plants were 'Simmonds' clonal scions grafted onto 'Waldin' rootstocks in 12.5-l containers, with a single dominant stem 1 m in height and 3 cm in diameter above the graft union. No indications of disease or beetle infestation were evident in any of these plants prior to their use in an experiment.

2.2 Experimental locations

Swamp bay experiments were conducted at the University of Florida in Gainesville. Due to space limitations, experiments 1 and 2 were split into two locations. Experiment 1 was conducted in an outdoor nursery under full sun conditions (mean temp = $19 \pm 3^\circ\text{C}$), and Experiment 2 was in a evaporative cooler pad and fan system, plastic-roofed greenhouse with supplemental lighting (16:8 diurnal light), set to 21°C day/ 18°C night. Due to superior growing conditions and plant availability, avocado experiments were conducted in Miami, FL. Two consecutive avocado experiments were conducted in a glasshouse without supplemental lighting and maintained at $25 \pm 2^\circ\text{C}$ at the University of Florida's Tropical Research and Education Center in Homestead FL.

2.3 Inoculum preparation

Isolates of *R. lauricola* that had been used in previous swamp bay and avocado experiments, respectively, were used in all studies. Isolate PL571 (GenBank JQ861956.1), isolated from an infected redbay and grown on cycloheximide-streptomycin malt agar (CSMA) (Harrington 1981), was used to inoculate swamp bay, and isolate RL4 (CBS 127349, GenBank HM446155), isolated from an infected avocado and grown on malt extract agar, was used to inoculate avocado. Conidia from 7- to 14-day-old cultures were harvested with sterile water and quantified with a haemocytometer. Serial dilutions were used to obtain concentrations of 1×10^5 , 1×10^4 , 1×10^3 and 1×10^2 conidia ml^{-1} , and inoculum viability for each spore suspension was assessed on CSMA. After 7 days on a laboratory bench, colonies of *R. lauricola* were counted to calculate the numbers of CFUs that were present in each dilution.

2.4 Host inoculations

A single 2-mm-diameter hole was drilled into the stem (15 mm deep) of each avocado at a 45° angle at 15 cm above the graft union, and a conidial suspension was placed into the wound by pipette (100 μl per plant at the treatment concentration). Due to the swamp bays' small stem diameter, inoculum volume was lowered and placed into two wounds. Drill wounds (7.5 mm deep) were placed 15 and 30 cm above the soil line (on opposite sides of stem), and the conidial suspension was added to the wounds by pipette, 25 μl per hole (50 μl total per plant) at the desired concentration. For both experiments/hosts, water-inoculated plants served as controls, and wounds were then wrapped in plastic laboratory film after inoculation. Treatments were replicated five (avocado) or six (swamp bay) times and completely randomized. Interval scales were used to rate external disease progress of avocado (0 = asymptomatic, 1 = 1–10%, 2 = 11–20%, 3 = 21–30%; ... 9 = 80–90% and 10 = 90–100% of the canopy with wilt or foliar dieback) on days 0, 7, 14, 20, 24, 28, 32 and 36. Due to swamp bays' smaller canopy structure, a reduced rating scale was used (0 = asymptomatic, 1 = 1–25%, 2 = 26–50%, 3 = 51–75% and 4 = 76–100% of the canopy with wilt) and was rated every week after inoculation for 140 days. Internal symptom development for both hosts was determined after an experiment was terminated or when a plant died; after bark was removed, discoloration of the entire above-ground vascular cylinder was assessed with the same scale per host; for example, 0–10 for avocado and 0–4 for swamp bay.

2.5 Pathogen recovery

At the conclusion of the experiments, plants were assessed for colonization by *R. lauricola* according to Ploetz et al. (2012). For swamp bay, a stem segment was removed at 50 cm above the soil line, and a section of two major roots (average depth below soil line = 7.5 cm) were randomly selected and removed per tree. Six pieces of vascular stem tissue (5 mm²) and six pieces of root tissue (5 mm²) were surface sterilized and placed on two plates each of the CSMA+ medium (Hughes et al. 2012; Ploetz et al. 2012). For avocado, six stem pieces of stem (5 mm²) from 20, 40 and 60 cm above the inoculation point were assayed for the presence of *R. lauricola* as above (Ploetz et al. 2012). Recovery of the pathogen from roots of artificially inoculated avocado trees is not detailed here, but typically occurs within 2–3 weeks (R. C. Ploetz, unpublished data). For each experiment, the number of plates on which *R. lauricola* was recovered was calculated for each treatment and replication.

2.6 Statistical analyses

For statistical analyses, disease severities were converted to proportions based on the mid-point of the scale ranges (i.e. 80–90% = 0.85). To determine the accuracy of treatment concentrations (agreement between conidium numbers and their viability) as well as treatment uniformity among all experiments (within and among both hosts), analyses of variance (ANOVA) were with SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). The area under the disease progress curve (AUDPC) using the mid-point rule method was calculated with Microsoft Excel (2010), according to Campbell and Madden (1990), as $\sum_{i=1}^{n-1} [(t_{i+1} - t_i)(y_i + y_{i+1})/2]$, where: t = time in days, y = proportion of symptomatic canopy (disease severity) and n = the number of observations. The SAS NLIN procedure was used to fit the data to various nonlinear models (Campbell and Madden 1990). The Gompertz model, which was selected based on superior goodness of fit, residual distributions and regression coefficient of determination (R^2) for the linear relation between observed and predicted data, was used to estimate the rate (r), asymptote (maximum mean disease severity) (K) and severity at time 0 (Y_0) for disease progress over time (Park and Lim 1985). A monomolecular model, where $y = A*(1-B*EXP[-CX])$, was selected based on goodness of fit, residuals and R^2 to estimate the effects of inoculum concentration and experiment on the rate of disease progress (Park and Lim 1985). ANOVA was performed for the parameter estimates using the GLM procedure followed by a multiple comparison of means according to Fisher's least-squared difference test (LSD) at $p = 0.05$. Fisher's exact test for the fungal isolation experiments was conducted using the FREQ procedure. Standard error (SE) was calculated as $[\text{std dev}/\sqrt{n}]$, where n = number of replications.

3 Results

3.1 Inoculum preparation and quantification

For experiments with both hosts, conidium viability was high (mean = 96%) and the numbers of CFUs on CSMA were similar to the concentration of conidia for the inoculum level used (no significant difference in the ANOVA test, $p = 0.94$). Results of pair-wise comparisons were also similar for CFUs among treatment concentrations for experiments with both hosts (no significant differences in ANOVA tests, $p = 0.17$ – 0.96).

3.2 Swamp bay

Statistical analysis revealed that the effects of inoculum concentration, experimental location and their interaction were significant to disease progress (Table 1). The interaction (inoculum concentration \times experimental location) was due to poor disease development in the first experiment (outdoor nursery) at 10² conidia treatment (Tables 1 and 2). With the exception of the rate of disease progression, where no interactions were found (Table 1), data are presented separately for both experiments. By 35 days after inoculation (DAI), all *R. lauricola* treatments had at least one plant with wilt symptoms (Fig. 1). Water controls remained healthy throughout the experiment (data not shown).

Table 1. Statistical significance values (p) for experiment, inoculum concentration and their interaction on laurel wilt disease parameters for swamp bay and avocado.

Host	Source of variation	d.f.	Internal severity	External severity	Rate	AUDPC
Swamp bay	Experiment (E)	1	0.0115	0.0006	0.001	0.0001
	Inoc. Conc. (C)	3	0.0001	0.0001	0.001	0.0001
	E \times C	3	0.0024	0.0001	0.060	0.0001
Avocado	Experiment (E)	1	0.334	0.173	0.337	0.532
	Inoc. Conc. (C)	3	0.001	0.001	0.375	0.001
	E \times C	3	0.870	0.992	0.569	0.373

d.f., degrees of freedom; swamp bay $n = 48$, avocado $n = 40$.

Table 2. Summary of disease development parameters for experiments 1 and 2 on swamp bay in study of disease progress over time following inoculation with *R. lauricola*¹.

	Inoculum concentration ²	Internal severity ³	External severity ⁴	AUDPC ⁵	Mortality (n = 6) ⁶	Days until death ⁷
Experiment 1	10 ²	0.30 ± 0.19 A	0.26 ± 0.16 A	21.84 ± 14.02 A	2	42, end
	10 ³	0.84 ± 0.04 B	0.91 ± 0.03 B	82.19 ± 11.24 B	6	42, 126
	10 ⁴	0.84 ± 0.04 B	0.87 ± 0.02 B	97.08 ± 3.91 B	6	35, 119
	10 ⁵	0.89 ± 0.01 B	0.89 ± 0.002 B	99.71 ± 1.37 B	6	28, 56
Experiment 2	10 ²	0.80 ± 0.05 B	0.89 ± 0.003 B	90.44 ± 5.97 B	6	28, 91
	10 ³	0.84 ± 0.04 B	0.88 ± 0.002 B	99.29 ± 1.51 B	6	28, 49
	10 ⁴	0.89 ± 0.01 B	0.89 ± 0.003 B	101.06 ± 1.58 B	6	28, 49
	10 ⁵	0.89 ± 0.01 B	0.89 ± 0.002 B	99.73 ± 0.87 B	6	28, 42

¹Potted swamp bay plants were artificially inoculated with various concentrations (conidia per plant) of *R. lauricola*.

²Inoculum concentration = number of conidia inoculated into each plant.

³Internal severity = proportion of xylem tissue with vascular discoloration.

⁴external severity = estimated asymptote of disease progress curves.

⁵AUDPC = area under the disease progress curve.

Values denote means ± standard errors.

⁶Mortality = number of plants that died of a total of six replicates in each experiment.

⁷Days until death = time for the first replicate to die, followed by the time until all replicates died; 'end' indicates that plants survived until the end of the experiment.

Means in columns followed by different letters are significantly different ($p = 0.05$), according to Fisher's LSD.

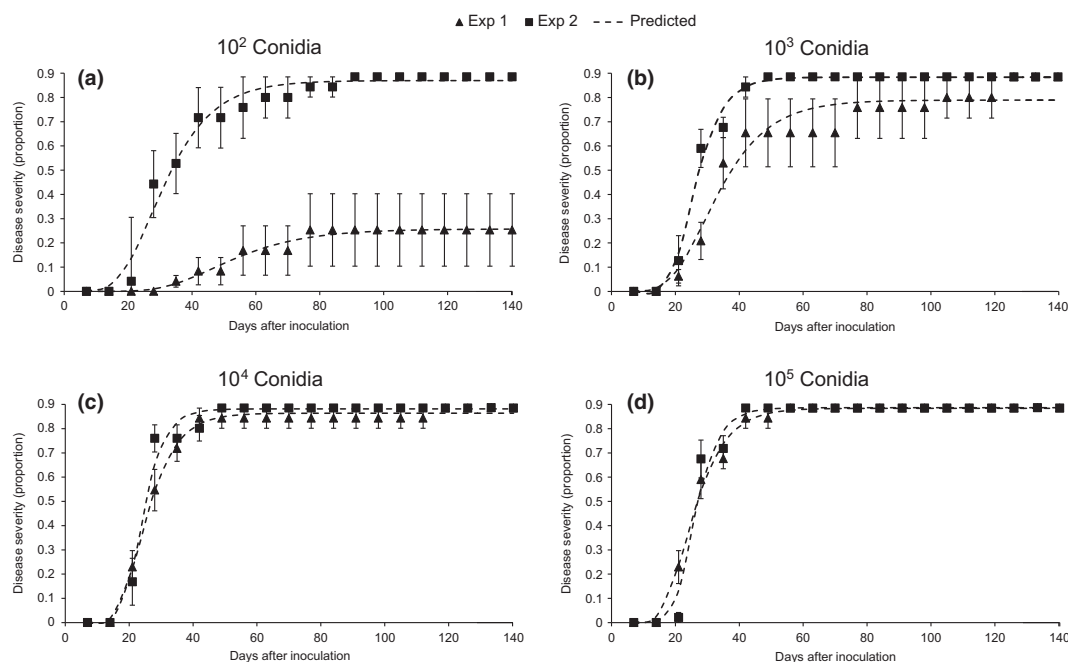


Fig. 1. Mean severity of laurel wilt as a proportion of the canopy with symptoms (\pm standard errors) over time for swamp bay. Curves are for observed data and predicted (Gompertz models, see Table 2) disease progress. *Raffaelea lauricola* inoculation concentration: a) 10² conidia, b) 10³ conidia, c) 10⁴ conidia, d) 10⁵ conidia.

In Experiment 1, only two of six plants developed wilt symptoms at 10² conidia per plant (Fig. 1a, Table 2). Consequently, disease progress data for this experiment were poorly fit to the Gompertz prediction model ($R^2 = 0.10$) and had a higher standard error (Fig. 1a compared with the second experiment ($R^2 = 0.78$)) (Fig. 1a, Table 3). In addition, the number of days to plant death was greater in this experiment with some plants dying after 140 DAI (Table 2). In contrast, disease severity in Experiment 2 increased rapidly from day 21 to day 77 in the 10² treatment, with mortality beginning at day 28 and progressing to 100% by day 91 (Fig. 1a, Table 2). Disease progress curves at 10³ conidia per plant were similar in both experiments, although all plants died sooner in Experiment 2 than Experiment 1 (49 vs. 126 DAI) (Fig. 1b, Table 2), and there was a better fit between observed and predicted disease severity in Experiment 2 ($R^2 = 0.94$) than in Experiment 1 ($R^2 = 0.59$) (Fig. 1b, Table 3). At higher inoculum concentrations, disease development and host mortality were

Table 3. Results of nonlinear regression analyses of laurel wilt disease severity on swamp bay over time following inoculation with varying levels of *Raffaelea lauricola* conidia¹.

Conidia/plant	Regression equation for disease severity over time ²	³ R ²
Experiment 1		
10 ²	$Y = 0.2575*(1.27E-9/0.2575)^{EXP(-0.444X)}$	0.10
10 ³	$Y = 0.7894*(3.33E-7/0.7894)^{EXP(-0.432X)}$	0.59
10 ⁴	$Y = 0.8639*(3.01E-15/0.8639)^{EXP(-1.0685X)}$	0.90
10 ⁵	$Y = 0.8841*(5.58E-12/0.8841)^{EXP(-0.9921X)}$	0.90
Experiment 2		
10 ²	$Y = 0.8697*(8.551E-7/0.8697)^{EXP(-0.6713X)}$	0.78
10 ³	$Y = 0.8839*(9.01E-28/0.8839)^{EXP(-1.1956X)}$	0.94
10 ⁴	$Y = 0.8817*(9.4E-55/0.8817)^{EXP(-1.5053X)}$	0.94
10 ⁵	$Y = 0.8867*(7.47E-55/0.8867)^{EXP(-1.3937X)}$	0.96

¹Potted swamp bay plants were artificially inoculated with different concentrations of conidia of *R. lauricola*.
²A Gompertz model was used ($y = K*(Y_0/K)^{EXP(-RX)}$), wherein Y = disease severity, K = asymptote, Y_0 = disease severity at time 0, R = rate of disease progression and X = time.
³R² = coefficient of determination of the predicted vs. observed data regression line.

more consistent. In Experiment 1 and Experiment 2, Gompertz curves were similar for 10⁴ (R² for experiments 1 and 2 = 0.90, 0.94) and 10⁵ conidia per plant (R² for experiments 1 and 2 = 0.90, 0.96), as determined by the high coefficients of determination obtained between observed and predicted disease severities (Fig. 1c,d, Table 3), as were the times that elapsed until inoculated plants died. Most plants died after 42 days in Experiment 1, and all plants died between 42 and 49 days in Experiment 2 (Fig. 1c,d, Table 2).

With the exception of the 10² treatment in Experiment 1, internal disease severities (0.30 for 10² vs. 0.80–0.89 for 10³ to 10⁵ of the xylem discoloration), external disease severities (0.26 for 10² vs. 0.87–0.91 for 10³ to 10⁵ of the canopy with symptoms) and AUDPCs (22 for 10² vs. 82–10 for 10³ to 10⁵) were statistically similar among treatments and experiments (Fig. 1, Tables 1 and 2). Among treatment classes, internal and external disease severities were similar ($p = 0.59$). Inoculum concentration and experiment both affected the rate of disease progress, and no interactions were detected (Table 1). When rate of disease progress over time was plotted against inoculum concentration (Log₁₀) and fitted to a monomolecular growth curve, mean rates were significantly higher for Experiment 2 than Experiment 1 (1.27 vs. 0.78) (Fig. 2a). Combined disease progressions for the 10³, 10⁴ and 10⁵ inoculum treatments were similar with rates of 1.07, 1.27 and 1.21, respectively, but the combined mean rate of the 10² treatment (0.61) was significantly lower than at the 10³–10⁵ conidia levels (Fig. 2b).

Isolation of *R. lauricola* from swamp bay stems was correlated with inoculum concentration ($n = 48$, $p = 0.001$); however, this relationship was due to the inability to isolate the pathogen from asymptomatic plants at the 10² concentration and its consistent isolation at higher concentrations (data not shown). The pathogen was recovered from the stem sapwood of all symptomatic plants, regardless of treatment concentration, but never from those without symptoms (data not shown). Fisher's exact test was used to examine associations between pathogen isolations from stem and root tissue, and from discoloured and non-discoloured root tissue. When *R. lauricola* was not isolated from stem tissue, it was not found in the root tissue (0 of 4 attempts), but when it was recovered from stem sapwood, it was isolated from 77% (34 of 44 attempts) of the corresponding root samples ($n = 48$, $p = 0.005$, data not shown). For the root vascular tissue, *R. lauricola* was isolated from 35% (6 of 17 attempts) of the non-discoloured samples, but from 90% (28 of 31) of the discoloured samples ($n = 96$, $p = 0.0001$, data not shown).

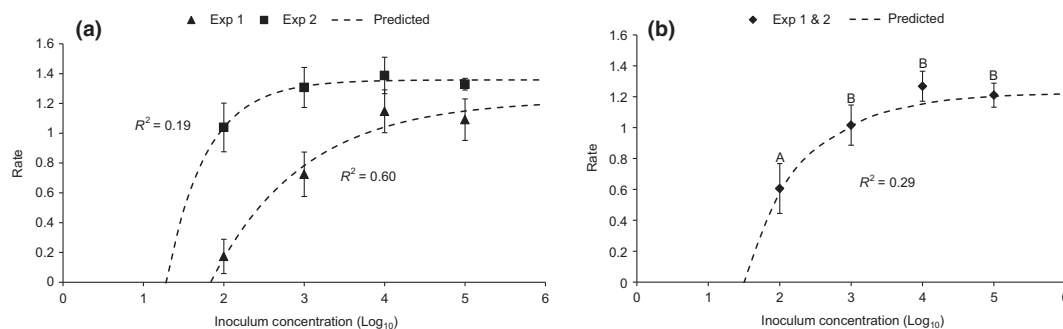


Fig. 2. Rate of laurel wilt disease progress over time following inoculation of swamp bay with *R. lauricola*. (a) The effect of the different experiments was fit to monomolecular curves. Mean rates were 0.78 for Experiment 1 and 1.27 for Experiment 2 ($p = 0.001$). (b) Combined effect of inoculum concentration (Log₁₀), fit to a monomolecular growth curve. Mean slope values for inoculum concentrations were 10² = 0.61, 10³ = 1.07, 10⁴ = 1.27 and 10⁵ = 1.21 ($p = 0.001$). Values/curves denoted by different letters are significantly different ($p = 0.05$). R² = coefficient of determination of the predicted vs. observed data regression line.

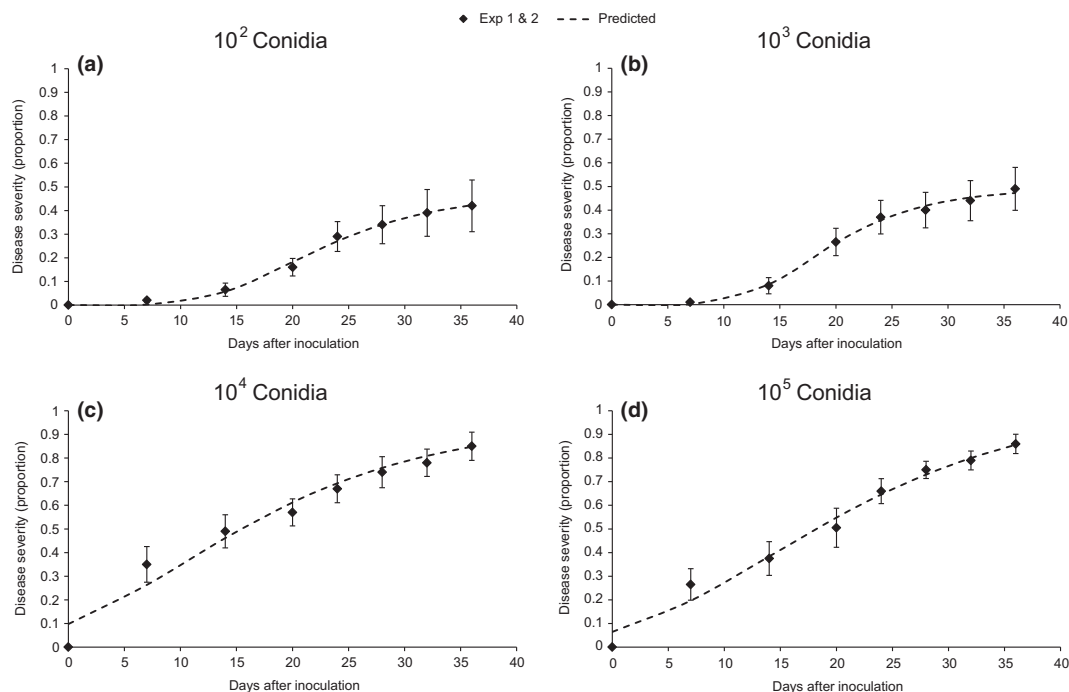


Fig. 3. Mean severity of laurel wilt as a proportion of the canopy with symptoms (\pm standard errors) over time for avocado. Curves are for observed data and predicted (Gompertz models, see Table 4) disease progress. *Raffaelea lauricola* inoculation concentration: a) 10^2 conidia, b) 10^3 conidia, c) 10^4 conidia, d) 10^5 conidia.

3.3 Avocado

Differences were not detected between disease severity data and disease progress curves in the two experiments, and no interactions between experiments and inoculum levels were found for any of the disease parameters (Table 1). Therefore, data sets for the experiments were combined.

By 7 DAI, some plants in the 10^4 and 10^5 treatments had begun to develop symptoms, and by 14 DAI, some plants in all inoculated treatments had symptoms (Fig. 3). Internal and external disease severities were similar when compared within treatments ($p = 0.095$) (Table 4). Based on internal and external disease severities and AUDPCs, significantly less disease developed in the 10^2 - and 10^3 -treated plants than the 10^4 - and 10^5 -treated ones. However, the rates of disease development were similar at all concentrations (Fig. 3, Table 4). There were no differences in plant mortality among the inoculated treatments of each experiment ($p = 0.48$) (Table 4). Only two, two, five and four of ten total plants died, at the respective

Table 4. Summary of disease development parameters for experiments 1 and 2 (combined) on avocado in study of disease progress over time following inoculation with *R. lauricola*¹.

Inoculum concentration ²	Internal severity ³	External severity ⁴	Rate ⁵	AUDPC ⁶	Mortality (n = 10) ⁷	Days until death ⁸
10^2	0.41 \pm 0.11 A	0.46 \pm 0.12 A	0.12 \pm 0.03 A	6.28 \pm 1.39 A	2	36, end
10^3	0.48 \pm 0.10 A	0.53 \pm 0.10 A	0.18 \pm 0.02 A	7.74 \pm 1.54 A	2	32, end
10^4	0.82 \pm 0.05 B	1.04 \pm 0.14 B	0.19 \pm 0.05 A	19.25 \pm 2.09 B	5	24, end
10^5	0.83 \pm 0.04 B	0.96 \pm 0.07 B	0.13 \pm 0.02 A	17.36 \pm 1.71 B	4	20, end

¹Potted avocado plants were artificially inoculated with various inoculum concentrations (conidia per plant) of *R. lauricola*.

²Inoculum concentration = number of conidia inoculated into each plant.

³Internal severity = proportion of xylem tissue with vascular discoloration.

⁴External severity = estimated asymptote of disease progress curves.

⁵Rate = Gompertz model *R* value from the NLIN procedure.

⁶AUDPC = area under the disease progress curve.

Values denote means \pm standard errors.

⁷Mortality = number of plants that died of 10 replicates.

⁸Days until death = time for the first replicate to die; 'end' indicates that plants in each treatment survived until the end of the experiment.

Means in columns followed by different letters are significantly different ($p = 0.05$), according to Fisher's LSD.

Table 5. Nonlinear regression analyses of laurel wilt disease severity over time following inoculation of avocado with varying concentration of *Raffaelea lauricola*^a.

Conidia/plant	Regression equation for external disease severity over time ²	³ R ²
10 ²	$Y = 0.4746*(6.28E-7/0.4746)^{EXP(-0.1325X)}$	0.39
10 ³	$Y = 0.4931*(4.49E-9/0.4931)^{EXP(-0.1686X)}$	0.50
10 ⁴	$Y = 0.9577*(0.0974/0.9577)^{EXP(-0.815X)}$	0.68
10 ⁵	$Y = 1.0478*(0.0643/1.0478)^{EXP(-0.073X)}$	0.73

¹Potted avocado plants were inoculated with different concentrations of conidia of *R. lauricola*.
²A Gompertz model was used ($y = K*(Y_0/K)^{EXP[-RX]}$), wherein Y = disease severity, K = asymptote, Y_0 = disease severity at time 0, R = rate of disease progression and X = time.
³R² = coefficient of determination of the predicted vs. observed data regression line.

10², 10³, 10⁴ and 10⁵ concentrations, and mortality began after 36, 32, 24 and 20 days (Table 4). R²s for linear regressions of the observed vs. predicted data from the Gompertz model increased as did inoculum concentration (Table 5). Water controls remained healthy throughout the experiment (data not shown).

Isolation of *R. lauricola* from stems was correlated with inoculum concentration, with lower isolation frequencies obtained for the 10²-treated plants than those receiving higher inoculum concentrations (n = 117, p = 0.0001, data not shown). The pathogen was isolated from 94% of the stained sapwood, and the height above the inoculation point (20, 40 and 60 cm) did not affect the isolation of *R. lauricola* in inoculated plants (n = 117, p = 0.84, data not shown). Disease did not develop in, and the pathogen was not recovered from, water-inoculated plants.

4 Discussion

The present results indicate that *R. lauricola* is highly virulent on swamp bay, with 10² conidia approaching the minimum number needed to cause disease in this host. Small-scale investigations found uniform rates of disease progression and external severities among *R. lauricola* isolates gathered from its invaded range, with genetic analysis revealing uniformity among isolates (Hughes 2013).

Although it is not clear why a higher rate of laurel wilt development was observed in the greenhouse (Experiment 2) vs. outdoor nursery (Experiment 1), differing environments in these experiments may be responsible. Study plants in Experiment 2 experienced higher temperatures and longer day lengths compared to those in Experiment 1. Differences in the development of Dutch elm disease were previously reported for studies comparing pathogen-inoculated plants in controlled vs. field conditions (Kais et al. 1962; Smalley and Guries 1993). Sutherland et al. (1997) reported that ambient temperature and hours of daylight were positively related to the defoliation of elms inoculated with *Ophiotoma novo-ulmi* Brasier. Green et al. (1985) noted highly susceptible elm clones had different internal responses (xylem discoloration) to aggressive (now *O. novo-ulmi*) and non-aggressive (*O. ulmi* [Buism] Nannf.) strains depending on temperature, with the aggressive strain yielding more internal symptoms at low temperatures and the non-aggressive strain preferring a higher temperature regime. Although interactions between environment and the development of these diseases are incompletely understood, temperature and day length may play important roles for both Dutch elm disease and laurel wilt.

Laurel wilt developed in avocado at the lower inoculum concentrations, with disease severity and mortality increasing with rising concentrations. Results from our inoculated potted plants showed these low-level inoculations can lead to a partial crown wilt or defoliated stems while other parts of the canopy seemed unaffected. However, casual observations have showed that large field trees usually develop severe symptoms, while Ploetz et al. (2012) found avocado stem diameter positively correlated with external disease severity, a phenomenon that suggests that age/size of the avocado may play a role in disease development. In addition, the development of mild-to-moderate symptoms caused by low-level inoculations may entice additional attacks by *X. glabratus* and the various other generalist ambrosia beetle species that seek stressed plants and utilize avocado for brood in south Florida, possibly resulting in further decline of the tree (Carrillo et al. 2012). Thus, inoculation concentrations that lead to immediate mortality may not be necessary in locations where other pests/pathogen can exploit the stress induced by laurel wilt. Nonetheless, more information is needed on the amounts of inoculum and numbers of infection points that are needed to kill, or cause this disease on, larger trees in the field.

The findings reported here relate to the level or concentration of conidia required to cause disease. Because as few as 100 conidia cause lethal laurel wilt, mass attack by the beetle(s) and high titres of the pathogen may not be necessary to kill tree species that are susceptible to this disease. This is unusual among diseases that have ambrosia beetle vectors (Ploetz et al. 2013). Knowledge of the amount of inoculum necessary for disease in other ambrosia beetle–fungal symbiont systems is scarce because disease development is more reliant upon vector mass attack than the single- or few-point inoculations as in laurel wilt. For example, for the oak wilt diseases in Japan and Korea (both transmitted by *Platypus* ambrosia beetles) the *Raffaelea* infections within the tree are restricted in their internal colonization and thus host mortality would not occur without mass attack (Kim et al. 2009; Takahashi et al. 2010).

The pathogen isolation experiments with swamp bay revealed the ability of *R. lauricola* to move downward into the root system of this host. Pathogen recovery from roots was strongly associated with its recovery from stems, as well as stem and root discoloration. Ploetz et al. (2012) found substantial basipetal movement of this pathogen in avocado, with additional isolations from infected roots capturing *R. lauricola* (R. C. Ploetz, personal communication). Transmission of the

Dutch elm disease pathogen and the US oak wilt pathogen (*Ceratocystis fagacearum* [Bretz] J. Hunt) occurs through functional root grafts as well as by insect vectors (Gibbs 2001). Although there is no empirical evidence for root graft transmission of *R. lauricola*, observations in sassafras and avocado provide strong anecdotal support for this type of spread (C. Bates and R.C. Ploetz, personal communications). In commercial avocado plantings in Florida, root grafting was demonstrated previously by the movement of glyphosate; when the removal of alternate trees in a row was attempted with this herbicide, entire rows of trees were killed (J.H. Crane, personal communication). Recently, healthy avocado trees adjacent to laurel wilt-affected trees were observed to succumb to the disease over a few months. The combination of evidence (*R. lauricola* has been previously isolated from roots of infected potted and field avocado trees + glyphosate movement via root grafts + directional mortality without evidence of ambrosia beetle attack) suggests that root graft transmission of *R. lauricola* is occurring in avocado (R. C. Ploetz, personal communication). Thus, management of root graft transmission of this pathogen has become a primary concern in affected avocado orchards, with further research needed to substantiate and possibly halt this method of movement. Although this study shows the advance of *R. lauricola* into the roots of swamp bay, attempts to locate root-grafted swamp bay and/or redbay in the wild have failed (D. Spence, personal communication). Our results indicate the successful downward movement of *R. lauricola* into the root system; however, without evidence of root grafting, this may be an unlikely transmission avenue for swamp bay or redbay.

Results from the present study suggest that relatively few conidia of *R. lauricola*, possibly transmitted by a single or few ambrosia beetles, would be needed to induce laurel wilt in a susceptible host. However, much more work is needed to fully understand how the vector(s) interact with healthy hosts and why *R. lauricola* causes such a devastating disease. Although numbers of *R. lauricola* CFUs that are found in mycangia have been established for various beetle species (A. Campbell, unpublished data, Harrington and Fraedrich 2010; Harrington et al. 2011; Carrillo et al. 2014), it is not known how these numbers relate to subsequent infection and disease development in susceptible hosts. Information is also needed on the avenue by which beetles introduce inoculum during their first interactions with a healthy host tree, whether the transfer of *R. lauricola* from mycangia to the host tree is active or passive, and whether the inoculum that initiates systemic infection comes directly from mycangia or whether substantial colonization in galleries is needed before the disease process begins. Moreover, it is not known to what extent environmental conditions and host vigour determine the amount of attack by ambrosia beetles, infection by *R. lauricola* and development of laurel wilt. The successful management of this disease will depend on substantially more information in these and other areas (Mayfield et al. 2009; Ploetz et al. 2011).

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