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Response of swamp bay, *Persea palustris*, and redbay, *P. borbonia*, to *Raffaelea* spp. isolated from *Xyleborus glabratus*

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Summary

Laurel wilt is a devastating invasive disease of members of the Lauraceae plant family. It is caused by the fungus *Raffaelea lauricola*, which is a nutritional symbiont of its ambrosia beetle vector, *Xyleborus glabratus*. In the United States, six *Raffaelea* spp., in addition to *R. lauricola*, have been recovered from mycangia of *X. glabratus*. We compared the response of two laurel wilt suspects, swamp bay (*Persea palustris*) and redbay (*Persea borbonia*), to five of these species, another undescribed *Raffaelea* sp., and *R. lauricola*. Six weeks after inoculation, only *R. lauricola* caused significantly greater symptoms than water inoculations. The fungi varied in their ability to move systemically and be recovered from the host at the end of the experiment. Stem hydraulic conductivity was decreased by *R. lauricola*, but none of the other taxa. Although the roles these fungi play in the life cycle of *X. glabratus* are not known, they do not appear to be pathogens on these host tree species.

1 | INTRODUCTION

Laurel wilt is a lethal disease of redbay (*Persea borbonia* (L.) Spreng.), swamp bay (*Persea palustris* (Raf.) Sarg.) and other members of the Lauraceae family (Fraedrich et al., 2008; Mayfield, Smith, Hughes, & Dreaden, 2008; Smith et al., 2009). It is caused by the fungus *Raffaelea lauricola* Harrington, Fraedrich, and Aghayeva (2008), which is a symbiont of the redbay ambrosia beetle, *Xyleborus glabratus* Eichh., its primary vector (Fraedrich et al., 2008). *Xyleborus glabratus* is native to Asia (Wood & Bright, 1992), but laurel wilt has only been reported in the south-eastern United States (Fraedrich et al., 2008).

Most ambrosia beetle symbionts are saprophytes, colonizing dead and dying wood. Those that are phytopathogens (e.g. *Raffaelea quercivora* Kubono et Shin. Ito) or suspected of being a phytopathogen (e.g. *R. quercus-mongolicae* K.H. Kim, Y.J. Choi & H.D. Shin) cause significant damage to host trees but, unlike *R. lauricola*, are not systemic and require mass attack by their vectors and multiple inoculations to kill trees (Kim, Choi, Seo, & Shin, 2009; Kubono & Ito, 2002; Ploetz, Hulcr, Wingfield, & de Beer, 2013; Torii et al., 2014).

Several *Raffaelea* spp. have been isolated from mycangia of *X. glabratus* in the south-eastern United States, although at lower concentrations (based on colony-forming units (CFUs)) than *R. lauricola* (Campbell, Ploetz,

Dreaden, Kendra, & Montgomery, 2016; Harrington & Fraedrich, 2010). Some of the same species have also been recovered from *X. glabratus* in Asia (Harrington et al., 2011). Although these fungi are associated with a major invasive forest pest, their ecological roles are not known.

To better understand the roles these *Raffaelea* spp. may play in laurel wilt disease, those recovered from *X. glabratus* in Florida were inoculated into *P. borbonia* and *P. palustris*. We examined their abilities to cause wilt symptoms, discolour sapwood, systemically move in inoculated plants and impact stem hydraulic conductivity. In addition, we were interested in whether results for these taxa were related to their phylogenetic relatedness to *R. lauricola*.

2 | MATERIALS AND METHODS

Isolates of seven *Raffaelea* taxa were used in inoculation experiments. Five that were recovered during a study of mycangial contents of *X. glabratus* in Florida (*Raffaelea subalba* T. C. Harrin., Aghayeva & Fraedrich CBS139933, *R.* cf. *subalba* CBS140119 (a possible new species), *Raffaelea arxii* Scott & du Toit CBS139940, *Raffaelea fusca* T. C. Harrin., Aghayeva & Fraedrich CBS139934, *R. subfusca* T. C. Harrin., Aghayeva & Fraedrich CBS139936) were

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identified by analysing 18S, 28S rDNA and beta-tubulin sequences (Campbell et al., 2016). In addition, an isolate of *R. lauricola*, PL571, and an undescribed close relative, *Raffaelea* sp. PL1004 (Dreaden, Davis, Harmon et al., 2014; Dreaden, Davis, de Beer et al., 2014), were tested (Table 1).

Two experiments were conducted, experiment 1 with *P. palustris* and experiment 2 with *P. borbonia*. These closely related tree species were chosen due to their high susceptibility to laurel wilt. They were obtained from commercial nurseries in 4-L pots and had mean stem diameters of approximately 1.5 cm (at 7 cm above soil line), and mean heights of approximately 1.5 m. They were watered and fertilized regularly, were actively growing at the time of inoculations, and were kept in greenhouses located in Gainesville, Florida for the duration of the study (experiment 1, July–August, 16:8 diurnal light and 21°C day/18°C night, and experiment 2, March-May, natural light and 21°C). Treatments in each experiment were completely randomized.

In experiment 1, eight treatments (seven fungal isolates and water control) were replicated three times. Inoculum was produced on malt extract agar amended with cycloheximide and streptomycin CSMA (Harrington, 1981). Conidia were harvested by flooding plates with sterile water and diluted to 1,000 per μl after quantification with a hemacytometer. Two 2.8-mm-diameter holes, 0.75 cm deep, 7 cm above the soil line and opposite each other, were drilled at a downward 45° angle into stems. Each hole received 50 μl of inoculum (50,000 conidia), or water for water-inoculated controls and was sealed by wrapping the stem with Parafilm. Inoculum viability was assessed by plating 20 μl of the inoculum on CSMA and checking for germination and growth after 4 days.

Six weeks after inoculation, trees were evaluated for external symptom development and rated on a 0–4 scale based on the percentage of canopy showing wilt symptoms (0: 0%; 1: 1–25%; 2: 26–50%; 3: 51–75%; or 4: 76–100%). Internal symptom development was calculated as the proportion of the stem that was discoloured (longitudinal length of discoloured area/tree height). In addition, 2-cm-long sections of stem were removed from 2.5 and 20 cm above the inoculation point and the top of plants, and five 0.5-cm^2 pieces from each section were surface disinfested in 4% sodium hypochlorite, plated on CSMA within 6 hr of harvesting and monitored for 2 weeks for fungal growth. Subcultures of fungi recovered from stem sections were grouped based on colony morphology, DNA was extracted from one isolate of each morphotype and a portion of the 18S rDNA was amplified and sequenced as described by Dreaden, Davis, Harmon et al. (2014). The 18S sequences were then aligned with sequences from the original isolates used for inoculation, and isolate identity was confirmed with 100% sequence homologies.

The proportion of total stem length that was discoloured was analysed using mixed models (Proc Mixed, SAS 9.3) to determine whether internal symptom development differed by treatment. A mean separation table was constructed using differences of least squares means. Data were log transformed to normalize distribution. External symptom development in trees was compared using a Kruskal–Wallis ANOVA. This nonparametric version of a one-way ANOVA was chosen to account for non-normal data distribution. No post hoc tests or mean separation were carried out.

In experiment 2, nine treatments (seven fungi, water and non-treated controls) were replicated four times. Internal and external symptom development in the *R. lauricola* and water-inoculated trees were measured 6 weeks after inoculation, and isolations were made from stem sections 1.5–2 cm above the inoculation points, as described above. External symptom development was measured as per experiment 1, while internal symptom development was the proportion of stem area that was discoloured (manually delineated and measured using ImageJ (Schneider, Rasband, & Eliceiri, 2012)). This proportion was the mean (discoloured area/total cross-sectional stem area) of both ends of the stem section (at 2 and 22 cm above the inoculation point), which gave a single representative value for each tree.

TABLE 1 *Raffaelea* taxa, isolates and their recovery (proportion of trees) from above the inoculation point in experiments 1 and 2

Taxon	Isolate	GenBank accession[a]	Isolation Source	Location	Recovery Expt 1 2.5 cm	Expt 1 20 cm	Expt 2 2 cm
R. subalba	CBS139933	KP164576	*Xyleborus glabratus*	Highlands County, FL	1/3	0/3	2/4
R. cf. *subalba*	CBS140119	KP164577	*X. glabratus*	Miami-Dade County, FL	0/3	0/3	1/4
R. arxii	CBS139940	KP164578	*X. glabratus*	Miami-Dade County, FL	3/3	0/3	1/4
R. subfusca	CBS139936	KP164579	*X. glabratus*	Miami-Dade County, FL	3/3	0/3	2/4
R. fusca	CBS139934	KP164580	*X. glabratus*	Alachua County, FL	0/3	0/3	2/4
Raffaelea sp.	PL1004[b]	KF026302	*Persea americana*	Homestead, FL	1/3	0/3	3/4
R. lauricola	PL571[b]	JQ861956	*Persea borbonia*	Middleburg, FL	3/3	2/3	4/4

[a]18S rDNA gene regions were deposited in GenBank.
[b]From authors collections.

The remaining treatments (*R. subalba*, *R.* cf. *subalba*, *R. arxii*, *R. fusca*, *R. subfusca*, *Raffaelea.* sp., and the non-treated control) were processed 8 weeks after inoculation. Processing the treatments in two batches enabled samples to be processed the same day they were harvested. Treatments with fewer than three replications were excluded from statistical analysis of internal discoloration. However, due to the absence of within-treatment variation, treatments with fewer than three replications were included in the analysis of external symptoms and hydraulic conductivity. Statistical analyses for proportion of discoloured stems and external symptoms were conducted as in experiment 1.

In experiment 2, sapwood-specific stem hydraulic conductivity (k, kg_{water} s^{-1} $m^{-2}_{sapwood}$ MPa^{-1} m) measurements were recorded from 20-cm-long stem sections (2–22 cm above the inoculation point). The methodology described in Gonzalez-Benecke, Martin, and Peter (2010) was used with degassed, sterile filtered 20 mM KCl solution. Initial "native" conductivity (k_{nat}) was determined as the first measurement immediately after sample collection. After k_{nat} determinations, maximum conductivity (k_{max}) was determined for the same segments after they were vacuum infiltrated with solution for 24 hr to refill embolized xylem vessels. Simple linear regressions were performed to determine the relationship between proportion of discoloured stem area (i.e. explanatory variable) and k_{nat} and k_{max} (i.e. response variables). To compare k_{nat} and k_{max} for the *R. lauricola* treatment to the water control, independent group *t* tests were conducted with the Cochran approximation (to account for unequal variance). Pooled *t* tests were run to compare the k_{nat} and k_{max} of each treatment. Seven trees (two *R.* c.f. *subalba*, two *R. subfusca*, one *R. fusca* and two non-inoculated controls) that were infested with *Xylosandrus compactus* had discoloured xylem surrounding the galleries, lower xylem conductivity (data not shown) and, thus, were not analysed.

3 | RESULTS

Trees inoculated with *R. lauricola* started to develop external symptoms after 3 weeks in both experiments and, with the exception of one tree in experiment 2 on which one branch remained alive, were dead by the end of an experiment. Neither the trees inoculated with other *Raffaelea* taxa nor the water-inoculated controls developed external symptoms in either experiment. The nonparametric ANOVA found treatment effects for external symptoms in both experiments 1 and 2 ($p = .002$, DF = 7 and $p < .001$, DF = 8, respectively).

The amount of sapwood that was discoloured varied among the *Raffaelea* taxa (Fig. 1a–f, shows images taken at the end of an experiment and how trees responded to inoculation with *R. lauricola* (c, d) and other *Raffaelea* spp. (e, f)). Statistically significant differences in the ability of taxa to cause discoloration were found in experiment 1 ($p = .0001$, F = 49.26, Num DF = 7, Den DF = 16), with *R. lauricola* causing the greatest discoloration and the water inoculation the least. The remaining taxa caused minor discoloration. Differences were also seen in experiment 2 ($p = .029$, F = 3.29, Num DF = 5, Den DF = 17); however, none of the isolates, including *R. lauricola*, differed from the water-inoculated control (Figs 1 and 2). Differences in the proportion of discolored sapwood were only statistically significant between trees inoculated with *R. lauricola* and *R.* sp. PL1004 (Fig. 2). No taxon was recovered from tree tops and only *R. lauricola* was recovered from 20 cm above the inoculation point in experiment 1 (Table 1).

In experiment 2, treatments affected both k_{nat} and k_{max} (ANOVA, $p = .034$, F = 2.69, Num DF = 8, Den DF = 20 and $p = .037$, F = 2.64, Num DF = 8, Den DF = 20, respectively). k_{nat} and k_{max} for the *R. lauricola* treatment were not different ($p = .996$, t = −0.01, DF = 6), reflecting that the plants were well watered, before stem segment excision and indicating that xylem obstructions, rather than embolisms, were responsible for reduced conductivity. The *R. lauricola* and water control treatments were different for k_{nat} and k_{max} at $p = .073$ and .098, respectively. There was a significant, negative relationship between discolored stem area and k_{nat} ($p < .001$, DF = 28; $R^2 = .44$, $y = −0.056x + 0.130$), as well as k_{max} ($p = .004$, DF = 28; $R^2 = .27$, $y = −0.058x + 0.189$).

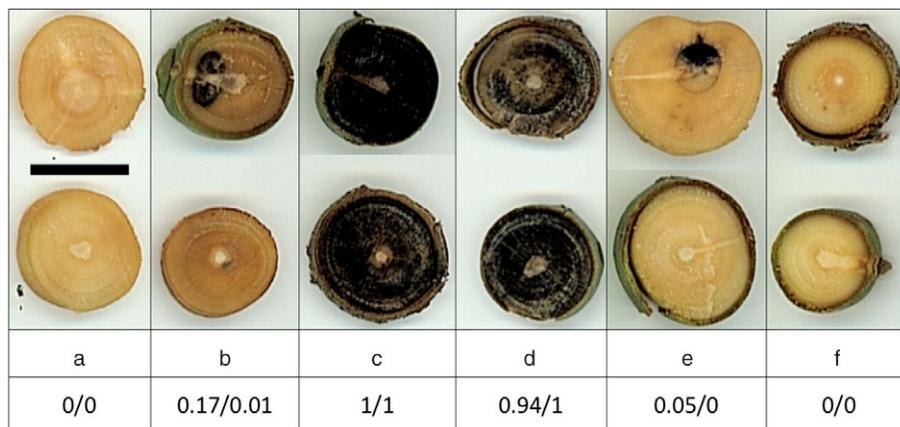| a	b	c	d	e	f
0/0	0.17/0.01	1/1	0.94/1	0.05/0	0/0

FIGURE 1 Cross sections of stems from 2 and 22 cm, unordered, above the inoculation points from experiment 2. a and b: water-treated controls. c and d: *Raffaelea lauricola*-treated stems. e and f: *Raffaelea subalba*-treated stems. Proportion of the discoloured stem area (discoloured area/total cross-sectional stem area) for the upper/lower images are at the bottom of the figure. The scale bar is 1 cm
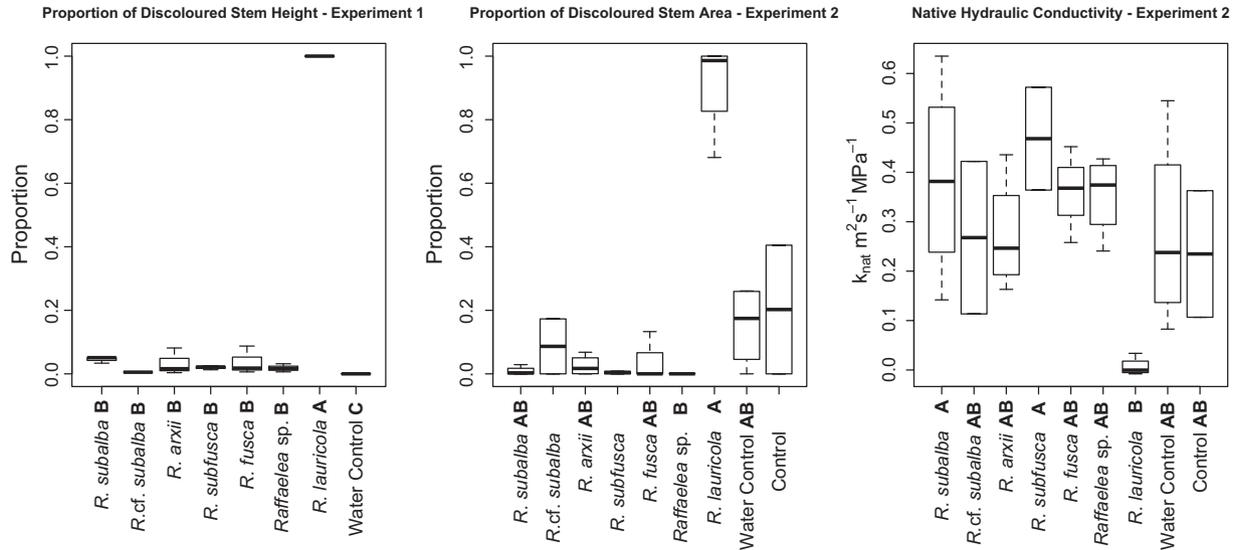
FIGURE 2 Boxplots with proportion of discoloured internal stem length (discoloured length/tree height) from experiment 1, left, proportion of discoloured internal stem area (discoloured area/total cross-sectional stem area) from experiment 2, centre, and native hydraulic conductivity (k_{nat}), right, after inoculation with seven *Raffaelea* taxa, water control, and non-inoculated control. Stem sections for k_{nat} measurements were 20 cm long from 2 to 22 cm above the inoculation points from experiment 2. Whiskers represent minimum and maximum values. Bold font represents significant differences at *p* = .05. Due to damage by *Xylosandrus compactus*, some treatments in experiment 2 had only two replications and were not included in the statistical analysis and mean separation for proportion of discoloured stem area, centre

4 | DISCUSSION

With the exception of *R. lauricola*, no *Raffaelea* taxon recovered from *X. glabratus* had been tested for pathogenicity on redbay or swamp bay prior to this study. Five taxa from *X. glabratus* mycangia in Florida and another taxon recovered from *Persea americana* did not cause external symptoms on these trees. These results were not surprising as ambrosial symbionts are typically not pathogenic (Ploetz et al., 2013). Although the ecological role of the other mycangial fungi is not known, they do not appear to be pathogens on these hosts or to be important in the ongoing laurel wilt epidemic in the south-eastern United States.

The small amount of discoloured stem sapwood that was caused by some of the other taxa in this study are similar to those caused by the *Raffaelea* spp. that cause Japanese and Korean oak wilts. In the latter diseases, vertical and horizontal movement of the pathogens is limited to the areas adjacent to beetle galleries, and thousands of attacks are needed to kill mature trees (Kim et al., 2009; Kubono & Ito, 2002; Ploetz et al., 2013). It is not known whether discoloration caused by the other *Raffaelea* taxa in this study would have expanded further if the experiment had continued longer than the 6–8 weeks used in our studies, although lesions caused by *R. quercivora* (Japanese oak wilt) stop expanding 2 weeks after inoculation (Murata, Matsuda, Yamada, & Ito, 2009). Additional work is warranted to determine whether tree mortality would occur on these or other hosts after inoculations of longer duration or multiple times with the other *Raffaelea* taxa. The ability of the other *Raffaelea* taxa to move in the host and survive the duration of the experiments was limited insofar as only *R. lauricola* was recovered from 20 cm above the inoculation point. It is possible

that with multiple attacks on trees by multiple species of ambrosia beetles these fungi could be re-inoculated many times, leading to a situation similar to the oak wilts in eastern Asia. Nonetheless, in this study, systemic infection and lethal wilt occurred only after inoculation with *R. lauricola*.

The amount of discoloured sapwood varied among the *Raffaelea* taxa. Statistically significant differences were found in experiment 1 between the ability of *R. lauricola* to cause discoloration versus that caused by different taxa. However, in experiment 2 significant differences in discoloration (alpha = 0.05) only occurred between *R. lauricola* and *R.* sp. PL1004-treated stems. The differences in the amount of discoloured sapwood from experiments 1 and 2 are likely because proportion of discoloured stem length and proportion of discoloured stem area was measured in experiments 1 and 2, respectively. In experiment 1, all of the *R. lauricola* inoculated trees had internal discoloration the length of their stems but in experiment 2 more variation in discoloured stem area and less differences between treatments were found, Figs 1 and 2. Discoloration surrounding the wounds created by the inoculation method was observed in the water-inoculated trees, Fig. 1. In one of the non-inoculated control trees, discoloration that appeared to originate from a previous wound near the base of the stem was found and extended up the stem into the section measured, Fig. 2. This discoloration that seems to be associated with wounds, both previous and from the inoculation method, might explain the results in experiments 1 and 2 and illustrates a possible limitation of using sapwood discoloration as a measure of symptom development.

Discoloration appears to be a general host reaction to vascular wilt pathogens (Beckman, 1987). In *P. americana*, vascular discoloration that followed inoculation with *R. lauricola* increased as did non-functional

xylem (Inch & Ploetz, 2011). In the present work, there was an inverse relationship between vascular discoloration and xylem hydraulic conductivity in *P. borbonia*. Thus, vascular discoloration is related to xylem dysfunction in trees affected by this pathogen.

The limited sample size and missing data, due to unexpected damage caused by *X. compactus*, limited our ability to detect differences in hydraulic conductivity between treatments. However, trees inoculated with *R. lauricola* showed reduced k_{nat} and k_{max}, which were not different from each other. As tyloses occur in conduit cells that were previously air-filled (Tyree & Zimmermann, 2002), the inability of vessels to recover hydraulic conductivity in trees inoculated with *R. lauricola* may indicate that tyloses induced by infection reduced conductivity, as suggested by Inch, Ploetz, Held, and Blanchette (2012). Tyloses are induced by many vascular wilt fungi (Beckman, 1987), including those formed in *Ulmus* spp. after infection by *Ophiostoma novo-ulmi* (D'Arcy, 2000).

The seven *Raffaelea* taxa used in this study came from different clades in the genus (Dreaden, Davis, de Beer et al., 2014). However, only *R. lauricola* caused significant symptoms on the tested hosts. The *Raffaelea* sp. isolate PL1004, which is phylogenetically close to *R. lauricola* (Dreaden, Davis, de Beer et al., 2014), was not pathogenic to *P. borbonia* and *P. palustris* in this study nor to avocado (*P. americana*) in previous work (R.C. Ploetz, unpublished data). Thus, it was not possible to examine whether there was a phylogenetic signature for symptom induction (e.g. *R. lauricola* vs the other taxa). Until better understandings of pathogenicity among these fungi are available, predicting which taxa are pathogenic will continue to rely on artificial inoculation studies.

Lateral transfer is common in ambrosia beetles (Carrillo et al., 2014; Hulcr & Cognato, 2010); for example, *R. lauricola*, *R. ellipticospora*, *R. fusca* and *R. subfusca* have been recovered from *X. glabratus* in Asia, whereas *R. arxii*, *R. subalba* and *R.* cf. *subalba* were also recovered from *X. glabratus* in the United States (Campbell et al., 2016; Harrington & Fraedrich, 2010; Harrington et al., 2011). Whether they would pose a threat in other ambrosia beetle species (vectors) and to other host trees is not known. Understanding how and why *R. lauricola* causes laurel wilt on its hosts, but its close relatives do not, would appear to be a fertile area for future research.

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