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RESEARCH ARTICLE

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Effect of *Acaromyces Ingoldii* Secondary Metabolites on the Growth of Brown-Rot (*Gloeophyllum Trabeum*) and White-Rot (*Trametes Versicolor*) Fungi

Rabiu Olatinwo[a], Chi-Leung So[b] and Thomas L. Eberhardt[c]

[a]USDA Forest Service, Southern Research Station, Pineville, LA, USA; [b]School of Renewable Natural Resources, LSU AgCenter, Baton Rouge, LA, USA; [c]USDA Forest Service, Forest Products Laboratory, Madison, WI, USA

ABSTRACT

We investigated the antifungal activities of an endophytic fungus identified as *Acaromyces ingoldii*, found on a loblolly (*Pinus taeda* L.) pine bolt in Louisiana during routine laboratory microbial isolations. The specific objectives were to determine the inhibitory properties of *A. ingoldii* secondary metabolites (crude extract) on the mycelial growth of a brown-rot fungus *Gloeophyllum trabeum* and a white-rot fungus *Trametes versicolor*, and to determine the effective concentration of *A. ingoldii* crude preparation against the two decay fungi *in vitro*. Results show the crude preparation of *A. ingoldii* from liquid culture possesses significant mycelial growth inhibitory properties that are concentration dependent against the brown-rot and white-rot fungi evaluated. An increase in the concentration of *A. ingoldii* secondary metabolites significantly decreased the mycelial growth of both wood decay fungi. *G. trabeum* was more sensitive to the inhibitory effect of the secondary metabolites than *T. versicolor*. Identification of specific *A. ingoldii* secondary metabolites, and analysis of their efficacy/specificity warrants further study. Findings from this work may provide the first indication of useful roles for *Acaromyces* species in a forest environment, and perhaps a future potential in the development of biocontrol-based wood preservation systems.

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Antifungal index; endophyte; fungal inhibition; wood decay

1. Introduction

Acaromyces ingoldii is an endophytic fungus belonging phylogenetically to the Cryptobasidiaceae, Exobasidiomycetidae (Ustilaginomycetes, Basidiomycota), and has been isolated from diverse habitats worldwide, from mite cadavers from the coastal plain of Israel [1–4], to marine sediment in the South China Sea [5]. Boekhout et al. [1] reported a high mortality rate among several mite species after inoculation with *A. ingoldii*. Subsequent studies [2,3] confirmed that *A. ingoldii* possesses potent biocontrol capabilities against mite species and showed that toxic chemicals secreted by the fungus were lethal to mites. Since marine-derived fungi have yielded a variety of biologically active compounds, Gao et al. [5] conducted *in vitro* testing of specific compounds isolated from *A. ingoldii* against tumor cells and demonstrated significant tumor growth inhibition.

Recently, *A. ingoldii* was identified among the fungi found in the nesting cavities of the red-cockaded woodpecker (RCW; *Leuconotopicus borealis* Vieillot), inside the heartwood of living longleaf pine (*Pinus palustris* Mill.) trees. Given *A. ingoldii* toxicity towards mites [3], it was suggested that its presence in the nesting cavity could attack either feather mites on the birds, or alternatively, mites that feed on fungi that facilitate cavity excavation through wood decay [6]; the possibility also exists that *A. ingoldii* may directly inhibit the growth of fungi that are "detrimental" to the birds. Laboratory studies with fungal cultures have shown secretions from *A. ingoldii* inhibit the growth of phytopathogens found in soil, foliage and fruit [4].

In our laboratory, *A. ingoldii* was isolated and identified during a routine laboratory screening for fungal species associated with bark beetles found in the pine forests of the southeastern US. The source of the *A. ingoldii* was a loblolly (*Pinus taeda* L.) pine bolt taken from a beetle-infested tree in the Kisatchie National Forest (Louisiana). The *A. ingoldii* isolate in culture was found to secrete dark-pigmented secondary metabolites into the culture media. Given the aforementioned growth inhibitory activities, particularly those involving fungi, it remained to be determined if these secreted metabolites affect the growth of wood decay fungi. The brown-rot fungi, a representative example being

CONTACT Rabiu Olatinwo rabiu.o.olatinwo@usda.gov

Gloeophyllum trabeum, mainly attack softwoods (conifers) by typically degrading wood polysaccharides (cellulose and hemicelluloses), and modifying the lignin, which remains as a polymeric residue in the decaying wood [7]. White-rot fungi, a representative example being *Trametes versicolor*, mainly attack hardwoods, and typically degrade all the major components of wood including cellulose, hemicelluloses, and lignin [8]. Natural products from plants and other sources may unlock access to novel fungicides and potentially serve as alternatives to synthetic pesticides for controlling wood decay fungi [9].

From an ecological perspective, evaluating the fungicidal or fungistatic properties of the *A. ingoldii* isolate against wood-decaying fungi could be useful in the elucidation of its role in wood decay environments in which *A. ingoldii* is found, specifically, the nesting cavities of the RCW. Alternatively, such an assessment provides an initial step in the development of biocontrol-based wood preservative systems. Our objectives were to (1) determine the inhibitory activities of *A. ingoldii* secondary metabolites (crude extract) on the mycelial growth of wood decay fungi and (2) evaluate the sensitivity of *T. versicolor* and *G. trabeum* to the crude extract in assessing if there is any species specificity in the inhibitory activities of *A. ingoldii*.

2. Materials and methods

2.1. Fungi

The brown-rot (*Gloeophyllum trabeum* Mad-617, ATCC-11539) and white-rot (*Trametes versicolor* Mad-697, ATCC-42462) strains used in this study were provided by the USDA Forest Products Laboratory (Madison, WI, USA). Cultures were grown and maintained on a medium containing 2% malt extract, 1.5% agar, and 0.005% yeast extract. *Acaromyces ingoldii* R-8 was isolated in 2014 from a bolt collected during an investigation of fungal species associated with bark beetles infesting loblolly pine trees on the Kisatchie National Forest (Rapides Parish, LA, USA). The identification of the isolate R-8 was confirmed by PCR amplification and analysis of the internal transcribed spacer (ITS) region from genomic DNA extracted from a 10-day-old culture grown on potato dextrose agar, PDA (EMD Chemical Inc. Gibbstown, NJ, USA). PCR amplification was conducted using the forward primer ITS1F (5′CTTGGTCATTTAGAGGAAGTAA′3) [10] and reverse primer ITS4R (5′-TCCTCCGCTTA TTGATATGC′3) [11]. The sequenced and amplified PCR product from the ITS region (GenBank Accession No. KT998902), with 98% similarity with the CBS 10050 strain (GenBank Accession No. NR_073342.1), was submitted to the National Center for Biotechnology Information (NCBI) GenBank database.

Analysis of chemical compounds in the secondary metabolites of *A. ingoldii R-8* crude preparation was conducted by the Natural Products Discovery Group, the University of Oklahoma, Norman OK (http://npdg.ou.edu/).

2.2. Antifungal assays

Experiments on the inhibitory effect of *A. ingoldii* R-8 secondary metabolites were conducted using a crude preparation from a liquid culture of the fungus. The liquid medium was prepared by dissolving potato dextrose broth, PDB (2.4 g, Sigma-Aldrich, St. Louis, MO, USA) in de-ionized water (100 ml) in an Erlenmeyer flask (250 ml) capped with aluminum foil. Autoclaved PDB medium was allowed to cool for 2–3 hours before being inoculated with one agar plug (5 mm) from a 10-day-old maintenance agar plate of *A. ingoldii* R-8; then, the liquid culture was shielded from light and incubated on the bench at room temperature (RT, *ca.* 25 °C) without shaking for 28 days (duration based on preliminary experiments) and then processed further to generate the crude preparation containing secreted secondary metabolites. Under aseptic conditions, the liquid culture was decanted into sterile 15 ml conical tubes (Falcon Becton Dickson, NJ, USA), and centrifuged at $3000 \times g$ for 5 min; the supernatant was then filtered using a sterile Millex ® GP 0.22 µm syringe filter (Millipore, Dublin, Ireland) to eliminate any remaining suspended spores. The effect of the *A. ingoldii* R-8 secondary metabolites on the mycelial growth, for both *G. trabeum* and *T. versicolor,* was evaluated at different concentrations of the crude preparation in two experimental setups.

In the first experiment, the mycelial growths of the two wood decay fungi were screened to determine fungistatic activities of the crude preparation at five concentrations (100, 10, 1, 0.1 and 0.01%, v/v) of *A. ingoldii* R-8 secondary metabolites obtained by serial dilution of the spent PDB medium with fresh PDB medium followed by dispensing into the wells of a sterile standard size 24-well culture plate (Falcon Becton Dickson, NJ, USA). Undiluted PDB medium dispensed into separate wells served as the control. Four replicates of each concentration (2 ml volume per well, leaving enough space for fungal growth) and the PDB medium control was inoculated individually with $4 \times 4\ mm^2$ square plug of actively growing *T. versicolor* mycelium. The same concentration assay was prepared for *G. trabeum*. Culture plates were incubated for four weeks at RT and visual assessments of culture plates for mycelial growth

were conducted daily. The 24 well plate experiment was then repeated.

In the second experiment, the mycelial diameter growth was measured to determine the antifungal index of *A. ingoldii* secondary metabolites in a narrower range of concentrations. To do this, the potato dextrose agar (PDA) medium was amended with six different concentrations (10, 5, 2.5, 1, 0.5, and 0.1% v/v) of *A. ingoldii* crude preparation in 100 × 15 mm sterile polystyrene petri dishes (Falcon Becton Dickson, NJ, USA). Five replicates were prepared for each concentration of the secondary metabolite preparation and the PDA control (0% v/v). Each petri dish was inoculated at the center with a 5 mm actively growing *G. trabeum* mycelial plug; the same setup was conducted for *T. versicolor*. Culture plates were incubated at RT. Mycelial growth diameter (cm) on each individual plate was measured daily by visual assessments using a dissecting microscope (AmScope SM-1BZ-FRL). Measurements continued until fungal mycelia reached the edges of the petri dishes for at least one of the controls, then, the antifungal index was determined, expressed as the percentage of fungal growth inhibition by the following equation:

$$Antifungal\ Index(AI,\ \%) = (1 - D_a/D_c) \times 100$$

where D_a is diameter radial growth of mycelium on the amended plate, and D_c is diameter radial growth of mycelium on the control plate.

2.3. Statistical analyses

The mean comparison of antifungal indexes were conducted in SAS-JMP v13 (SAS Inc., NC, USA). Significant differences ($p < .05$) were determined by using the Student's t-test.

3. Results and discussion

Chemical analysis of secondary metabolites from the *A. ingoldii R-8* crude preparation found three compounds; cryptosporin, coryoctalactone D, (Figure 1) and a third compound similar to cryptosporin that was not yet fully solved. Results from the 24-well culture plate experiment (Figure 2) showed that after 21 days there was no growth for either the brown-rot (*G. trabeum*) or white-rot (*T. versicolor*) decay fungi in wells with ≥1% or ≥10% concentrations of the secondary metabolites (crude extract), respectively. Thus, the presence of *A. ingoldii* secondary metabolites demonstrated growth inhibitory effects on wood decay fungi. Based on these results, the highest concentration of *A. ingoldii* preparation used in the PDA medium was 10%.

Results from the PDA medium amended with *A. ingoldii* crude preparation, in petri dishes, showed significant inhibition of *G. trabeum*. The mycelial growth reached the edge of the petri dish in both the control and 0.1% relative concentration after 12 days. However, growth of *G. trabeum* was completely inhibited at 5 and 10%. Inhibition of *T. versicolor* mycelial growth diameter was observed at relative concentrations of 0.5% and above. After 10 days, white-rot mycelial growth reached the edge

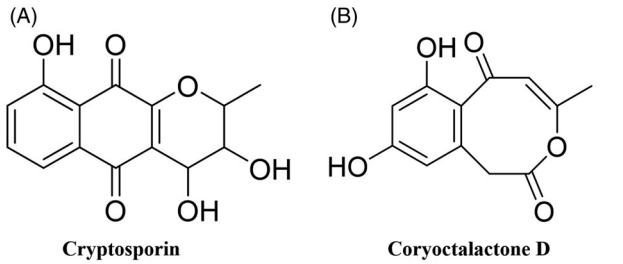

Figure 1. Chemical structures of two compounds; (A) cryptosporin and (B) coryoctalactone D were identified among *A. ingoldii* secondary metabolites.

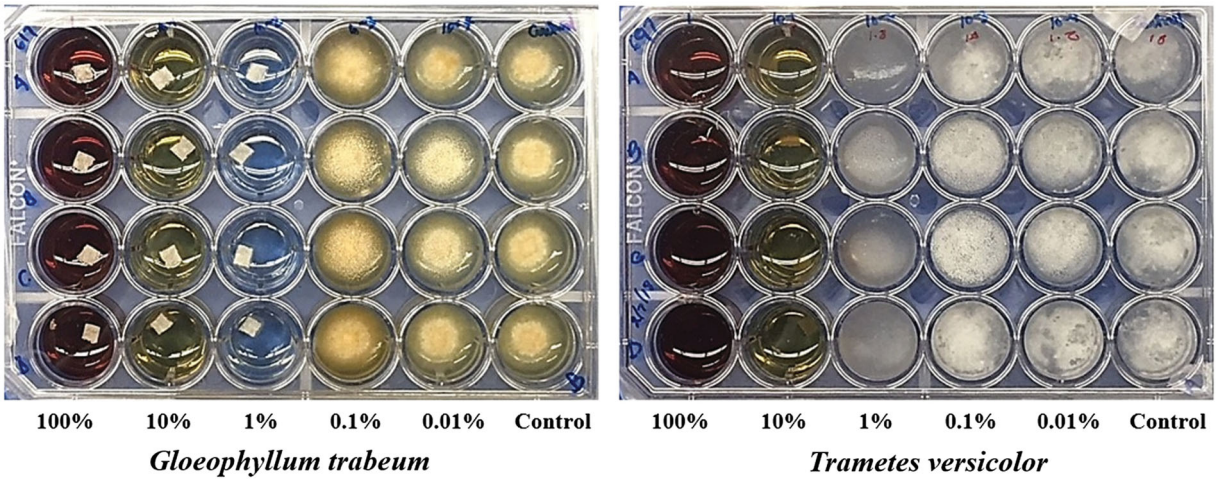

Figure 2. Effect of *A. ingoldii* secondary metabolites concentrations on growths of *G. trabeum* (Mad-617) and *T. versicolor* (Mad-697) in 24-well potato dextrose broth (PDB) assay.

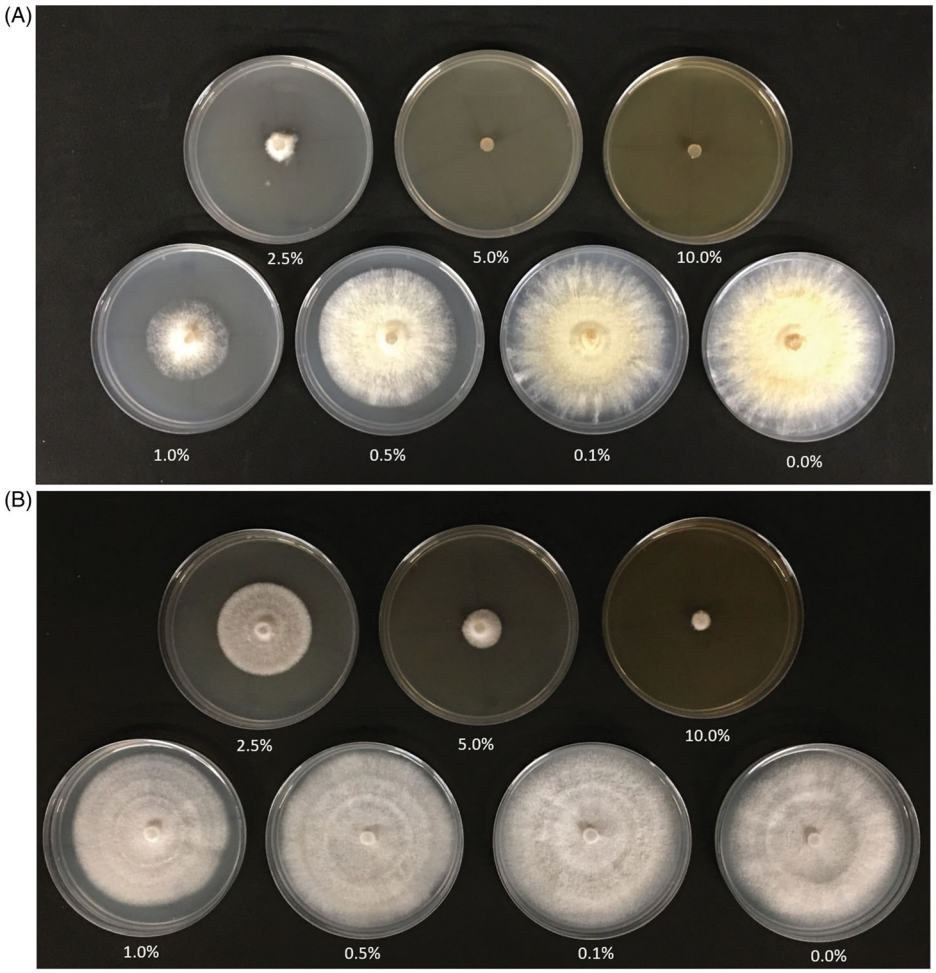

Figure 3. Effect of *A. ingoldii* secondary metabolites concentrations on growths of (A) *G. trabeum* (Mad-617) and (B) *T. versicolor* (Mad-697) on amended potato dextrose agar (PDA) medium with six different concentrations (10, 5, 2.5, 1, 0.5, and 0.1% v/v) of A. ingoldii crude preparation and the unamended PDA control (0%) after 10 days.

Table 1. Differences in the antifungal index (%) of *A. ingoldii* secondary metabolites on *G. trabeum* (Mad-617) and *T. versicolor* (Mad-697) at different concentrations of amended potato dextrose agar (PDA).

Species	Mean Antifungal Index (%) at amended PDA concentrations*					
	10.0%	5.0%	2.5%	1.0%	0.5%	0.1%
Gloeophyllum trabeum	100.0 a	100.0 a	85.8 a	53.5 a	19.8 a	2.3 a
Trametes versicolor	91.3 b	81.8 b	50.3 b	17.0 b	3.8 b	0.0 a
p value	.0006	.0001	.0007	.0003	.0054	.0705

*Column levels not connected by same letter are significantly different according to the Student's *t*-test.

of the petri dish in the control; growth was observed at all concentrations, including 10%. Statistically significant differences in the mycelial growth inhibition (antifungal index) observed at most relative concentrations *of A. ingoldii* extract showed *G. trabeum* growth was more sensitive to the secondary metabolites than *T. versicolor*.

In side-by-side comparisons, antifungal properties of *A. ingoldii* secondary metabolites have a greater effect on *G. trabeum* than *T. versicolor* at any given concentration (Table 1). Specifically, the antifungal index effect of *A. ingoldii* secondary metabolites on *T. versicolor* growth at 0.1% relative concentration was 0%, compared to 2.3% for *G. trabeum*. While at 0.5% relative concentration, the inhibitory effect on *T. versicolor* and *G. trabeum* mycelial growths were 3.8% and 19.8%, respectively. This increases to 17.0% and 53.5% at 1%, and 50.3% and 85.8% at 2.5%, respectively. Although not completely inhibited, the antifungal index effect on *T. versicolor* growth reached 91.3% at 10%. While at 5 and 10%, the inhibition of *G. trabeum* was complete (antifungal index = 100%). Using the lower secondary metabolite concentrations, the growth patterns of the fungi during the experiment (Figure 4) were seemingly linear to the point of reaching maximum growth (edge of plate).

In this study, the crude preparation of *A. ingoldii* from liquid culture showed significant mycelial growth inhibitory properties (antifungal index) that is concentration dependent against the brown-rot and white-rot fungi evaluated. An increase in the

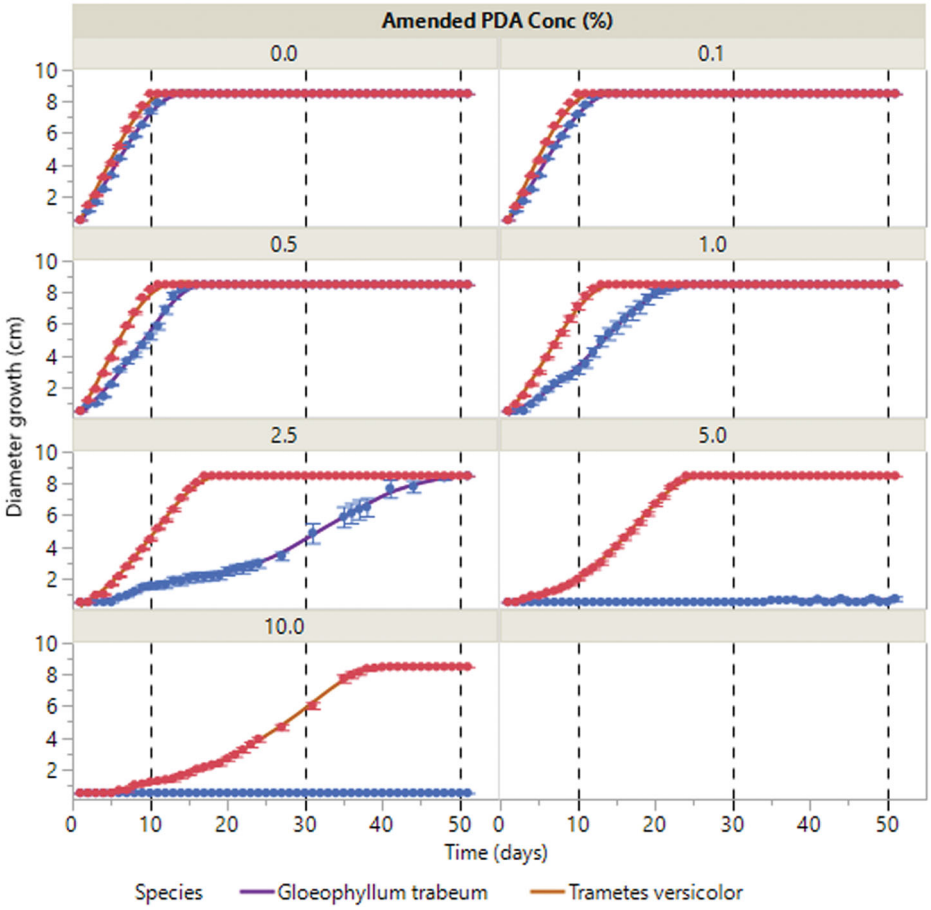

Figure 4. Growth rate of *G. trabeum* (Mad-617) and *T. versicolor* (Mad-697) on potato dextrose agar (PDA) medium amended with six different concentrations (10, 5, 2.5, 1, 0.5, and 0.1% v/v) of *A. ingoldii* crude preparation.

concentration of *A. ingoldii* secondary metabolites significantly decreased the mycelial growth of both wood decay fungi. Olatinwo and Fraedrich [10,11] found that a crude preparation from *A. ingoldii* inhibited the laurel wilt pathogen, *Raffaelea lauricola, in vitro*. Laurel wilt is an important disease that affects members of the Lauraceae including sassafras (*Sassafras albidum*) [12]. Similarly, a crude extract from the basidiomycete *Quambalaria cyanescens* was also found to have antifungal activities [13]. In that study, chromatography was used to isolate 3 napthoquinone-derived compounds. In the aforementioned study by Gao et al. [5] on *A. ingoldii* isolates, secondary metabolites included 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, which is one of three compounds identified from chemical analysis of the secondary metabolites from the *A. ingoldii* crude preparation in the present study. The specific contributions of cryptosporin and coryoctalactone D in the observed antifungal activities will be investigated in future studies.

Results show that the brown-rot fungus (*G. trabeum*) was more sensitive to the inhibitory effect of the secondary metabolites than the white-rot fungus (*T. versicolor*). This result is intriguing in terms of the discovery of *A. ingoldii* in the nesting cavities excavated in longleaf pine trees by the RCW [6]. It remains to be determined if the greater sensitivity of the brown-rot fungus is by default, based on the fact the brown-rot fungi are normally associated with the decay of softwood species; or, if this apparent greater sensitivity of a brown-rot fungus is simply a matter of chance. Another fungus associated with softwood species, the pathogen *Porodaelalea pini*, was among the 5 most common taxa found in the nesting cavities [6]. If the frequency of this heart rot fungus is an indication of limited inhibition by *A. ingoldii* and its metabolites, then the fungal interactions that occur in nesting cavities are indeed complex. It has been hypothesized that *A. ingoldii* could attack fungi detrimental to the woodpeckers [6]. We are not aware of any study suggesting that brown-rot fungi present in nesting cavities would be a competitive detriment to pathogens such as *P. pini*. Also, it is seemingly counterintuitive to have fungal interactions in nesting cavities that inhibit the growth of brown-rot fungi with the capacity to facilitate RCW excavations.

Finally, the results from the current study indicate that compounds contained in the secondary metabolites of an *A. ingoldii* crude extract may have useful applications in protection and preservation of

wood products. Indeed, research on biocontrol measures has shown crude preparations of fungal metabolites to be antagonistic to wood decay fungi and molds [14,15].

4. Conclusions

Differences in the inhibition of brown-rot and white-rot fungi by *A. ingoldii* secondary metabolites were observed, but the significance to fungal interactions in nature remains to be resolved. Additional investigations are also needed to determine the specific compositions of the secondary metabolites to enable further analysis of the efficacy, specificity, and other benefits that *Acaromyces* species may offer in the development of biocontrol-based wood preservation systems.

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Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Rabiu Olatinwo http://orcid.org/0000-0002-4075-243X

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