Research

An *Acaromyces* Species Associated with Bark Beetles from Southern Pine Has Inhibitory Properties Against *Raffaelea lauricola*, the Causal Pathogen of Laurel Wilt Disease of Redbay

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Abstract

Laurel wilt is a destructive disease of redbay (*Persea borbonia*) and other species in the laurel family (Lauraceae). It is caused by *Raffaelea lauricola*, a fungal symbiont of the redbay ambrosia beetle, *Xyleborus glabratus* (Coleoptera: Curculionidae), cointroduced into the United States around 2002. During assessments of fungi associated with bark beetles from loblolly pine, an unknown fungus was isolated that appeared to have broad-spectrum antifungal activities. In this study, we identified the unknown fungus and determined the inhibitory effect of its secondary metabolites on *R. lauricola*. DNA analysis identified the fungus as *Acaromyces ingoldii* (GenBank accession no. EU770231). Secondary metabolites produced by the *A. ingoldii* completely inhibited *R. lauricola* mycelial growth on potato dextrose agar (PDA) plates preinoculated with *A. ingoldii* and reduced *R. lauricola* growth significantly on malt extract agar plates preinoculated with *A. ingoldii*. *R. lauricola* isolates inoculated on PDA plates 7 days after *A. ingoldii* were completely inhibited with no growth or spore germination. Direct evaluation of *A. ingoldii* crude extract on *R. lauricola* spores in a multi-well culture plate assay showed inhibition of spore germination at 10% and higher concentrations. Secondary metabolites from *A. ingoldii* could be potentially useful in managing the future spread of laurel wilt.

Keywords: biocontrol, integrated pest management, diagnostics, trees, *Acaromyces ingoldii*, *Persea borbonia*

Laurel wilt is an important disease that affects members of the Lauraceae including avocado (*Persea americana*), redbay (*Persea borbonia*), and sassafras (*Sassafras albidum*) (Fraedrich et al. 2008). The causal pathogen of the disease is *Raffaelea lauricola*, a fungal symbiont associated with *Xyleborus glabratus* (Coleoptera: Curculionidae), the primary insect responsible for disease transmission (Harrington et al. 2008). The first report of the disease in the United States was coincidental with the introduction of *X. glabratus* near Savannah, Georgia, around 2002. The ambrosia beetle and the fungus were likely introduced into the United States from Asia via solid wood packing material (Harrington et al. 2008). The unusual attraction of *X. glabratus* to healthy trees combined with its unique association with the *R. lauricola* and the highly virulent nature of this pathogen make it possible for a single infection to kill a healthy tree (Fraedrich et al. 2008). Since 2002, *X. glabratus* and laurel wilt disease have become established across the Atlantic coastal plains in South Carolina, Georgia, Florida, and Alabama (Bates et al. 2013; Fraedrich et al. 2008; Mayfield et al. 2008; Peña et al. 2012; Smith et al. 2009a, 2009b) and in recent years have spread into northern Louisiana (Fraedrich et al. 2015), Texas (Menard et al. 2016), and Arkansas (Olatinwo et al. 2016), threatening susceptible hosts across the southern forests.

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Because of the rapidly expanding distribution of laurel wilt in the United States, the ecological impacts of the disease on forest ecosystems, and the economic impacts of this disease on avocado production, it is urgent and critical to explore potential control measures. Current disease management strategies for laurel wilt are very limited and generally ineffective for long-term management of the disease. Macro-infusion of redbay trees with the fungicide propiconazole can protect trees from laurel wilt for limited durations (Mayfield et al. 2008), making it essential to reinject trees every year or two. In commercial avocado groves, although the primary vector, *X. glabratus*, is uncommon (Carrillo et al. 2012; Menocal et al. 2018), laurel wilt can still spread via root transmission or lateral transfer by other ambrosia beetles (Carrillo et al. 2014; Ploetz et al. 2017). Although sanitation practices may help to limit disease spread in avocado orchards, these practices have not been effective in natural and urban forests (Hughes et al. 2015). The use of microorganisms to inhibit and control *R. lauricola* and *X. glabratus* has attracted some attention in recent years. Dunlap et al. (2017) found three *Paenibacillus* species and a *Bacillus* species were biologically active against *R. lauricola*, and Carrillo et al. (2015) demonstrated that an entomopathogenic fungus, *Beauveria bassiana* (Ascomycota: Hypocreales), was a potential biological control agent for *X. glabratus*.

During investigations of the fungal species associated with bark beetles from loblolly pine (*Pinus taeda* L.) in central Louisiana, an unknown fungus was discovered that exhibited broad-spectrum antifungal activities against many other fungi occurring in pine wood including species in the genera *Ophiostoma*, *Fusarium*, *Pestalotiopsis*, and *Trichoderma* (Olatinwo, unpublished). Preliminary investigations on the inhibitory properties of secondary metabolites

from the unknown fungus led to our question of whether the fungus could exhibit similar fungistatic activity against *R. lauricola.* Hence, the primary goals of this study were (i) to identify the unknown fungus associated with the bark tissue of a loblolly pine, (ii) to evaluate possible inhibitory effect of the secondary metabolites produced by the fungus on the mycelial growth and spores germination of *R. lauricola*, and (iii) to determine efficacy of the secondary metabolites on *R. lauricola* in an in vivo experiment. This study is part of a larger effort to identify secondary metabolites with potent antifungal activity against *R. lauricola* that could be potentially useful within an integrated pest management program.

Identification of Fungal Isolates with Inhibitory Activities

The unidentified isolate (isolate R-8) was obtained during an investigation of fungal species associated with bark beetles from bolts of loblolly pine on the Kisatchie National Forest (Rapides Parish, LA) in October/November 2014. The isolate was maintained on potato dextrose agar (PDA) (39.0 g of PDA, EMD Chemical, Burlington, MA) and malt extract agar (MEA) (33.6 g of MEA, MP Biomedicals, Santa Ana, CA). Cultures of isolate R-8 consistently produced dense mycelium and noticeable dark-pigmented secondary metabolites that diffused through both culture media and were often observed within 7 to 10 days (Fig. 1).

The identity of isolate R-8 was confirmed by polymerase chain reaction (PCR) amplification and analysis of the internal transcribed spacer (ITS) region from genomic DNA sample extracted from a 10-day-old culture grown on PDA. Extraction was conducted using the QIAGEN DNeasy Plant Tissue Mini extraction kit (QIAGEN, Valencia, CA) following the manufacturer's instructions, and the extracted DNA was stored at –20°C and used as template in the PCR amplifications.

PCR amplification was performed in a 10-µl reagent mixture contained 5 µl of TopTaq PCR Master Mix (Qiagen), 1.5 µl of a 5-µM solution of the forward primer ITS1F (5′-CTTGGTCATTTAGAG GAAGTAA-3′) (Gardes and Bruns 1993) and reverse primer ITS4R (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990), 1 µl of 10× CoralLoad, and 1 µl of the DNA template, and amplification was performed using an Eppendorf Mastercycler Pro PCR machine. The amplification protocol consisted of initial denaturation at 95°C for 3 min, followed by 35 cycles of 35 s denaturation at 95°C, 55 s annealing at 58°C, and 1 min extension at 72°C, and a final extension at 72°C for 10 min. Gel electrophoresis was performed to examine amplified products by loading 5 µl of PCR products on 1% agarose gels. The agarose was stained with ethidium bromide after 20 min of electrophoresis, and the resulting bands were visualized under ultraviolet illumination. PCR products were purified and sequenced at Genewiz (South Plainfield, NJ) (https://www.genewiz.com/).

The DNA sequence of the ITS region from isolate R-8 was compared with ITS sequences in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sixteen sequences (all available in GenBank) were included in multiple alignments and phylogenetic analysis (Table 1). Sequence alignments, editing, and analyses were conducted in MEGA 7.0 (Kumar et al. 2016). Molecular phylogenetic analysis and the evolutionary history was inferred by using the maximum likelihood method based on the Tamura–Nei model (Tamura and Nei 1993).

The sequenced ITS region from the unknown isolate R-8 (GenBank accession no. KT998902) and two other similar isolates, R-1 (GenBank accession no. KT998901) and R-10 (GenBank accession no. KT998903), found on loblolly pine bark had 98% similarity to *Acaromyces ingoldii* (GenBank accession no. NR07332, CBS 110050) that was originally isolated from citrus rust mites on grapefruit in Israel (Boekhout et al. 2003) (Table 1, Fig. 2).

To our knowledge, this would be the first known report of *A. ingoldii* isolates from loblolly pine.

Boekhout et al. (2003) described *A. ingoldii* as an anamorphic fungus, belonging phylogenetically to the Cryptobasidiaceae, Exobasidiomycetidae (Ustilaginomycetes, Basidiomycota) with septate hyphae, usually with the cytoplasm retracted in cells separated by lysed cells, and with sterigma-like outgrowths frequently occurring near the septa, giving rise to chains of fusiform blastoconidia. The fungus was further described as having thin aerial mycelium made up of blastoconidia, which gives the colony a velvety-pruinose appearance (Boekhout et al. 2003). Although *A. ingoldii* does not form blastoconidia in liquid media Sztejnberg et al. (2004), blastoconidia were obtainable when distilled water was added to Petri dishes with artificial media on which the fungus had developed.

Acaromyces is a monotypic yeast-like genus morphologically similar to "smut" fungi in the genera *Pseudozyma* and *Meira* (Exobasidiomycetes, Ustilaginomycotina), the latter of which is also associated with mites. Although phylogenetic analyses place *Meria* and *Acaromyces* within the Exobasidiales, *Acaromyces* spp. are placed in the family Cryptobasidiaceae, whereas *Meria* spp. are in the Brachybasidiaceae (Boekhout et al. 2003; Rush and Aime 2013).

Inhibition of *R. lauricola* Mycelial Growth

Four *R. lauricola* isolates were used in the study. Two isolates (LA1 and LA2) were obtained from sapwood tissues of a sassafras tree with laurel wilt near Bernice, Louisiana, in 2014 (Fraedrich et al. 2015), one isolate (TX1) was collected from a redbay tree in 2015 near Lumberton, Texas (Menard et al. 2016), and an isolate (HH5) was collected from redbay in 2005 at Hilton Head, South Carolina (Fraedrich et al. 2008).

The inhibitory activity of *A. ingoldii* (isolate R-8) against *R. lauricola* isolates was evaluated on PDA and repeated on MEA assays in plastic Petri dishes (Fisherbrand; 9-cm diameter) in the laboratory. Three treatments were evaluated: (i) *Raffaelea* plated 7 days after isolate R-8 was inoculated with 50 µl of spore suspension (~2.0×10^4 CFU/ml) around the perimeter of the culture plate to allow inward diffusion of secondary metabolites into the agar (Fig. 1); (ii) *Raffaelea* agar plug plated simultaneously with isolate R-8 (no secondary metabolites secreted into the agar prior to pairing); and (iii) *Raffaelea* agar plug plated alone (control). Each treatment had four replicates. Assay plates were incubated at ambient temperature of ~25°C, and mycelial diameter growth for the *R. lauricola* isolates was measured and recorded daily for 2 weeks. The rate of mycelial growth for each of the four isolates was subsequently calculated. An analysis of variance (ANOVA) of the factorial experimental data and the mean comparison among treatments, isolates, and assay media using standard error of mean (SEM) were conducted in SAS-JMP version 11 (SAS, Cary, NC).

The mycelial growth of *A. ingoldii* was noticeably faster on PDA than MEA (Fig. 3), and production of secondary metabolites, observed as a dark-pigmented compound released into agar, was consistently observed on both MEA and PDA media at 10 to 15 days after inoculation. However, secondary metabolite production was initiated 2 to 3 days sooner on PDA compared with MEA, perhaps owing to differences in the nutrient composition of the two media. The rate of *R. lauricola* mycelial growth differed among the three treatments evaluated on the two culture media (MEA and PDA); however, the pattern of mycelial growth responses to treatments was consistent for all four *R. lauricola* isolates (Fig. 3, Tables 2 and 3). Compared with other treatments, mycelial

growth for all *R. lauricola* isolates was completely inhibited on PDA and significantly reduced on MEA when secondary metabolites of *A. ingoldii* were present in the agar at the time that the fungi were plated (treatment A; Fig. 3). Among the *R. lauricola* isolates, growth of HH5 (interestingly, the oldest isolate) was faster than the three other *R. lauricola* isolates, whereas isolate TX1 was slightly slower than isolates LA1 and LA2 (Fig. 3). Results showed the same pattern of inhibitory activity was observed among all isolates

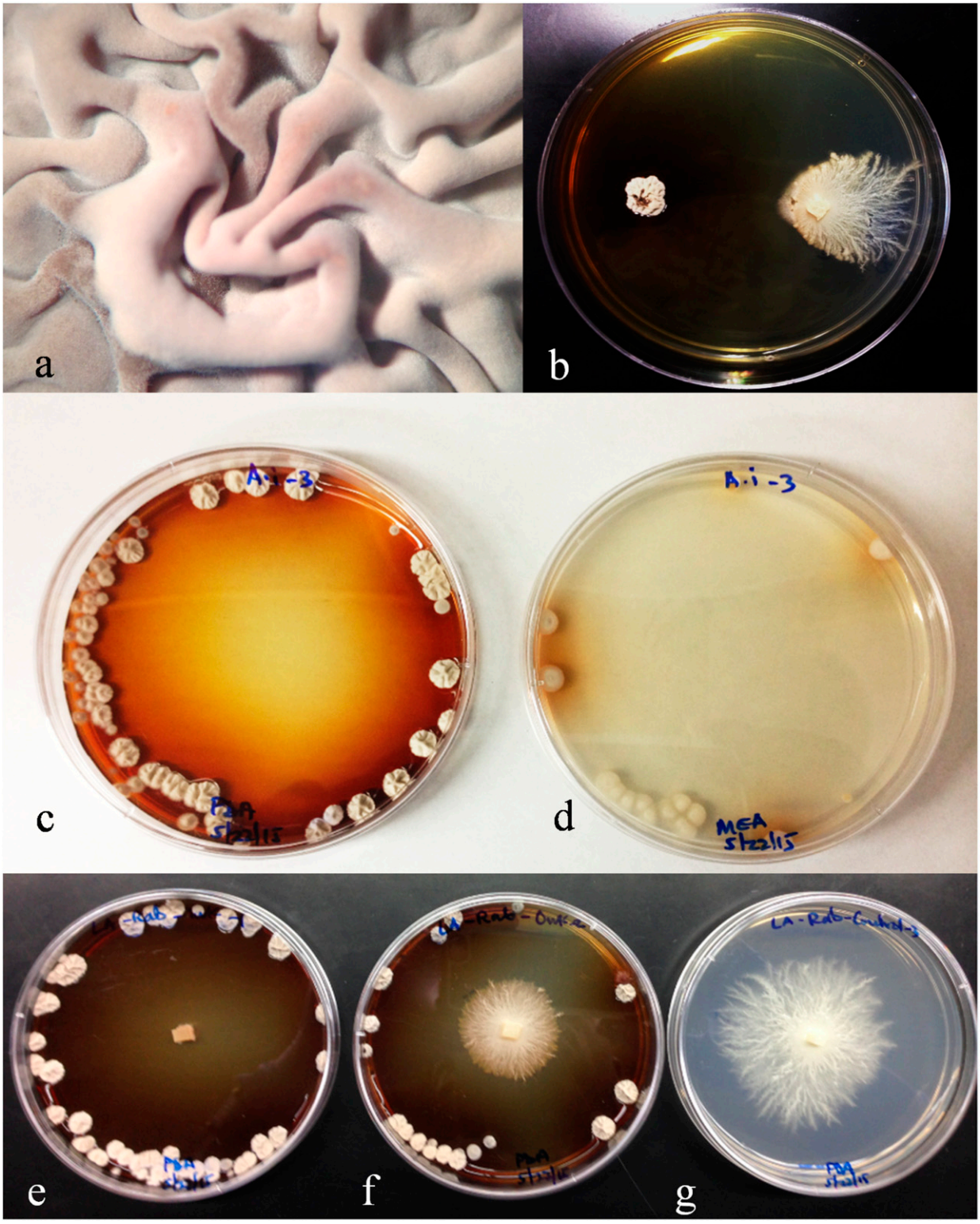

FIGURE 1

a, A culture of *Acaromyces ingoldii* (isolate R-8) mycelium observed under the dissecting microscope; **b,** inhibition of *Raffaelea lauricola* isolate by *A. ingoldii* secondary metabolites on potato dextrose agar (PDA); **c,** brown discoloration in PDA associated with diffusion of secondary metabolites from *A. ingoldii* on the PDA; **d,** growth of *A. ingoldii* and associated discoloration on malt extract agar; **e,** complete inhibition of *R. lauricola* (isolate LA) plated on PDA 7 days after *A. ingoldii*. **f,** partial inhibition of *R. lauricola* plated simultaneously with *A. ingoldii* on PDA; and **g,** *R. lauricola* isolate LA without *A. ingoldii* on a PDA.

(i.e., no growth of *R. lauricola* on PDA when *A. ingoldii* is previously established; reduced growth on PDA when *R. lauricola* and *X. ingoldii* are established at the same time). On MEA, *R. lauricola* isolates show reduced growth when *A. ingoldii* is previously established but no reduction in growth when *R. lauricola* and *A. ingoldii* are established at the same time (Tables 2 and 3).

There was no indication of direct competition between *A. ingoldii* and *R. lauricola* isolates when the fungi were plated simultaneously (treatment B); however, subsequent secondary metabolite production and diffusion through the agar significantly reduced the rate of mycelial growth (fungistatic effect) of all *R. lauricola* isolates on PDA. Plating *A. ingoldii* and *R. lauricola* simultaneously on MEA had no subsequent effect on mycelial growth of *R. lauricola* (Table 3). Secondary metabolites produced by *A. ingoldii* consistently and significantly reduced mycelial growth of *R. lauricola* with complete inhibition of mycelial growth on PDA. Initial *R. lauricola* mycelial growth on MEA was subsequently halted after 2 to 3 days owing to the fungistatic effect of secondary metabolites released into the agar by the *A. ingoldii* (Table 3).

Inhibition of *R. lauricola* Spore Germination

The effect of *A. ingoldii* (isolate R-8) secondary metabolites on the germination of *R. lauricola* spores was evaluated using different concentrations of crude extract obtained from liquid culture prepared by dissolving 2.4 g of potato dextrose broth (PDB) (Sigma-Aldrich, St. Louis, MO) in 100 ml of sterile deionized water in a 250-ml Pyrex Erlenmeyer flask (Pyrex, Greencastle, PA). Autoclaved PDB mixture was allowed to cool for 2 to 3 h and then inoculated with isolate R-8 agar plug. The culture was incubated at room temperature (~25°C) for 28 days, after which crude extract was decanted into sterile 15-ml conical tubes (Falcon Becton Dickson, Franklin Lakes, NJ) and centrifuged at 3,000 × *g* for 5 min. The supernatant was filtered using the Millex GP 0.22 μm syringe filter (Millipore, Carrigtwohill, Ireland) to eliminate any remaining suspended spores. For the spore germination test, five concentrations (100, 10, 1, 0.1, and 0%) of extract was obtained by serial dilution of filtrate with PDB in a sterile 96-well culture plate (Eppendorf AG, Hamburg, Germany). Sixteen replicates of each concentration (100-μl volume per well) were inoculated individually with 10 μl of *R. lauricola* (TX1) spore suspension. The assay plate was incubated at room temperature, and spore germination was evaluated after 72 h.

Direct exposure of *R. lauricola* spores to 10% or higher concentrations of *A. ingoldii* crude extracts resulted in complete

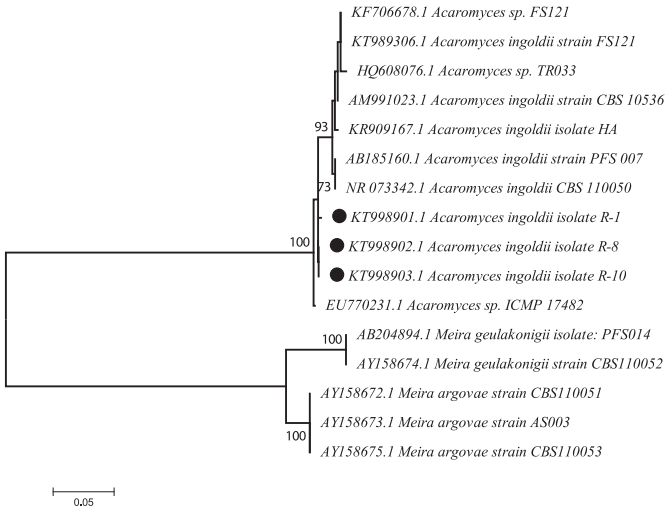

FIGURE 2

Molecular phylogenetic analysis by the maximum likelihood method. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura–Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (−1,252.7) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with superior log likelihood value. The tree was drawn with branch lengths measured in the number of substitutions per site. The analysis involved 16 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There was a total of 431 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7 (Kumar et al. 2016).

TABLE 1
Internal transcribed spacer (ITS) region sequences included in the phylogenetic analysis of the *Acaromyces ingoldii* isolate R-8 and closely related sequences in GenBank

GenBank number	Organism	Strain ID	Host/source	Origin
KT998901	*Acaromyces ingoldii*	R-1	*Pinus taeda*, Louisiana	U.S.A.
KT998902	*Acaromyces ingoldii*	R-8	*Pinus taeda*, Louisiana	U.S.A.
KT998903	*Acaromyces ingoldii*	R-10	*Pinus taeda*, Louisiana	U.S.A.
HQ608076	*Acaromyces sp.*	TR033	*Trachymyrmex septentrionalis* nest, Texas	U.S.A.
KT989306	*Acaromyces ingoldii*	FS121	Marine sediment, South China Sea	China
KF706678	*Acaromyces ingoldii*	FS121	Marine sediment, South China Sea	China
AM991023	*Acaromyces ingoldii*	CBS 10536	*Mangifera* sp.	Vietnam
KR909167	*Acaromyces ingoldii*	HA	*Vitis* sp. (grapevine wood)	France
NR073342	*Acaromyces ingoldii*	CBS 110050; AS001	Citrus rust mite on grapefruit	Israel
AB185160	*Acaromyces ingoldii*	PFS 007	Japanese pear fruit	Japan
EU770231	*Acaromyces sp.*	ICMP 17482	*Vitis* sp. (grapevine trunk)	New Zealand
AB204894	*Meira geulakonigii*	PFS014	Japanese pear fruit	Japan
AY158674	*Meira geulakonigii*	CBS110052; AS004	Citrus rust mite on grapefruit	Israel
AY158672	*Meira argovae*	CBS110051; AS002	Citrus rust mite on pummelo	Israel
AY158673	*Meira argovae*	AS003	Citrus rust mite on grapefruit	Israel
AY158675	*Meira argovae*	CBS110053; AS005	Carmine spider mite on castor bean	Israel

inhibition of *R. lauricola* spore germination and hyphae growth in culture plate assays, whereas spore germination and hyphae growth were observed at concentrations between 0 and 1% (Fig. 4).

Efficacy Against *R. lauricola* on Redbay Seedlings

The effect of different concentrations of *A. ingoldii* secondary metabolites extract on *R. lauricola* was evaluated on redbay seedlings in a growth chamber experiment (Fig. 5). Six treatments

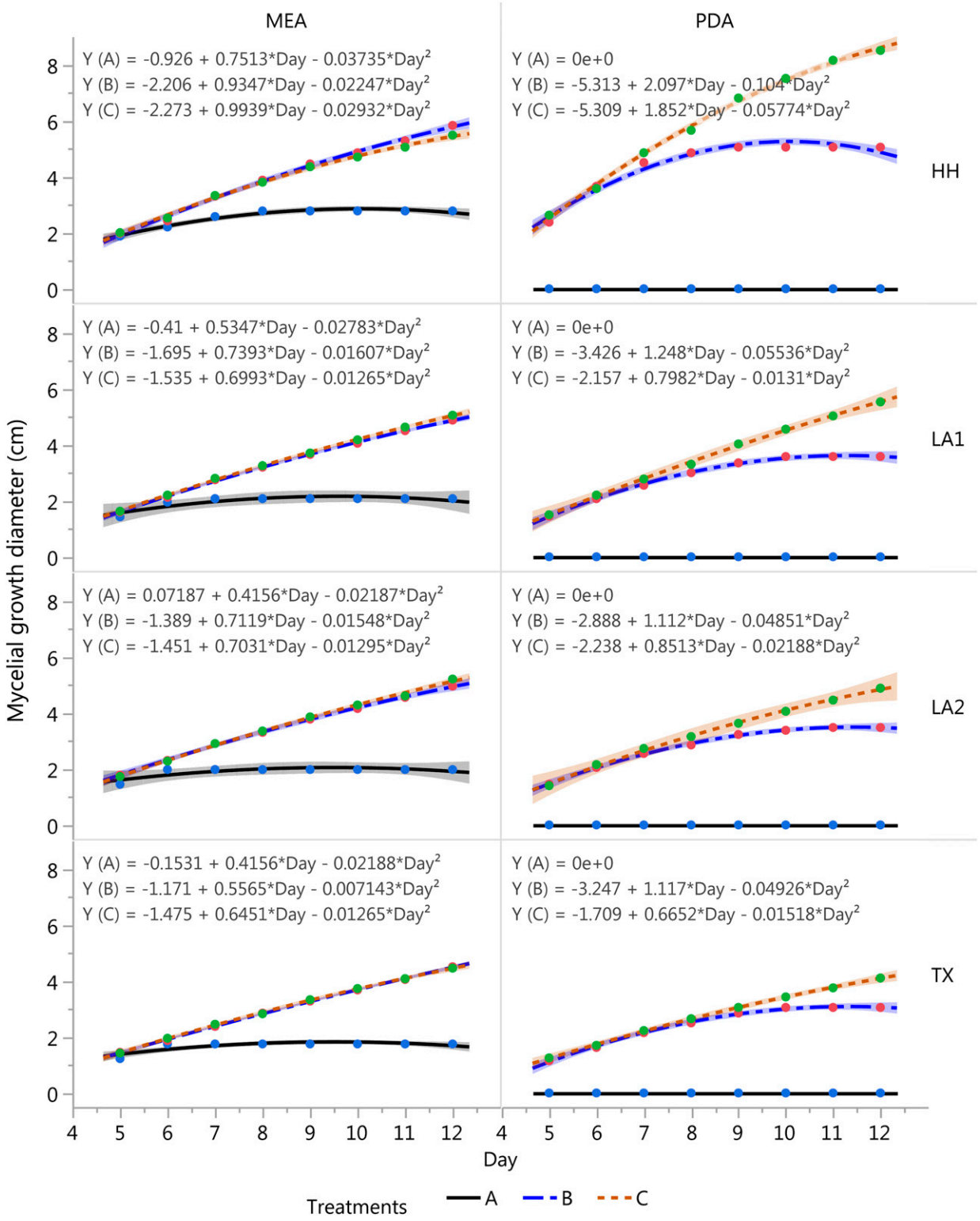

FIGURE 3

Acaromyces ingoldii treatment effects on the rate of mycelial growth of four *Raffaelea lauricola* isolates on malt extract agar and potato dextrose agar. Treatment A = *A. ingoldii* established in plate margins 7 days prior to inoculation of plates with *R. lauricola*; treatment B = plates simultaneously inoculated with *A. ingoldii* and *R. lauricola*; and treatment C = plates inoculated only with *R. lauricola*.

evaluated were (a) inoculation with only *R. lauricola* spores suspended in sterile water (RL + 0%), (b) inoculation with *R. lauricola* spores suspended in 10% extract (RL + 10%), (c) inoculation with *R. lauricola* spores suspended in 20% extract (RL + 20%), (d) inoculation with only sterile water (control), (e) inoculation with 10% extract, and (f) inoculation with 20% extract. Each treatment had four replicates, and the *R. lauricola* spore concentration used for inoculation was 20 µl of spore suspension (~1.0×10^5 CFU). The evaluation of laurel wilt symptoms on redbay seedlings was conducted by visual assessment 6 weeks postinoculation, based on four symptom categories: (i) no symptoms, (ii) chlorotic leaves, (iii) partial wilt of plants involving one or more branches, and (iv) total wilt of plants. The number of seedlings exhibiting symptoms per treatment was recorded and compared across the six treatments.

Results showed that redbay seedlings inoculated with *R. lauricola* spores suspended in 10% or 20% solutions of the *A. ingoldii* secondary metabolites did not prevent the development of laurel wilt in redbay plants. After 6 weeks, three out of four redbay seedlings inoculated with only *R. lauricola* (RL + 0%) wilted, and the fourth seedling developed noticeable symptoms including chlorotic leaves and wilting in lower branches. All four redbay seedlings inoculated with *R. lauricola* spores suspended in 10% *A. ingoldii* extract (RL + 10%) were symptomatic and completely wilted after 6 weeks, but only two of four seedlings inoculated with *R. lauricola* spores suspended in 20% *A. ingoldii* extract (RL + 20%) wilted, and the remaining two seedlings had very mild symptoms. These results suggest that that higher concentration of the extract (i.e., 20%) was better than lower concentration (10%); however, both concentrations were not lethal to *R. lauricola* and offered no significant long-term protections to seedlings evaluated in this study.

Fungistatic effects of the extract against *R. lauricola* spores that were observed during in vitro tests were not observed when *R. lauricola* with extracts were inoculated into seedlings, suggesting that *R. lauricola* can overcome any initial inhibition and still kill a plant. The extract dosage and mode of application may have been inadequate for several reasons, including further dilution of the extract within the xylem that provided a less than optimal concentration as spores moved beyond the inoculation point or the possible loss of potency of the extract within the host over time. These factors could have rendered the *A. ingoldii* crude extract to be less effective against *R. lauricola* for inhibition to occur. However, the results also demonstrate the resilience of *R. lauricola* as a pathogen, indicating a consistent and considerable level of exposure to a potent active ingredient would be needed to prevent infection and laurel wilt disease establishment.

It is possible that a prophylactic treatment of redbay seedlings with live *A. ingoldii* spores several weeks ahead of a *R. lauricola* infection could offer an enduring protection as opposed to a single application of extract. In fact, the use of *A. ingoldii*, an endophytic fungal antagonist, may be more effective as a biocontrol agent through natural production of metabolites as it grows within the host tissue in response to an intruding *R. lauricola* pathogen. Hence, additional studies will be required to examine the best approach to

TABLE 3
Effect of *Acaromyces ingoldii* treatments on mycelial growth of four *Raffaelea lauricola* isolates on malt extract agar (MEA) and potato dextrose agar (PDA) after 12 days

Isolate	Treatment[y]	Growth on MEA, mean ± SEM (cm)[z]	Growth on PDA, mean ± SEM (cm)[z]
HH5	A	2.8 ± 0.1 a	0.0 ± 0.0 a
	B	5.8 ± 0.2 b	5.1 ± 0.1 b
	C	5.5 ± 0.1 b	8.5 ± 0.0 c
LA1	A	2.1 ± 0.2 a	0.0 ± 0.0 a
	B	4.9 ± 0.1 b	3.6 ± 0.1 b
	C	5.1 ± 0.1 b	5.5 ± 0.3 c
LA2	A	2.0 ± 0.2 a	0.0 ± 0.0 a
	B	5.0 ± 0.2 b	3.5 ± 0.1 b
	C	5.2 ± 0.1 b	4.9 ± 0.4 c
TX1	A	1.8 ± 0.1 a	0.0 ± 0.0 a
	B	4.5 ± 0.0 b	3.1 ± 0.1 b
	C	4.5 ± 0.1 b	4.1 ± 0.2 c

[y] Treatments: A = *R. lauricola* plated 7 days after *A. ingoldii* (isolate R-8) was inoculated with 50 µl of spore suspension (~2.0×10^4 CFU/ml) around the perimeter of the culture plate to allow inward diffusion of secondary metabolites into the agar; B = *R. lauricola* agar plug plated simultaneously with *A. ingoldii* (no secondary metabolites secreted into the agar prior to pairing); and C = *R. lauricola* agar plug plated alone (control). Each treatment had four replicates.

[z] Mean and standard error of mean (SEM) not followed by same letter are significantly different within column (i.e., within medium type).

TABLE 2
Analysis of variance (ANOVA) and effect tests of *Acaromyces ingoldii* (isolate R-8) on the mycelial growth of four *Raffaelea lauricola* isolates after 12 days on malt extract agar and potato dextrose agar

Source	DF[z]	Sum of squares	Mean square	*F* ratio	Prob. > *F*
ANOVA					
Model	23	444.1	19.3	216.0	<0.0001
Error	72	6.4	0.1		
Cumulative total	95	450.5			
Effect tests					
Treatments (T)	2	328.9		1,840.0	<0.0001
Media (M)	1	19.3		215.5	<0.0001
Isolates (I)	3	34.5		128.7	<0.0001
T × M	2	34.0		190.2	<0.0001
T × I	6	10.4		19.3	<0.0001
M × I	3	4.4		16.5	<0.0001
T × M × I	6	12.6		23.5	<0.0001

[z] DF = degrees of freedom.

optimize the efficacy of *A. ingoldii* in future greenhouse and field application experiments.

Potential Application in Disease Management

Findings from this study suggest potential uses for the secondary metabolites produced by *A. ingoldii* against *R. lauricola*, the cause of laurel wilt. Previous observations, along with the results from the current study, highlight the importance of secondary metabolites associated with *Acaromyces* species on other organisms. We observed that secondary metabolites produced by *A. ingoldii* (isolate R-8) consistently and significantly reduced the mycelial growth of *R. lauricola* isolates. We also observed 100% mycelial growth inhibition in all four *R. lauricola* isolates evaluated against R-8 secondary metabolites on PDA and found a significant reduction in the mycelial growth of the four isolates on MEA after 12 days, when *R. lauricola* was plated 7 days after *A. ingoldii* (isolate R-8) was inoculated. Faster production of secondary metabolites on PDA than MEA most likely accounted for the early inhibition of mycelial growth on PDA. The rapid mycelial growth of *R. lauricola* that was initially noted on MEA was subsequently halted after 2 to 3 days owing to lagging fungistatic effects of secondary metabolites being produced by the *Acaromyces* isolate R-8. Direct competition between *A. ingoldii* and *R. lauricola* was minimal on PDA and absent MEA.

A. ingoldii has been reported across diverse habitats and environments worldwide (Table 1). In fact, the isolate evaluated in the current study was discovered from a loblolly pine bolt during assessments of fungi associated with bark beetles, suggesting *A.*

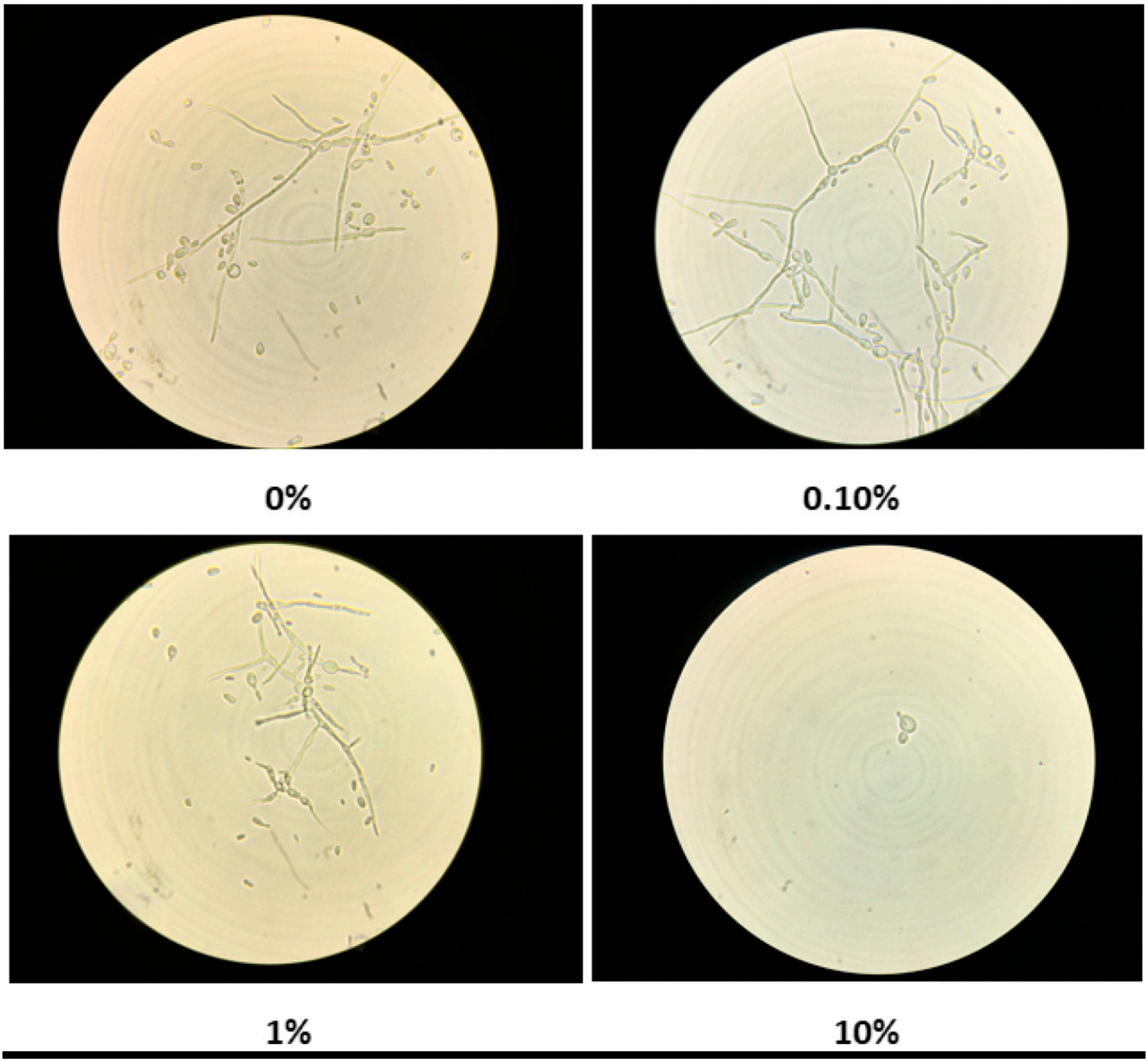

FIGURE 4

Microscopic assessments of the effect of varying concentrations of *Acaromyces ingoldii* crude extract (0 to 100%) on spore germination of *Raffaelea lauricola* (isolate TX1) after 72-h incubation in a 96-well culture plate. No spore germination was found in the crude extract at 10 and 100% concentrations.

ingoldii could be associated with galleries of bark beetles that colonize southern pines, a new niche for *A. ingoldii* that has never been reported. Is the fungus protecting the beetles from mites, or could the fungus be detrimental to the beetles? Currently, the role the fungus might be playing is unclear; hence, further investigation would be needed. Interestingly, the fungus has been also isolated from nesting cavities of the red-cockaded woodpecker (RCW; *Leuconotopicus borealis* Vieillot), an endangered bird species in the southeastern United States that specializes in excavating cavities into the heartwood of living pines. Jusino et al. (2015) found that cavities being excavated by RCWs have shown distinct fungal communities that commonly include *A. ingoldii*, which may be beneficial to the woodpeckers by parasitizing mites or inhibiting fungi that could be detrimental to the RCW.

FIGURE 5

a, *Raffaelea lauricola* spores suspended in different concentrations of *Acaromyces ingoldii* (isolate R-8) secondary metabolites crude extract; and **b,** some redbay seedlings inoculated with *R. lauricola* spores in the growth chamber.

Historically, *Acaromyces* species have been thought to be pathogens of various mite species. Bekker (1940) described *Acaromyces glycyphagi* as a parasite of the mite *Glycyphagus destructor* (Schrank), and it was associated with a high rate of mite mortality. Boekhout et al. (2003) reported a high mortality rate among several mite species after inoculation with *A. ingoldii.* Subsequent studies (Gerson et al. 2008; Paz et al. 2007) confirmed that *A. ingoldii* possessed potent biocontrol capabilities against mite species and showed that toxic chemicals secreted by the fungus were lethal to mites. Paz et al. (2007) also found *A. ingoldii*, and two other fungi, *Meira geulakonigii* and *Meira argovae*, caused high mortality in five herbivorous mite species within 1 to 2 weeks after application.

A. ingoldii has been found to inhibit the growth of other phytopathogenic fungi, and it was suggested that competition for hydrocarbons could be responsible for the inhibitory activity, although the antagonistic effect exhibited by *A. ingoldii* against mites and fungi has also been attributed to secreted toxins, as confirmed by exposing sclerotia of *Sclerotinia sclerotiorum* and *S. rolfsii* to crude extracts of the fungus (Gerson et al. 2005; Kushnir et al. 2011; Sztejnberg et al. 2004). *A. ingoldii* was associated with pears in Japan, where it was reported to be causing a stain in immature fruit (Yasuda et al. 2005); however, there was no indication that the fungus was responsible for any damage. The fungus has been also associated with other fruit tree crops in France (Travadon et al. 2016), Israel (Boekhout et al. 2003), New Zealand (https://www.ncbi.nlm.nih.gov/nuccore/EU770231), and Vietnam (https://www.ncbi.nlm.nih.gov/nuccore/AM991023) and was found in marine sediment in the South China Sea (Gao et al. 2016). One could argue that with its ability to establish in such diverse habitats and with its unique inhibitory properties as observed, *A. ingoldii* might potentially be a good biocontrol agent.

The preliminary analysis of secondary metabolites from *A. ingoldii* in the current study yielded cryptosporin, coryoctalactone D, and a third compound similar to cryptosporin not yet fully identified (Olatinwo, unpublished). Gao et al. (2016) showed that secondary metabolites from some *A. ingoldii* isolates have yielded a new naphtha-[2,3-b] pyrandione analog, acaromycin A, and a new thiazole analog, acaromyester A, as well as the previously known compound (+)-cryptosporin. Interestingly, studies with these metabolites have shown they have significant activities against tumor cells (Gao et al. 2016).

The current study represents a preliminary effort to identify microorganisms with natural antifungal compounds with fungicidal/fungistatic activities against *R. lauricola*, which could be potentially useful as part of integrated management program to limit further spread of laurel wilt. The fungistatic effect of *A. ingoldii* demonstrated in this study and on a broad range of fungi observed in the laboratory (Olatinwo, unpublished) suggest the secondary metabolites present in the crude extracts may have potential utility in managing *R. lauricola* and perhaps laurel wilt disease in high-value susceptible host tree species such as in commercial avocado groves in California and Florida. At this point, successful application strategies and tactics using the secondary metabolites are unknown and will depend on many factors. Further investigation will be needed to characterize specific composition of the *A. ingoldii* secondary metabolites and to examine the efficacy, specificity, effective deployment methods, and other potential benefits that the *Acaromyces* species may offer for potential disease management.

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