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Identification of the Achilles heels of the laurel wilt pathogen and its beetle vector

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Abstract
Ambrosia beetles harbor fungal symbionts that serve as food sources for larvae and adults. These beetles lay their eggs along tunnels in xylem sapwood, which is the substrate for fungal growth. Symbiotic fungi of the genus Raffaelea found in invasive and indigenous ambrosia beetles include the highly virulent plant pathogen Raffaelea lauricola affecting members of the Lauraceae family. R. lauricola is responsible for the deaths of > 500 million trees since 2005. Infection by as few as 100 spores can kill a healthy tree within months. Our data show that R. lauricola is cold-adapted with optimal growth between 16 and 26 °C, with little to no growth at temperatures ≥ 30 °C. The fungus is halophilic and shows a dramatic decrease in growth at pH ≥ 6.8. Fungicide resistance profiling revealed sensitivity of R. lauricola to prochloraz, dichlorofluanid, most conazoles, dithiocarbamates, and zineb (zinc fungicide), whereas the related species Raffaelea arxii showed more limited fungicide sensitivity. Entomopathogenic fungi potentially useful for beetle control were generally highly resistant to most fungicides tested. Coupling pH decreased the concentration for 95% inhibition of fungal growth (IC₉₅) of the most potent R. lauricola fungicides by 3–4-fold. Use of avocado bark plug insect bioassays revealed that commercially available Beauveria bassiana can be used as a biological control agent capable of effectively killing the beetle vectors. These data provide simple and practical recommendations to specifically target R. lauricola while having minimal effects on other symbiotic and entomopathogenic fungi, the latter of which can be used to manage the beetle vectors.

Keywords Laurel wilt pathogen · Fungicide · Environmental signal/stress response · Raffaelea lauricola · Entomopathogenic fungi

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Introduction

Many ambrosia beetles (Curculionidae: Scolytinae and Platypodinae) have co-evolved with a collection of specific symbiotic fungi that are carried to host trees in specialized insect structures termed mycangia (Gohli et al. 2017; Harrington 2005). During excavation of host trees by burrowing beetles, the fungi are seeded on the plant host, growing in the xylem sapwood and heartwood. Eggs are laid along tunnels in xylem galleries, and beetle larvae and adults graze on the fungi growing in the xylem (Harrington 2005). For ambrosia beetles, the fungi are the major if not the sole source of food and belong to the genera Ambrosiella and Raffaelea, which are closely related to Ceratocystis and Ophiostoma from which they likely evolved (Harrington et al. 2010). Although these fungi grow only on the xylem substrates of trees, many of the beetle fungal symbionts are not considered particularly virulent plant pathogens. An exception to this is the highly virulent Raffaelea lauricola, vectored by the redbay ambrosia beetle, Xyleborus glabratus, both of whom originated in Asia (Harrington et al. 2011) and were first detected in the USA around the turn of the twenty-first century (Harrington et al. 2011; Rabaglia et al. 2006). Since then, the vector and associated pathogen have spread throughout the southeastern USA, where more than 300 million trees of redbay (Persea borbonia) have been killed (Ploetz et al. 2017a). Aside from redbay, additional economically important susceptible Lauraceae members include swampbay, camphor, sassafras, spicebush, and avocado (Campbell et al. 2016; Fraedrich et al. 2015, 2016). R. lauricola has been isolated from other (indigenous) ambrosia beetles, likely as a result of acquisition of the fungus from infected trees (Carrillo et al. 2014; Ploetz et al. 2017b). In addition, soil and/or root transmission between trees may also help spread the disease-causing pathogen.

Histological examination of R. lauricola infection of avocado revealed tylose development and xylem blockage. External symptoms appear only after severe internal pathology that includes alterations in the rates of leaf gas exchange rates and xylem sap flow (Inch et al. 2012; Ploetz et al. 2012b, 2015). The fungus can be particularly virulent, with the inoculation of only 100 fungal conidia needed for killing the host, likely via the disruption of proper xylem functioning (Hughes et al. 2015; Inch and Ploetz 2012). Importantly however, systemic infection by the fungus does not appear to progress into the fruit pulp (mesocarp) or seed (endocarp) (Ploetz et al. 2012a). Despite the severity of the disease and its spread, little is known concerning basic physiological growth and stress resistance parameters of the fungus, even though such knowledge can have direct impacts on disease mitigation and management strategies. Several reports have examined the sensitivity of R. lauricola to a number of fungicides (Ploetz et al. 2017c). Redbay trees treated via root flare injections of propiconazole inhibited the development of crown wilt for at least 30 weeks after artificial inoculation with R. lauricola (Mayfield et al. 2008a). Twenty different fungicides were examined in vitro, with propiconazole found to be particularly effective in inhibiting fungal growth (Ploetz et al. 2011). Additional antifungal compounds including various demethylation inhibitors (DMIs), and quinone outside and inside inhibitors, also showed promise. In greenhouse studies, the effectiveness of some DMIs and thiobenzadole in controlling laurel wilt were demonstrated. Soil drench and topical branch or trunk applications of propiconazole and trimadimenol were also effective in suppressing the disease in small potted avocado trees. However, these application methods failed to move sufficient amounts of fungicide into the xylem of larger fruit-bearing trees in the field (Ploetz et al. 2017c). Microinjection and microinfusion of fungicides has been tested, but these data have not translated to viable control in the field (Ploetz et al. 2017c).

Here, we report on the systematic fungicide and stress profiling of R. lauricola revealing targets for laurel wilt disease mitigation and control. Our data show unique stress and growth optima for R. lauricola that are distinct from the beetle symbiotic and entomopathogenic fungi, including commercially available mycopesticides that contain Beauveria bassiana. We further show that B. bassiana can cause significant mortality of the Florida avocado beetle vector of R. lauricola, Xyleborus bispinatus when the entomopathogenic fungus is applied to the bark.

Materials and methods

Microbial strains and media

R. lauricola (CBS 127349, Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, The Netherlands) and Raffaelea arxii (CBS 139941) strains were kindly provided by R. Ploetz (UF-TREC, Homestead, FL). B. bassiana 0062 (CGMCC7.34, China General Microbiological Culture Collection Center) was obtained from the Biotechnology Research Center at Southwest University (Chongqing, China). B. bassiana 6660, B. bassiana 8710, B. bassiana GHA, Metarhizium anisopliae 511, Isaria farinosa 7075, and I. farinosa 11,833 were obtained from Dr. R. Humber (ARSEF Collection of Entomopathogenic Fungal Cultures, Ithaca, NY). Fungal strains were cultured on potato dextrose broth/agar (PDB/PDA), Czapek-dox broth/agar (CZB/CZA), and malt extract broth/agar (MEB/MEA) as needed. Spore concentrations were measured using a hemocytometer and adjusted as needed (typically to 10^5–10^8}
spores/ml). Fungicides were purchased from Sigma-Aldrich (St. Louis, MO, USA). Media and all other reagents unless otherwise noted were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

**Fungicide susceptibility assays**

Two different assays were used to determine the minimum inhibitory concentrations of the fungicides. In the first (diffusion disc assay), fungal spore suspensions (1 × 10^5/ml) were mixed into PDA when the temperature of the media was < 50 °C. The media containing the fungal spores was immediately poured into Petri dishes. After solidification, sterilized filter paper discs (5 mm diameter) were placed on the surface of the agar plates. Then, a series of dilutions of each fungicide (20 μl) was inoculated onto the filter paper discs, and agar plates were sealed with Parafilm and incubated at 26 °C for 7 days. Minimum inhibitory concentrations were calculated based on the zones of growth inhibition around the fungicide-impregnated discs. In the second assay, the IC₅₀ and IC₉₅ values of the fungicides were determined using the following protocol: 1 μl of 1 × 10⁸/ml spore suspension was inoculated onto the center of PDA or pH buffered PDA plates containing a concentration range (0.5–5000 ng/ml) of each respective fungicide. Plates were sealed and incubated at 26 °C for 12 d, after which the diameters of the fungal colonies on the plates were quantified. Inhibition parameters IC₅₀ and IC₉₅ were calculated using IBM SPSS 19 (IBM, 2010, Armonk, NY) Statistics. A preliminary experiment used to define the parameters of fungicide susceptibility (Supplemental Table S1) was performed using three technical replicates, and the entire experiment was repeated three times with independent batches of spore suspensions unless otherwise noted.

For propidium iodide (PI) staining, R. lauricola conidia were inoculated at a final concentration of 10⁷ spores/ml into PDB and PDB supplemented with 0.1 μg/ml prochloraz (PCZ), propiconazole (PPC), or tebuconazole (TBC), respectively, and incubated at 26 °C. Aliquots (100 μl) of propagules were removed from the liquid culture at 12, 18, 24, 36, and 48 h post-inoculation, and the samples were stained with 0.25 mM PI (final concentration) at room temperature for 5–10 min, after which samples were observed under an EVOS fluorescent digital inverted microscope (Thermo Fisher Sci., Waltham, MA, USA) at × 60 magnification.

**Phenotypic characterization of fungal strains**

Oxidative, osmotic, and cell wall perturbing stress phenotypes were examined after inoculation (1 μl of 1 × 10⁸ spores/ml) onto the center of PDA plates supplemented with various concentrations of calcofluor white (500–1000 μg/ml), Congo Red (100–500 μg/ml), H₂O₂ (0.5–6.0 mM), paraquat (0.1–1.0 mM), SDS (0.02–0.06%), menadione (0.1–1.0 mM), NaCl (0.5–1.0 M), or sorbitol (1.0–1.5 M). For examination of the effects of pH on vegetative growth, fungal strains were inoculated as above on PDA plates (base pH = 5.6), buffered to pH values ranging from 5.0 to 10.0 using either 2-(N-morpholino)ethanesulfonic acid (MES, pH 5.0–7.0), Tris (pH 7.0–10.0), or phosphate (pH 5.5–8.0)-based buffer systems. Growth temperature profiling was performed via inoculation of PDA plates sealed with Parafilm and then subsequently incubated at different temperatures: 15, 20, 26, or 32 °C. Inoculated plates were incubated for 12 days, after which the diameters of the colonies on the plates were measured (plates were photographed at 14 days).

Antagonism assays were performed as follows: 1 μl of R. lauricola, R. arxii, or B. bassiana conidia suspension (1 × 10⁸/ml) was pairwise inoculated onto two spots of PDA plates. As controls, 1 μl of each conidial suspension was solely inoculated onto one spot of the PDA plates. The inoculated plates were sealed, incubated at 26 °C, and photographed at 10 days.

**In vivo avocado bark plug bioassays**

Adults of X. bispinatus were obtained from naturally infested avocado, Persea americana Mil., “Lula,” logs (8) at the University of Florida Tropical Research and Education Center (TREC), Homestead, FL. To simulate the field efficacy of B. bassiana when X. bispinatus is exposed to the fungus applied to tree bark, an in vivo avocado bark plug bioassay was designed and constructed as follows. In each 15-ml conical disposable plastic Fisherbrand® centrifuge tube (ThermoFisher Sci.), a 2.5 cm × 13.0 cm strip of brown paper towel folded in half four times was placed inside and pushed to the bottom using the flat end of the spatula so that the folded paper towel filled the conical end at the 1.5 cm volume line. To maintain high relative humidity inside the tube, 400 μl of distilled water was pipetted on to the paper towel. Using a cork borer, a 531 mm² bark plug (height = 5 mm, diameter = 13 mm) from a Lula avocado bolt (obtained from TREC) was removed and immediately placed inside the centrifuge tube. Using a blunt poker, the plug was removed from the cork borer and with the bark side up was pushed down the tube using a stainless steel precision 15-cm-long straight forceps tweezer until it touched the moistened paper towel.

A 4.0 × 10⁶ spores/mL suspension of B. bassiana GHA strain was prepared by adding 0.1 ml of BotaniGard® ES to 100 mL of sterile distilled H₂O. Seventy-five microliters of the suspension was directly pipetted on the bark plug secured in the tube and allowed to soak for 15 min with the tube open. A control treatment of 75 μL of sterile distilled H₂O was applied.
likewise to bark plugs. An individual beetle was gently placed near the top of each tube by using a moistened camelhair brush. The tube was slightly tilted so that the beetle could gently slide down the tube until it reached the bark. Upon reaching the bark surface of the plug and observing that the beetle was freely moving on the surface, the tube’s screw cap was put in place. Tubes were randomized in a Styrofoam rack according to the treatments tested and placed into an environmentally controlled chamber set at 25 °C in total darkness for 24 h. After this time, the caps were replaced with Parafilm and then placed upright in a Styrofoam rack.

Beetles were checked daily post-exposure under a binocular dissecting scope to determine if they were alive or dead. If the beetle was not visible, the tube was tapped on a hard surface several times until movement was apparent. If no movement was visible after tapping, then the beetle was presumed dead. Using a permanent marking pen, a specific colored dot was marked on the Parafilm corresponding to the day’s color code. Dead beetles remained for several days in the tube with high moisture until mycosis was apparent. Bioassay experiments were conducted twice, and there were ten replicates per treatment. The median survival times (days) of the beetles in the two treatments were compared using the Kaplan-Meier survival analysis followed by a log-rank $\chi^2$ test (SAS JMP Pro 13 for Windows 2013, Statistical Discovery™, SAS Campus Drive, Cary, NC).

**Data availability** All data are available in the text and/or Supplementary Information sections.

**Results**

*R. lauricola* is highly susceptible to select fungicides whereas entomopathogenic fungi are largely resistant

Two insect symbiotic fungi, *R. lauricola* and *R. arxii*, and six entomopathogenic fungal strains, which included three *B. bassiana*, one *M. anisopliae*, and two *I. farinosa* strains, were used in this study (Supplemental Fig. S1). Forty-three potential fungicides were tested in a preliminary screen for their ability to inhibit growth of the insect symbiotic (*R. lauricola* and *R. arxii*) and pathogenic (*B. bassiana*, *M. anisopliae*, and *I. farinosa*) fungi (Supplemental Table S1). *R. lauricola* was particularly susceptible to prochloraz and most of the conazole fungicides. Most of the fungal strains showed broad fungicide resistance patterns, with little to no inhibition of growth (concentration for 50% inhibition of growth, IC$_{50}$ ≥ 1000 μg/ml) for aliphatic nitrogen (butylamine), aromatic (biphenyl, cresol), benzimidole precursor (thiophanate methyl), organophosphorus (edifenphos, iprobenfos, and inezin), triazole (bitertanol and flurotrimazole), zinc (zineb and ziram), and copper (copper hydroxide and copper sulfate) fungicides. In addition, little sensitivity was observed when exposed to jojoba and neem natural oil fungicides or to boric acid, salicylic acid, quinone, or KHCO$_3$ at the concentrations examined. The entomopathogenic fungi were largely resistant to the dithiocarbamate (dazomet, mancozeb, and maneb) fungicides, whereas moderate (100–300 μg/ml) to high (500–900 μg/ml) concentrations of these fungicides inhibited the growth of the *Raffaelea* species. Most of the entomopathogenic fungal strains were resistant to several amine fungicides including dimoxystrobin, furalaxy, matalaxy, banalaxy, caboxin, flutolanil, salicylanilide, fenfuram, benzohydroxamic acid, cyazofamid, and iprovalicarb; however, moderate susceptibility (IC$_{50}$ between 200 and 600 μg/ml) was observed for *B. bassiana* and *I. farinosa*, but not *M. anisopliae*, to dichlofluanid. In addition, most of the entomopathogenic strains were sensitive to prochloraz (IC$_{50}$ < 100 μg/ml); however, *R. arxii* was moderately resistant.

Among the phthalimide fungicides tested, the *Raffaelea* species and entomopathogenic fungi were resistant to phthalimide, with moderate sensitivity to captan for the *Raffaelea* species and *B. bassiana* and *I. farinosa* (Supplemental Table S1). Differential resistances were observed among the conazole fungicides tested. The entomopathogenic fungi were resistant to triadimefon and fluconazole, with the *B. bassiana* strains being resistant to etaconazole and penconazole, but slightly susceptible to tebuconazole. Two of the three *B. bassiana* strains were resistant to propiconazole, with a slight susceptibility observed for one strain. Similarly, slight susceptibility was seen for the *M. anisopliae* strain towards propiconazole and tebuconazole. In contrast, moderate sensitivity was seen for the two *I. farinosa* strains towards etaconazole, penconazole, and propiconazole. *R. lauricola*, however, was very sensitive to most of the conazole fungicides, whereas *R. arxii* was generally resistant to these compounds.

Compounds (7 in total) showing IC$_{50}$ values < 100 μg/ml against *R. lauricola* were tested to obtain accurate IC$_{50}$ and IC$_{95}$ values (Supplemental Fig. S2 and Table 1). Prochloraz was the most potent fungicide against *R. lauricola*, resulting in IC$_{50}$ and IC$_{95}$ values of 5 and 7 ng/ml, respectively. Prochloraz inhibition value was ~ 1500 ng/ml for *R. arxii* and 100–1000 ng/ml for most of the entomopathogenic fungi. Propiconazole, tebuconazole, penconazole, and etaconazole were also potent against *R. lauricola*, with IC$_{50}$ values ranging from 14 to 83 ng/ml and IC$_{95}$ = 32–102 ng/ml. These compounds required 100–1000× greater concentrations to inhibit the growth of *R. arxii* and the entomopathogenic fungal strains. The IC$_{50–95}$ values for zineb and maneb were in the 1–10 μg/ml range for *R. lauricola*, indicating they were potent, albeit requiring a much higher concentration than the other fungicides described previously. The IC$_{50–95}$
values for the latter two compounds were within the 2–500 μg/ml range for the other fungi tested. Death of *R. lauricola* by the three most effective fungicides, prochloraz, propiconazole, and tebuconazole, was confirmed via propidium iodide staining that revealed a lag period of ~18 h, followed by rapid death of the fungal cells, with > 95% killed within ~36 h (Fig. 1).
**R. lauricola** displays a unique neutral and alkaline pH sensitivity and is adapted for growth at lower temperatures

To better understand the environmental conditions under which *R. lauricola* is capable of growing, experiments to determine the optimal pH and temperature profiles for fungal growth were performed. Growth temperature profiling of the *Raffaelea* species revealed a broad temperature optimum for *R. lauricola* between 20 and 26 °C, with robust growth even at 15 °C but a sharp decrease at 32 °C (Fig. 2A, B). In contrast, *R. arxii* showed equivalent growth at 26–32 °C, but reduced growth at 20 °C, and markedly slower growth at 15 °C. Optimal growth pH profiling revealed a dramatic reduction in growth of *R. lauricola* under neutral to alkaline conditions (> pH 6.8), as compared to un-buffered PDA that has a base pH = 5.6, and PDA buffered at pH 6.4. In phosphate buffer, an ~80% growth inhibition was seen at pH 7.0, with little to no growth at pH ≥ 7.2. A similar trend was seen in Tris buffer, with ~75% growth inhibition observed at pH 8.0. In contrast, *R. arxii* and *B. bassiana* could grow in media at pH 9.0 (Fig. 2C, E, Supplemental Figs. S3 and S4). As *R. lauricola* acidifies the media during growth, buffering capacity was found to be important with at least 60 mM of phosphate buffer required for ~50% growth inhibition at pH 6.8, and > 90% inhibition of growth seen using 100 mM phosphate buffer, pH > 7.2 (Fig. 3).

The response of the *Raffaelea* species and *B. bassiana* to oxidative, osmotic, and cell wall stress was assessed via amendment of PDA with various compounds (Fig. 4A, B). Both *R. arxii* and *R. lauricola* were more resistant to sodium dodecyl sulfate (SDS) and Congo Red than *B. bassiana*, with the *Raffaelea* species capable of growing in 0.04% SDS and 500 μg/ml Congo Red, conditions in which *B. bassiana* showed little to no growth. Little to no growth of the *Raffaelea* species occurred in 0.06% SDS and at 1000 μg/ml Congo Red. In contrast, *B. bassiana* was significantly more resistant to H₂O₂ and grew at concentrations as high as 4 mM. Both *R. lauricola* and *B. bassiana* grew in the presence of Calcofluor White as high as 1000 mg/ml, whereas *R. arxii* growth was inhibited at 750 mg/ml, with little to no growth at 1 mM paraquat (Fig. 4).

**Increased efficacy of fungicides against *R. lauricola* at neutral pH**

To determine if combining pH modification with the fungicide could increase the effectiveness of the fungicide in targeting *R. lauricola*, IC₉₅ values for prochloraz, propiconazole, and tebuconazole were assayed in PDA amended with 80 mM phosphate buffered at pH = 6.6, 6.8, and 7.0 (Table 2). Compared to unamended PDA, the IC₉₅ for prochloraz decreased ~3-fold at pH 6.6–7.0, from 11.6 to 3.8 ng/ml. The
IC₉₅ for propiconazole and tebuconazole showed pH dependency, decreasing ~2-fold (i.e., increased effectiveness) at pH 6.6 and ~3-fold at pH 7.0 (from 60–60 to 15–16 ng/ml at pH 7.0).

**B. bassiana** can be used to stop beetle proliferation in the tree and shows antagonism with **R. lauricola**

Insect bioassays were performed using a commercially available formulation of **B. bassiana** as detailed in the Materials and Methods section. Kaplan-Meier survival analysis (censored at day 10) revealed **B. bassiana** mediated mortality of *X. bispinatus* adults as seen in median survival time of nine 7.9 ± 0.4 days whereas in the control group, survivorship in the control was > 85% (at 10 days) and an accurate LT₅₀ could not be calculated (log-rank \( X^2 = 4.9, P = 0.027, df = 1 \)) (Fig. 5).

To examine any effects **B. bassiana** may have on the growth of the *Raffaelea* species, in vitro co-inoculation experiments were performed. These data revealed compatibility...
Fig. 3 Growth response of *R. lauricola* to pH and phosphate buffer concentration. One microliter of *R. lauricola* conidia suspension (1 × 10⁸ spores/ml) was inoculated onto the center of PDA plates buffered with various concentrations of phosphate (20, 40, 60, 80, and 100 mM) with varied pH values (6.4, 6.6, 6.8, 7.0, 7.2, 7.5, and 8.0). The inoculated plates were incubated at 26 °C, and the plates were photographed after 14 days of growth (A), and the diameters of the colonies were quantified at 12 days of growth (B). Experiments contained three technical replicates and the entire experiment was repeated at least three times. Error bars = ± SE. Note that the symbols in panel B encompass the error bars.
between R. lauricola and R. arxii, but some antagonism between the Raffaelea species and B. bassiana, with R. lauricola growing faster than B. bassiana under the conditions examined (Fig. 6).

### Discussion

Since its introduction in the early twenty-first century, laurel wilt disease has spread at an unprecedented rate, ravaging

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**Fig. 4** Stress responses of R. lauricola and R. arxii. (A, B) Representative images and quantification of growth of R. lauricola and R. arxii in the presence of SDS, Congo Red, H₂O₂, calcofluor white, and paraquat, respectively. Experiments contained three technical replicates and the entire experiment was repeated at least three times. Error bars = ± SE

<table>
<thead>
<tr>
<th>Medium</th>
<th>IC₉₅/SE</th>
<th>Prochloraz (ng/ml)</th>
<th>Propiconazole (ng/ml)</th>
<th>Tebuconazole (ng/ml)</th>
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<tr>
<td></td>
<td></td>
<td>R. lauricola</td>
<td>R. arxii</td>
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<tr>
<td>PDA (pH = 5.6)</td>
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<td>1581.96</td>
<td>58.63</td>
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*SE:* standard error
forestlands and in some places almost eliminating indigenous members of the Lauraceae family (Harrington et al. 2008, 2011; Kendra et al. 2013). The causal agent of the disease, a fungal symbiont of *X. glabratus*, is associated with at least nine ambrosia beetle species, the main ones being *Xyleborus volvulus*, *Xyleborus ferrugineus*, and *X. bispinatus* (Carrillo et al. 2014; Hanula et al. 2008; Ploetz et al. 2017b). In Florida, the apparent main vector capable of transmitting the pathogen to avocado appears to be *X. bispinatus* (Saucedo et al. 2017). Although *X. glabratus* is also capable of attacking and infecting avocado (Mayfield et al. 2008b), this beetle is rare in commercial avocado crops (Carrillo et al. 2012; Kendra et al. 2017). There is also significant concern should the beetle/pathogen reach susceptible trees in regions yet unaffected, e.g., California (Fraedrich 2008). Although important information concerning aspects of the taxonomy, phylogenetics, and (expanding) distribution of the pathogen and associated beetles has been gathered, little is known concerning basic physiological parameters of the fungus that can help shed light on the reasons and mechanisms that underlie its particularly lethal infection and virulence. Conidial development was examined in a number of *Raffaelea* species including *R. arxii*, but not in *R. lauricola* (Gebhardt and Oberwinkler 2005). The efficacy of propiconazole and several other fungicides was tested against *R. lauricola*, but a systematic comparative survey has not been reported (Mayfield et al. 2008a; Ploetz et al. 2011). Aspects of the disease progression as well as plant responses to the pathogen, especially in avocado, have been examined (Hughes et al. 2015; Ploetz et al. 2012b, 2015), and detection methods for monitoring the incidence and spread of the disease have been developed (Dreaden et al. 2014).

Here, we performed a systematic examination of the fungicide and stress resistances of the plant pathogen *R. lauricola* and the related non-pathogenic *R. arxii*, as well as a wide range of entomopathogenic fungi, including the commercialized fungal species *B. bassiana* and *M. anisopliae*, the latter representing important tools for insect biological control that are compatible with organic farming, can be incorporated into integrated pest management (IPM) practices, and are actively studied as potential agents for control of ambrosia beetles (Glare et al. 2012; Ortiz-Urquiza and Keyhani 2016; Ortiz-Urquiza et al. 2015). In addition to the previously examined propiconazole, our data revealed five other fungicides with high activity against *R. lauricola*, including an amine fungicide, prochloraz, which displayed an IC₉₅ 4–5-fold lower than propiconazole, as well as other conazoles (tebuconazole, etc.).
penconazole, and etaconazole), and the triazole, bitertanol. Intriguingly, *R. arxii* as well as the entomopathogenic fungi showed for the most part IC₅₀ values at least 100-fold (and in many instances > 10³-fold) higher than *R. lauricola*, indicating significantly greater levels of resistance. In the case of the entomopathogenic fungi, this has important applied relevance in that use of these fungicides may be compatible with application of the entomopathogen agent for simultaneous targeting of the beetle and the fungal pathogen in laurel wilt disease control efforts.

Complementary to the fungicide profiling, our data revealed a number of other weaknesses that can be easily translated into providing immediate actionable potential targets for laurel wilt control. Notably, *R. lauricola* is cold-adapted and displays robust growth from 15 to 26 °C (2–3-fold greater than *R. arxii*), with reduced growth at 32 °C and little to no growth at temperatures > 37 °C. These temperature data provide critical insights into disease spread and possible avenues for mitigation of this plant pathogen, and they are significant in that they indicate that temperate regions are likely to be more susceptible to the spread of laurel wilt. In addition, our data indicate that for economically important crops such as avocado that are grown in warmer climates (Florida and California), control and sanitation efforts should be focused during the cooler months of the season. However, a standardized study is needed to determine how changes in the ambient temperature affect the temperature in the tree xylem where *R. lauricola* grows, and if laurel wilt incidence decreases during the summer months. Our data indicate that as temperature decreases, the fungus has the potential to spread rapidly.

Fungal growth pH profiling revealed another potential weakness of *R. lauricola*. Unlike *R. arxii* and the entomopathogenic fungi examined, which grow at initial external media pH values ≥ 9 in standard media, *R. lauricola* showed a sharp decrease in ability to grow beginning at pH ~ 6.6 (50% reduction in growth as compared to pH 5.6), with little to no growth seen at pH > 8.0. Experiments performed to test if formulations combining pH adjustment with selected fungicides may offer more effective control showed 3–4-fold increase in effectiveness of prochloraz, propiconazole, and tebuconazole simply by mixing the fungicide in a pH 7 buffer. It should be noted that these experiments were not performed using higher pH values, e.g., pH 8, as this already results in > 90% inhibition of fungal growth in the absence of the fungicide; however, in terms of practical applications, fungicide formulations in buffers at pH 8 may be even more effective. Overall, prochloraz was > 10-fold more effective compared to propiconazole when pH adjustment was taken into account. Furthermore, considering the relative concentrations needed for killing the fungus, prochloraz would be substantially less expensive (~ 10-fold). However, this fungicide is not currently registered for use in the USA. Even if propiconazole were to be used, formulations taking into account pH, i.e., increasing the alkalinity of the fungicide containing solution, could potentially increase its effectiveness 3–4-fold.

Both the fungicides and the pH manipulation are compatible with the potential use of entomopathogenic fungi for beetle control. Our data show that the commercially available *B. bassiana* can cause significant mortality within an artificial avocado tree bark plug system, indicating that subsequent field studies should be performed. Both *B. bassiana* and *M. anisopliae* are known to be effective against several other ambrosia beetles (Castrillo et al. 2011), and our data extend these findings. We also observed competition between *B. bassiana* and *R. lauricola/R. arxii* (but compatibility between the *Raffaelea* species), as has been reported for entomopathogenic fungi and other ambrosia beetle fungal symbionts (Castrillo et al. 2016). Our data provide a number of simple recommendations that could have significant impacts in laurel wilt control via identification of unexpected “Achilles heels” of the laurel wilt pathogen, *R. lauricola*.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical approval** This article does not contain any studies with animals performed by any of the authors.

**References**


