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Fungal symbionts of bark and ambrosia beetles can suppress decomposition of pine sapwood by competing with wood-decay fungi

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ABSTRACT

Bark and ambrosia beetles inoculate dying trees with symbiotic fungi. The effects of these fungi on wood decomposition are poorly understood. We determined the effects of three widespread Ascomycota symbionts and one introduced Basidiomycota symbiont on the decomposition of loblolly pine (*Pinus taeda*) sapwood. Ascomycetes caused <5% mass loss and no visible structural degradation, whereas the basidiomycete *Flavodon ambrosius* caused nearly 15% mass loss and visible structural degradation similar to free-living wood-decay fungi. *Ophiostoma ips* and *Raffaelea fusca* reduced white- and brown-rot decay through competition with *Ganoderma curtisii* and *Phaeolus schweinitzii*, respectively. The inhibitory effects of *O. ips and R. fusca* on decay were negated when co-inoculated with *F. ambrosius* suggesting that the spread of this invader could influence forest carbon cycles. In contrast to the predominant forest biology narrative, the common and widespread ophiostomatalean symbionts of bark and ambrosia beetles studied here appear to delay, rather than facilitate tree biomass recycling.

1. Introduction

Wood comprises as much as 98% of the living biomass in forests (Fittkau and Klinge, 1973) and is mostly composed of lignocellulose which requires non-enzymatic and specialized enzyme suites for biological decomposition. The ability to decompose wood is possessed primarily by fungi within the phylum Basidiomycota (Blanchette, 1991; Floudas et al., 2012). Decay fungi are essential to forest productivity and biodiversity because they release the immense stores of energy and nutrients bound in wood to the surrounding biological community (Rayner and Boddy, 1988). Because of the ubiquity of woody biomass world-wide, changing wood decomposition rates could have global effects on nutrient cycling and carbon sequestration (Floudas et al., 2012; Hibbett et al., 2016). Wood decomposition is modulated by ecological interactions between decay fungi and other organisms (Hulme and Shields, 1970; Boddy, 2000), and small changes in fungal colonization processes can lead to several-fold changes in decay rates (Fukami et al., 2010; Cline and Zak, 2015).

Wood-boring bark and ambrosia beetles are widely believed to facilitate biomass recycling by inoculating dead and dying trees with saprotrophic fungi including ambrosia fungi and occasionally wood-decay fungi (Miller et al., 2016; Ulyshen, 2016). These beetles comprise more than 7000 species in the weevil subfamilies Platypodinae and Scolytinae that thrive throughout tropical and temperate regions (Kirkendall et al., 2015). They are often the first insects to colonize the wood, entering before the tree has died and having major impacts on fungal community development within wood (Leach et al., 1934; Persson et al., 2011; Strid et al., 2014; Skelton et al., 2019). The beetles have various relationships with the

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fungi they carry, ranging from incidental commensalism, to highly co-evolved and reciprocally obligate mutualisms (Harrington, 2005; Hulcr and Stelinski, 2017). Some beetles have specialized glandular organs called mycangia for transporting and nourishing particular lineages of nutritional fungi and have become entirely dependent on a fungal diet (Francke-Grosmann, 1956; Batra, 1963; Hulcr and Stelinski, 2017). Likewise, some fungi have evolved complete dependence on these beetles for dispersal and colonization of wood tissues (Francke-Grosmann, 1956; Batra, 1963; Six, 2003; Harrington, 2005). The few species of beetles and symbiotic fungi that kill healthy trees and impact agricultural and silviculture interests have been the center of intense research efforts. In contrast, we know little about the ecological roles of the thousands of other beetles and their fungi that do not kill trees but are ubiquitous and abundant on every continent except Antarctica.

In the southeastern United States, the phloem of stressed, declining, or recently dead pines (*Pinus* spp.) is typically infested by native bark beetles in the genera *Ips, Dendroctonus, Orthotomicus,* and *Hylastes*, and the xylem is colonized by ambrosia beetles in the genera *Xyleborus, Myoplatypus*, and *Gnathotrichus*. These beetles are primarily associated with saprotrophic Ascomycota in the orders Ophiostomatales (e.g. *Ophiostoma, Leptographium*, and *Raffaelea*), and Saccharomycetales (e.g. *Pichia, Candida,* and *Ambrosiozyma*), and incidental associations with various other fungi are common (Harrington, 2005; Hofstetter et al., 2015; Hulcr and Stelinski, 2017; Skelton et al. 2018, 2019).

Beetle-associated Ascomycota can utilize some of the major chemical components of wood including structural polysaccharides, but available evidence indicates that they generally do not cause extensive structural degradation or wood mass loss. Most saprotrophic fungi, including beetle-associated fungi, express extracellular enzymes capable of degrading the major carbohydrate constituents of plant cell walls such as cellulose and hemicellulose (Blanchette, 1991). Such enzymes have been detected in mixed fungal communities associated with laboratory colonies of the ambrosia beetle *Xyleborinus saxesseni* (Licht and Biedermann, 2012), as well as pure cultures of the beetle-associated tree pathogens *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* (Svaldi and Elgersma, 1982; Binz and Canevascini, 1996; Przybył et al., 2006), the ambrosia fungus *Phialophoropsis* (Lehenberger et al., 2019), ambrosia and non-ambrosial *Geosmithia* (Veselská et al., 2019), and are likely to occur in many other beetle-associated fungi. However, the structural carbohydrates of woody tissues are reinforced by an interspersed matrix of the structural polymer lignin. Carbohydrate-targeting enzymes cannot effectively decompose the carbohydrates of wood unless the lignin is decomposed, unbound, or modified, which requires specialized non-enzymatic and enzymatic processes borne by few fungal lineages, mostly in Basidiomycota (Blanchette, 1991; Worrall et al., 1997; Floudas et al., 2012; Hibbett et al., 2016). Consequently, relatively few fungi cause extensive wood decomposition. Congruently, experimental studies of common beetle-associated Ascomycota have shown little to no degradation of wood structure, marginal loss of wood hardness, and minimal mass loss (Miller and Elgersma, 1976; Seifert, 1993; Kasson et al., 2016), suggesting that these fungi depend on more labile carbohydrate sources such starches, sugars, and other extractives, rather than the structural elements that comprise the majority of wood biomass (e.g. Schirp et al., 2007). Saprophytic fungi that cannot decompose lignocellulose, including beetle-associates, may still access non-structural nutrients in the wood by spreading their hyphae through ray parenchyma cells and resin canals to become distributed in the sapwood. Although they do not decompose the woody cell wall, they can mechanically penetrate cell walls using fine penetration pegs (Eriksson et al., 1990).

Beetle-associated fungi could impede colonization and decomposition by more aggressive wood-decaying fungi by competing with pioneer decay fungi for labile carbohydrates or producing toxic secondary metabolites. It has been previously demonstrated that colonization of wood by non-beetle-associated Ascomycota reduces subsequent decay through competitive interactions with decay fungi (Hulme and Shields, 1970; Behrendt et al., 1995). Some of these species of Ascomycota have potential commercial applications for preventing wood rot (Schubert et al., 2008). Similarly, a recent field experiment utilizing beetle exclosure cages showed that high densities of ambrosia beetles caused reduced decay of pine sap wood, potentially as an effect of competition between their ascomycete symbionts and wood-decay fungi (Skelton et al., 2019). Ophiostomatales may also facilitate colonization of fungi that cannot tolerate extractives such as pitch and other resinous compounds in freshly exposed xylem. Ophiostomatales and some other early colonizing fungi not only tolerate these compounds but can decompose them over time (Blanchette et al., 1992).

The primary nutritional symbiont of beetles in the genera *Ambrosiodmus* and *Ambrosiophilus* is unusual among ambrosia fungi. It is a fungus in the phylum Basidiomycota that decomposes lignocellulose and causes extensive mass loss and softening of wood similar to known aggressive wood-decay fungi (Kasson et al., 2016). Several species of *Ambrosiodmus* are native to the southeastern USA, and at least three species are non-native. An Asian ambrosia beetle, *Ambrosiodmus minor,* was first detected in Florida by state monitoring efforts in 2011, and has since become one of the most frequently collected ambrosia beetle species across the state where it infests many species of hardwood and coniferous trees (Hulcr et al., 2018). Although there have been more than 50 species of non-native bark and ambrosia beetles to become established in the USA (http://www.barkbeetles.info; accessed Oct 15, 2018), *A. minor* is particularly likely to affect wood decomposition because of the combination of its increasing abundance and its symbiotic wood-decay fungus. Another common basidiomycete beetle-associate, *Entomocorticium* spp., could also contribute directly to wood decomposition. This is a primary nutritional fungus of several native species of two bark beetle genera that specialize on conifers, *Dendroctonus* and *Pityoborus*. The phylogenetic placement of *Entomocorticium* within the paraphyletic white-rot genus *Peniophora* (Hsiau and Harrington, 2003), and the production of extracellular cellulases and polyphenol oxidases suggest *Entomocorticium* may cause structural decomposition of wood (Hsiau and Harrington, 2003; Valiev et al., 2009). However, *in situ* observations of this fungus suggested that it does not cause any noticeable structural decomposition of sapwood (Whitney et al., 1987).

The objectives of this study were to examine the direct effects of native ascomycete associates, and the introduced basidiomycete associate on pine wood decomposition, and to assess secondary effects through competitive interactions with two common wood-decay fungi in pines of the southeastern USA. We hypothesized that native bark and ambrosia beetles can suppress early stages of decay by inoculating fresh wood with fungi that do not extensively decompose structural elements of wood, but instead compete with pioneer colonizing wood-decay fungi. We further hypothesized the inclusion of the non-native wood-decay ambrosia fungus *Flavodon ambrosius* could offset the inhibitory effects of native beetle-associated fungi on early decomposition through functional redundancy with free-living decay fungi.

2. Methods

Isolate Recovery and Identification: All beetle-associated fungal isolates were obtained from the University of Florida Forest Entomology Bark and Ambrosia Beetle collection and were originally

isolated from pine-infesting bark and ambrosia beetles at the Austin Carey Experimental Research Forest near Gainesville, Florida, USA (Table 1). Isolation from live beetles and culture methods followed Skelton et al. (2018). Identification of beetle-associated fungi was based on BLASTn comparison of the ribosomal large sub-unit (LSU) DNA sequence, using the primers LR0R and LR5 (Vilgalys and Hester, 1990). DNA extraction, PCR amplification, and sequencing were performed as described in Skelton et al. (2018). We chose four common and globally-distributed beetle-associated fungi to represent major groups of beetle associates in pines: *Ophiostoma ips* (Ophiostomatales) which is commonly found associated with many pine-infesting bark beetle species (Harrington, 2005), *Raffaelea fusca* (Ophiostomatales) and *Ambrosiozyma monospora* (Saccharomycetales) which are commonly associated with many pine and hardwood infesting ambrosia beetles in the southeastern USA (Skelton et al., 2019), and *F. ambrosius* which is specifically associated with ambrosia beetles in the related genera *Ambrosiodmus* and *Ambrosiophilus* (Kasson et al., 2016; Simmons et al., 2016; Li et al., 2017). All of the beetle-associated species used in this study have been found associated with ambrosia beetles at the collection site, and *O. ips* is also associated with bark beetles (Skelton et al., 2019). We also obtained cultures of two species of widely distributed and abundant fungi that are pioneer colonizers and aggressive decayers of pine wood to examine interactions between beetle-associated fungi and wood decay fungi; one white-rot fungus, *Ganoderma curtisii* f. sp. *meredithiae* a closely related and physiologically distinct species of *G. curtisii* (hereafter referred to as *G. curtisii*; Loyd et al., 2018a) and one brown-rot fungus (*Phaeolus schweinitzii*). The isolates were cultured from small pieces (<1 cm^3) of context tissue from inside the basidiomata of each wood-decay fungus as described in Loyd et al. (2018b). The cultures of *G. curtisii* and *P. schweinitzii* are archived and maintained at the Center for Forest Mycological Research Fungal Collection (CFMR) in Madison, WI.

Direct effects experiment: To determine the direct contribution of each beetle-associated fungus to decay of pine sapwood, we used a modified version of the standard wood decomposition protocol described in Loyd et al. (2018b). Fresh sapwood was obtained from the lower trunk of a 20 cm dbh loblolly pine (*Pinus taeda*) from the University of Florida Austin Cary Forest, near Gainesville, Florida, USA. The sapwood was cut into cubes (length, width, and height of 2.5 cm). Only wood displaying no signs of existing damage or disease was used. To simulate beetle galleries, 4 holes (3 mm diameter) were drilled evenly spaced through two faces of the cube to transect the grain of the wood, i.e. the simulated galleries were horizontal when the cube was held in its original orientation in the tree. The cubes were then dried to completion at 50 °C for 7 d, weighed for dry mass and stored in sealed plastic bags until used. Just prior to use, cubes were placed individually in 237 ml glass jars with enough distilled water to hydrate to the original water content of the fresh wood (42% water by mass), left overnight to absorb the water, and then autoclaved twice for 1 h at 121 °C and 103 kPa with 24 h inbetween autoclave cycles. After cooling, the cubes were inoculated with fungal spore suspension (described below), jars were loosely capped and incubated at 25 °C in the dark for 14 d to allow beetle-associated fungi to colonize the wood. Cubes were then transferred to microcosms. Microcosms consisted of 237 ml jars containing 120 ml of hydrated vermiculite, tamped level, with two hydrated wood feeder strips 1 cm by 4 cm by 0.2 cm (*Quercus nigra*) laid parallel across the surface of the media. Microcosms were autoclave sterilized as described above. Strips were added to keep the methodology consistent between this experiment and the "indirect effects" experiment described below. The inoculated cubes were placed on the wood feeder strips, and microcosms were incubated at 25 °C in the dark for 90 d. Cubes were then removed from microcosms, lightly brushed to remove vermiculite and fungal hyphae from the external surfaces, weighed for wet mass, dried to completion at 50 °C, weighed for final dry mass, and then stored in sealed plastic bags at −20 °C for later micrograph imaging.

Simulated beetle galleries were inoculated by pipetting 100 μl of a spore suspension containing 50 spores per μl into each gallery to deliver a spore load similar to that observed from naturally dispersing beetles. We inoculated 5 cubes per fungal isolate. Spore suspensions were made by placing a colonized agar wedge in 0.5 ml of sterile water and vortexing at 2000 rpm for 30 s, determining spore concentration by light microscopy and counting using a hemocytometer, and adjusting with sterile water to reach the target concentration. *Flavodon ambrosius* produces arthrospores in culture, which, along with some hyphal fragments, comprised the inoculum for that species. Five negative controls were inoculated with sterile water.

Indirect effects experiment: This experiment was designed to determine whether pre-colonization of wood with beetle-associated fungi would reduce subsequent decay from a common pine wood-decay fungus, *G. curtisii*. The experimental design is illustrated in Supplemental Fig. S1. Tester cubes and microcosms were prepared as described above with one difference: the wooden feeder strips in the microcosms were colonized with *G. curtisii* prior to the addition of the tester cubes. Feeder strips were inoculated by placing a colonized agar plug at one end of each strip and incubating in the microcosm at 25 °C in the dark for 14 d. In addition to each of the beetle-associated fungi treatments, we also added an "all fungi" treatment in which *F. ambrosius* was combined with all three fungi from all native beetles, with total spore solution volume and number of total spores held constant. Incubation and final mass were determined as described above. We included 7–8 replicates for each treatment combination.

Impact on wood structure: A representative wood sample of each independent fungus and fungal combination from the indirect effects experiment was processed for scanning electron microscopy to assess damage to the structural components of the wood. Small sections were cut from each sample wood block and infiltrated with TBS™ Tissue Freezing medium™ (Triangle Biomedical Sciences, Durham, NC, USA) under a vacuum and then mounted on brass stubs at −20 °C in a freezing microtome (International Equipment Company, Needham Heights, MA, USA). Samples were sectioned to produce a smooth transverse view of the wood cells, and dehydrated by placing in 20, 30, 45, 75, and 95% ETOH for 5 min each.

Table 1
Collection information for fungal isolates used in microcosm experiments.

Species	Isolate name	extracted from	Location
Ambrosiozyma monospora	JH_14703	*Xyleborus pubescens*	Gainesville, Florida, US
Flavodon ambrosius	MAJ001	*Ambrosiodmus minor*	Gainesville, Florida, US
Raffaelea fusca	JH_14643	*Xyleborus affinis*	Gainesville, Florida, US
Ophiostoma ips	JH_14624	*Ips grandicollis*	Gainesville, Florida, US
Ganoderma curtisii f. sp. meredithiae	UMNFL50	basidiomata	Sarasota, Florida, US
Phaeolus schweinitzii	320NC	basidiomata	Charlotte, North Carolina, US

Following dehydration, samples were mounted on aluminum stubs and coated with gold/palladium on a Cressington 108 auto sputter coater (Cressington Scientific Instruments, Watford, United Kingdom). Samples were examined using a Hitachi S3500N (Hitachi, Tokyo, Japan) scanning electron microscope.

Interaction detail experiment: While the experiments using tester cubes were designed to simulate interactions occurring within sap wood to determine their effects on biomass loss, they did not offer a good visual assessment of the interactions. To provide a better view of the interactions that caused significantly reduced decay in the indirect effects experiment, we devised a separate assay using tester strips embedded in water agar. Fresh pine sapwood (same as described above) was cut into 1 cm by 0.2 cm by 6 cm strips, with the grain of the wood running along the longest dimension. Strips were dried and weighed, rehydrated by soaking in distilled water for 24 h, bulk autoclave sterilized as described above, and then embedded in a 1.5% water agar in standard 100 mm plastic Petri dishes. After cooling, a rectangular section of agar was removed from each end of the embedded strips and replaced with colonized rectangular agar plugs, pressing the colonized surfaces of the plugs against the ends of the strips. Each strip was inoculated with a wood-decay fungus on one end, and the other end was inoculated with either *O. ips*, *R. fusca*, or a sterile agar plug (negative control). In addition to *G. curtisii* from the previous cube experiments, we also included a common pine-infecting brown-rot fungus (*P. schweinitzii*). Petri dishes were sealed with plastic film, incubated at 25 °C in the dark for 120 d. Plates were examined and photographed at 120 d to characterize the interactions between fungi. Fungal interactions were observed on the exposed top facing surfaces of the wood strips. Strips were then removed from agar, rinsed and brushed gently to remove agar and hyphae from surfaces, dried to completion at 50 °C, and weighed for final dry mass.

Statistical analyses: For all three experiments, our response variable was percent dry mass lost calculated as the difference between initial dry mass and final dry mass, divided by initial dry mass and multiplied by one hundred. For convenience, experimental design and sample sizes for all three experiments are illustrated in Supplemental Fig. S1. Treatment effects were tested using linear models, implemented by the *lm()* function in the stats package for R (R Developement Core Team, 2010). The assumption of equal group variance was tested using a Bartlett Test, implemented by the *Bartlett.test()* function. In the direct effects experiment, we used the negative control as the reference group in the linear model. In the indirect effects experiment, the group of tester cubes that received only *G. curtisii* was used as the reference group and compared to all other groups which had been pre-colonized with a beetle-associated fungus. We used a Kruskal-Wallis test (*Kruskal.test* function in the stats package for R) to compare the "all fungi" group to the reference group because a high degree of variance in that treatment violated the assumption of homogeneity of variance. For the interaction detail experiment, percent dry mass lost was modeled as a function of decay fungus (*G. curtissi* [reference group] or *P. schweinitzii*), initial strip dry mass, beetle-associated fungus treatment (*O. ips, R. fusca*, or negative control [reference group]), with interaction terms for initial mass and decay fungus, and beetle associate and decay fungus. Initial strip mass was included in the model because decay in this assay was greatest on surface of the strips that were exposed above the agar, thus percent mass loss was higher on smaller strips which have a greater surface area to mass ratio.

3. Results

Direct effects experiment: The introduced Asian basidiomycete, *F. ambrosius,* caused approximately three times more mass loss than any other beetle-associated fungus that was assayed (Fig. 1). All assayed fungi caused mass loss that was significantly greater than negative controls indicating that some fraction of wood biomass had been consumed by each fungus (Table 2). However, the ascomycete beetle-associated fungi (*Ophiostoma, Raffaelea,* and *Ambrosiozyma*) caused less than 5% mass loss on average, suggesting that they did not utilize the structural components of the wood but mainly consumed the available labile sugars and extractives. In contrast, *F. ambrosius* caused mass loss that was comparable to the common free-living white-rot wood-decay fungus *G. curtisii* (Fig. 1) and similar to previous reports that used similar methods to measure decay from these fungi (Kasson et al., 2016; Loyd et al., 2018b).

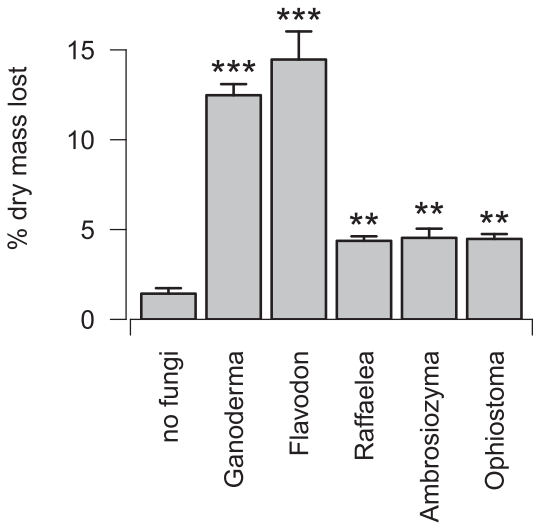

Fig. 1. Direct effects of beetle-associated fungi on decay of pine wood (as percent dry mass lost) over a 90 d microcosm experiment. All fungi assayed caused significantly more mass loss than negative controls (no fungi), however, *Flavodon ambrosius* was the only beetle-associated fungus to cause mass loss that was comparable to *Ganoderma curtisii,* a well-known decay fungus. Asterisks indicate significant difference from negative control group $^{**}p < 0.01$, $^{***}p < 0.001$.

Flavodon ambrosius was the only beetle-associated fungus to cause detectable structural damage to sapwood. Transverse sections of non-decayed wood (control) observed with SEM showed the typical arrangement of *P. taeda* wood cells with intact tracheids of both early and latewood (Fig. 3A). Similar to the negative control blocks, wood in decay microcosms colonized by the ambrosia fungi in the fungal phylum Ascomycota (*Ambrosiozyma, Ophiostoma,* and *Raffaelea*) revealed intact cells when transverse sections were observed with SEM (Fig. 3D–F). In contrast, transverse sections of *P. taeda* wood blocks that were colonized with the basidiomycetes *G. curtisii* or *F. ambrosius* showed evidence of simultaneous decay of

Table 2
Linear model for direct effects shown in Fig. 1 of beetle-associated fungi on mass loss in pine sapwood during a 90 d microcosm experiment. Residual standard error: 1.66 on 30 degrees of freedom, adjusted R-squared = 0.8973, $F_{5,30} = 62.16$, $p < 0.001$. * Treatments significantly different from the negative control reference group (no fungi).

coefficients	estimates	t-value	p-value
intercept	1.4325	2.431	0.021
A. monospora	2.9395	3.094	0.004*
F. ambrosius	13.0315	13.717	>0.001*
G. curtisii	11.0450	13.256	>0.001*
O. ips	3.0455	3.206	0.003*
R. fusca	3.1055	3.269	0.002*

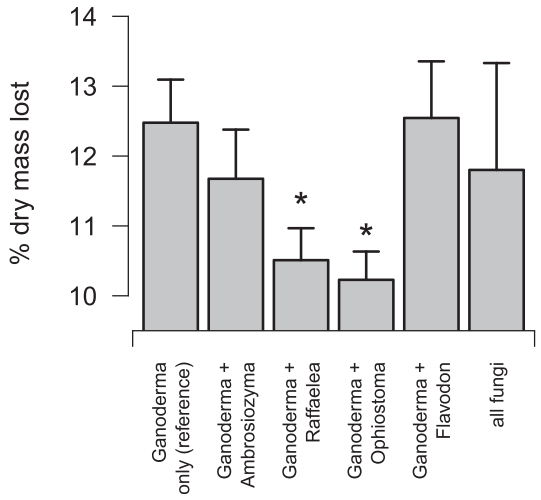

Fig. 2. Pre-colonization of two beetle-associated fungi had negative indirect effects on decay in pine wood by competing with the pine wood-decay fungus *Ganoderma curtisii*. Asterisks indicate treatments that were significantly different from the reference group which was wood only inoculated with *G. curtisii*, $p < 0.05$.

tracheid cells typical of white-rot decay fungi (Fig. 3B and C). The simultaneous decay of *P. taeda* wood caused by both *G. curtisii* and *F. ambrosius* was only observed in the earlywood cells, which tend to have larger cells with thinner cell walls compared to latewood cells (Fig. 3A).

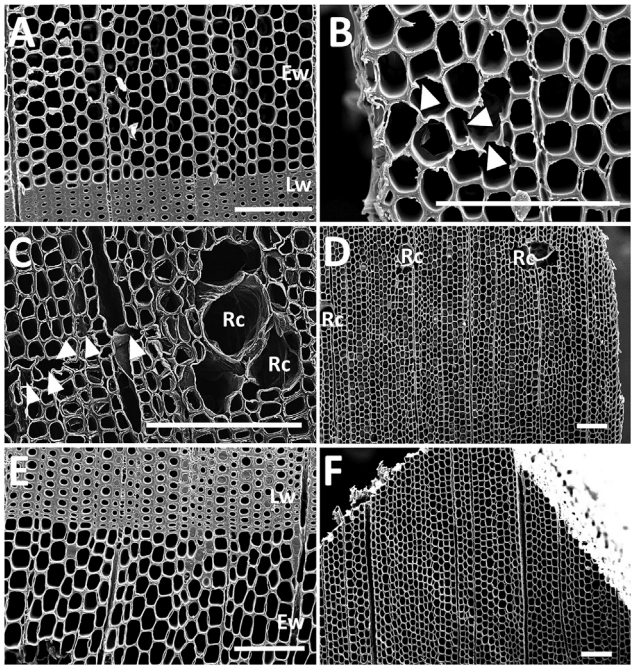

Fig. 3. Transverse cross sections of *Pinus taeda* wood blocks colonized with ambrosia fungi or the wood-decay fungus *Ganoderma curtisii* observed with scanning electron microscopy (bars = 200 μm). (A) Section of *P. taeda* wood not colonized with any fungi; (B) tracheids in the early wood simultaneously decayed by *G. curtisii* indicated by the arrowheads; (C) tracheids simultaneously decayed by the ambrosia fungus *Flavodon ambrosius,* indicated by the arrowheads. (D) Section of *P. taeda* wood colonized with *Raffaelea fusca* showing no evidence of decay, (E) section of *P. taeda* wood colonized with *Ambrosiozyma monospora* showing no evidence of decay, and (F) section of *P. taeda* wood colonized with *Ophiostoma ips* showing no evidence of decay. Ew = earlywood, Lw = latewood, Rc = resin canals. Note that higher magnification was used in A-C to show the details of the earlywood and latewood in the negative control and detail the type of decay caused by *F. ambrosius* and *G. curtisii.* D,F are shown at lower magnification to illustrate that there were no visible signs of decay across a wide field of view. High resolution versions are available in the electronic supplement and can be examined at magnification equivalent to other panels by digital zoom.

Indirect effects experiment: This experiment determined the effects of competition between beetle-associated fungi and a known wood-decay fungus (*G. curtisii*) on total decay (mass lost). This was achieved by comparing blocks inoculated with each beetle-associated fungus and *G. curtisii* to controls that were inoculated with only *G. curtisii*. The two ophiostomatalean fungi, *R. fusca* and *O. ips*, significantly reduced mass loss caused by *G. curtisii*. *Ganoderma curtisii* caused an average decrease of 12.5% in dry mass of tester cubes in the absence of beetle-associated fungi. This loss was significantly diminished by pre-inoculation with either *O. ips* or *R. fusca*, but not *A. monospora* or *F. ambrosius* (Table 3; Fig. 2). Pre-inoculating with all four beetle-associated fungi simultaneously resulted in higher variance in this group (Bartlett test; $K^2 = 20.665$, $p < 0.001$), but did not affect the mean mass loss compared to *G. curtisii* -only reference treatment (Kruskal-Wallis test; $X^2 = 0.21$, $p = 0.64$; Fig. 2). In both treatments involving *F. ambrosius* and *G. curtisii*, *F. ambrosius* visibly excluded *G. curtisii* from colonizing the tester cubes (Supplemental Fig. S2).

Interaction detail experiment: We observed multiple antagonistic interactions between the decay fungi and beetle-associated fungi. *Ophiostoma ips* grew relatively quickly at the onset of the experiment, but its growth was halted when it encountered either *P. schweinitzii* or *G. curtisii*, both of which continued to grow over *O. ips.* While there was no visible effect of *O. ips* on the growth of *G. curtisii*, the hyphal bundles of *P. schweinitzii* were thinner and sparser in the presence of *O. ips* compared to controls that only had *P. schweinitzii* (Fig. 4). *Raffaelea fusca* completely excluded colonization of wood from *G. curtisii,* causing *G. curtisii* to make mounding walls of hyphae at the zone of contact (Fig. 4). Because *R. fusca* was much slower growing, it colonized and defended relatively small sections of the tester strips, within approximately 1 cm of the inoculation point, but maintained its territory for the entire 120 d duration of the experiment and visibly prevented decay from *G. curtisii* within its territory (Fig. 4). There was no visible response of *P. schweinitzii* to *R. fusca*.

Overall, *P. schweinitzii* caused significantly more mass loss than *G. curtisii* in the wood strip assay (Table 4). There was also a significant interactive effect between *O. ips* and *P. schweinitzii* as *O. ips* reduced the decay caused by *P. schweinitzii*, congruent with visual observations of reduced hyphal growth of *P. schweinitzii* in the presence of *O. ips*. The mean percent mass lost due to *P. schweinitzii* was reduced from 15.79% to 14.01% when co-inoculated with *O. ips,* and 14.39% by *R. fusca,* though the effects of only *O. ips* were statistically significant (Table 4). In contrast to the cube experiments, there was no significant effect of either beetle-associated fungus on the decay caused by *G. curtisii* which probably reflected the overall poor performance of *Ganoderma* in this assay; an average of 4.83% mass was lost on strips compared to 12.5% on cubes.

Table 3
Effects of pre-inoculating pine sapwood cubes with beetle-associated fungi on decay caused by *Ganoderma curtisii* in a 90 d microcosm experiment. Effects are illustrated in Fig. 2. Residual standard error: 1.75 on 35 degrees of freedom, adjusted R-squared = 0.1749, $F_{4,35} = 3.067$, $p = 0.028$. * Treatments significantly different from the negative control reference group (*Ganoderma* only).

coefficients	estimates	t-value	p-value
intercept	12.4775	20.215	<0.001
A. monospora	−0.8025	−0.919	0.3642
F. ambrosius	0.0675	0.077	0.9388
O. ips	−2.2487	−2.576	0.014*
R. fusca	−1.9675	−2.254	0.036*

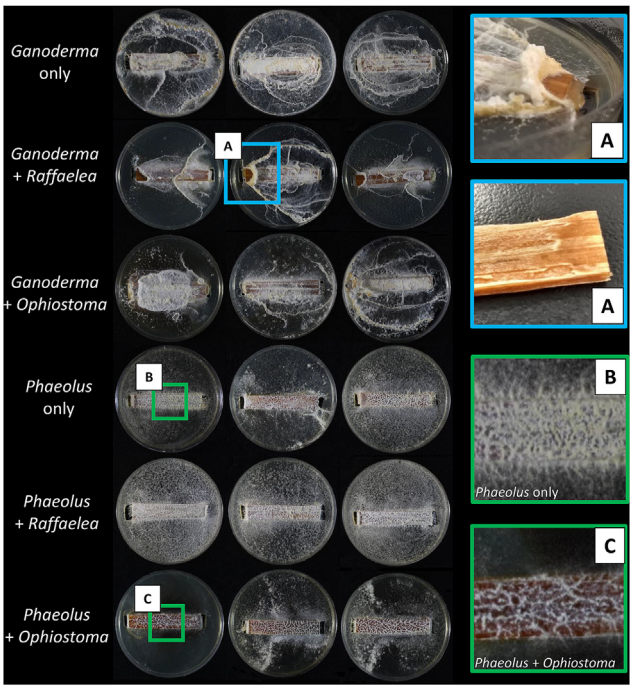

Fig. 4. Beetle-associated fungi inhibit decay of pine sapwood from a white-rot (*Ganoderma*) or brown-rot (*Phaeolus*) fungus. Three representative replicates are shown for each combination of fungi after 120 d incubation. (A) Blue boxes highlight the area where *Raffaelea fusca* has excluded *Ganoderma* from colonization and prevented decomposition of structural components on the same tester strip with mycelium intact (above) and after mycelium was removed (lower). Green boxes highlight differences in the density of hyphal bundles on representative tester strips that were inoculated with *Phaeolus* only (B) and *Phaeolus* plus *Ophiostoma ips* (C).

Table 4
Effects of co-inoculation of pine sapwood strips with beetle-associated fungi and two decay fungi, *Ganoderma curtisii* and *Phaeoleus schweinitzii,* on decay in a 90 d interaction detail experiment. Residual standard error: 2.38 on 44 degrees of freedom, adjusted R-squared = 0.8394, $F_{7,44} = 39.07$, $p < 0.001$.

coefficients	estimates	t-value	p-value
intercept	10.1104	5.339	<0.001
initial mass	−3.1860	−3.113	0.003*
decayer (*Phaeolus* vs. *Ganoderma*)	19.1531	7.206	<0.001
treatment *O. ips*	1.8526	1.587	0.119
treatment *R. fusca*	0.8479	0.711	0.480
initial mass: decayer	−6.0653	−3.897	<0.001*
decayer: *O. ips*	−4.6698	−2.909	0.005*
decayer: *R. fusca*	−1.7208	−1.047	0.301

4. Discussion

Bark and ambrosia beetles are widely believed to accelerate wood decomposition by introducing fungi during the early stages of saprotroph community assembly. However, the majority of fungi associated with these beetles are ascomycetes (Hofstetter et al., 2015; Hibbett et al., 2016; Hulcr and Stelinski, 2017), and the large majority of Ascomycota do not cause extensive wood decomposition under normal circumstances in most habitats (Floudas et al., 2012; Hibbett et al., 2016). Many Ascomycota, including lineages closely associated with bark and ambrosia beetles, possess enzymes for degrading the carbohydrates that comprise most of wood biomass (e.g. Licht and Biedermann, 2012; Lehenberger et al., 2019). However, most of the carbohydrate in wood is made inaccessible to enzymatic decomposition by a dense network of the structural polymer lignin, which must be decomposed, modified, or unbound to access the carbohydrates (Blanchette, 1991; Jeffries, 1991; Floudas et al., 2012). Consequently, fungi bearing adaptations for lignin decomposition or modification (i.e. the white- and brown-rot fungi in the Basidiomycota) have the greatest influence on the decomposition of woody biomass in forests. The results of the present study provide support for a new hypothesis; bark and ambrosia beetles suppress wood decomposition indirectly by introducing fungi that cause very little decomposition of wood but instead exclude or compete for labile resources with fungi capable of extensive decomposition. This finding is congruent with studies of other Ascomycota shown to reduce decay by competing with wood-decaying Basidiomycota (e.g. Hulme and Shields, 1970; Behrendt et al., 1995; Bruce et al., 1995).

The common and widespread ascomycete symbionts of bark and ambrosia beetles analyzed in the present study caused little mass loss and no visible decomposition of wood structure. We observed an average loss of less than 5% dry mass from each of the three ascomycete symbionts when inoculated as monocultures (Fig. 1). Although this was a significant loss compared to negative controls which had no fungi, the loss was not attributable to the decomposition of the structural components of the wood; scanning electron micrographs revealed no structural damage attributable to any ascomycete symbionts. Our results are congruent with previous work showing that ascomycete beetle associates cause relatively little mass loss or decomposition of wood structure when compared to common basidiomycete wood-decay fungi (Kasson et al., 2016).

Ascomycete symbionts of beetles interacted antagonistically with wood-decay fungi, limiting decay-fungi from colonizing experimental wood samples. Competition between non-decaying ascomycetes and wood-decaying basidiomycetes is well-known. For instance, the ascomycete *Trichoderma* is a proven biocontrol agent used to competitively exclude wood-decaying basidiomycetes through multiple competitive mechanisms (Hulme and Shields, 1970; Bruce et al., 1995). An even more relevant example is the effective use of a pigment-less mutant of *Ophiostoma piliferum* to competitively exclude the wood-decay polypore *Phlebiopsis gigantea*, as well as blue staining fungi in the order Ophiostomatales (Behrendt et al., 1995). These cases demonstrate that early colonization of wood by ascomycetes can inhibit subsequent colonization of pioneer decay fungi. These priority effects that exclude or suppress wood-decay fungi seem particularly relevant to beetle-associated fungi that arrive very early in saprotroph community assembly as a result of their symbiosis with bark and ambrosia beetles. Indeed, our results showed that some beetle-associated fungi can exclude some wood-decayers from colonizing portions of the wood, such as the ambrosia fungus *R. fusca* which prevented *G. curtisii* from colonizing portions of tester strips. In other cases, the hyphae of wood-decay and beetle-associated fungi intermingled, but the decay fungi showed reduced vigor. *Phaeolus schweinitzii* in the presence of *O. ips* had noticeably sparser mycelial growth than controls without *O. ips* (Fig. 4, inset C), and a slight inhibition of *G. curtisii* by *O. ips* was observed on agar media (data not presented). It is notable that both decay fungi colonized wood that was already colonized by *O. ips* and it is possible that the reduction in decay caused by *O. ips* may have diminished if our experiment had been conducted over a longer time period. Although our study included only a few strains of beetle-associated fungi, the results suggest that some Ascomycota associates of bark and ambrosia beetles have the potential to impede subsequent colonization of wood by wood-decaying basidiomycetes. Future efforts that include more fungal species and different ecological

contexts, including wood type, temperature, pH, etc., will help determine the generality of this phenomenon in nature.

The two beetle-associated Ophiostomatales examined in this study reduced decay from wood-decaying basidiomycetes. Wood cubes incurred significantly less mass loss from *G. curtisii* when they were pre-inoculated with either *O. ips* or *R. fusca* (Fig. 2). Areas in which *R. fusca* had excluded *G. curtisii* showed none of the structural decomposition that occurred where *G. curtisii* had colonized experimental wood strips (Fig. 4A). We also saw reduced decay from *P. schweinitzii* when co-inoculated with *O. ips* in the interaction detail experiment. Ophiostomatalean fungi can rapidly reduce non-structural carbohydrates such as sugar and extractives in wood (Wang et al., 2013), and competition between ascomycetes and basidiomycetes for non-structural carbohydrates can reduce decay rates in non-beetle-associated systems (Hulme and Shields, 1970). Thus, resource competition for labile carbohydrates is a plausible mechanism for the reduced decomposition from wood-decay fungi when they were co-inoculated with ophiostomatalean beetle associates. The exclusion of *G. curtisii* from the small areas of wood colonized by *R. fusca* suggests that this ambrosia fungus may compete through chemical or physical means, in addition to, or instead of competition for labile carbon resources. Furthermore, this result suggests that some ambrosia fungi may be good competitors for wood-based resources, even in the absence of their vectors which are widely believed to facilitate ambrosia growth mainly by removing competitors. It should be noted that our experiments were conducted under artificial laboratory conditions. Increased fungal diversity, stochastic environmental variables, interactions with other insects, and many other variables could influence the outcomes of the interactions that we observed in the laboratory. Consequently, future work using field experiments is needed. One such field experiment recently indicated that beetle-associated fungi indeed do reduce decay rate at early stages of decay in pine logs under natural conditions (Skelton et al., 2019).

In contrast to our findings, other studies of the effects of bark and ambrosia beetles on wood-decay generally conclude that the beetles facilitate wood decomposition (reviewed in Ulyshen, 2016). Much of the disparity between our study and others is a result of differences in perspective and interpretation, rather than conflicting experimental outcomes. These differences fall into three categories. First, many symbionts of bark and ambrosia beetles, commonly referred to as “blue-stain fungi”, cause extensive discoloration of sapwood and phloem surrounding beetle galleries. Observations of this discoloration is sometimes conflated with decomposition, either by the authors or subsequent readers of research articles that report dark staining as a consequence of beetle infestation (e.g. Leach et al., 1934; Švihra and Kelly, 2004). The results presented in this paper and others (Kasson et al., 2016), indicate that wood staining does not equate to wood decomposition; both *O. ips* and *R. fusca* caused discoloration of the wood with minimal loss of biomass and no signs of structural decomposition. Our results are also congruent with extensive forest products testing. Bark beetle outbreaks produce an abundance of harvestable wood that has been stained by their fungal associates. Consequently, there has been extensive effort to examine many physical and mechanical properties of wood that has been stained by beetle-associated fungi to determine its suitability for market. These studies have generally found marginal or no significant effects of beetle-associated blue-stain fungi on diverse measures of the structural integrity of wood (Lum, 2003; Lam et al., 2006; Lum et al., 2006; Mizell, 2007). Thus, we do not equate wood staining with wood decomposition.

The second source of disparity is guilt by association. Field experiments and surveys that relate wood-boring insects and decay often involve not only bark and ambrosia beetles, but other wood borers such as longhorn beetles (Cerambycidae) and termites. Several such studies provide compelling evidence that longhorn beetles cause significant losses in wood mass or density, however direct evidence for an association between bark and ambrosia beetles and wood decomposition is generally lacking or inseparable from the effects of other wood-boring insects (e.g. Leach et al., 1934; Leach and Orr, 1937; Edmonds and Eglitis, 1989; Müller et al., 2002; Angers et al., 2011). Longhorn beetles are much larger than bark and ambrosia beetles, and consume or eject proportionately larger volumes of wood, which leads to significant losses in wood mass through maceration. In contrast, even heavy infestations of the much smaller ambrosia beetles result in less than half of one percent loss in sapwood volume (Zhong and Schowalter, 1989). Our results in the present study and another recent study (Skelton et al., 2019) indicate that the negative effects on decomposition as a consequence of competition between beetle associates and wood-decay fungi are likely to offset the small amount of physical maceration of sapwood by ambrosia beetles, resulting in a net negative effect of ambrosia beetles on wood mass loss.

The third source of disparity between our conclusions and those of others is the intuitive but tenuous inference that increasing fungal diversity in decaying logs will necessarily result in increased decomposition of wood. Experimental and observational studies have firmly demonstrated that bark and ambrosia beetles cause several-fold increases in fungal diversity in freshly dead wood, including increased diversity in decay fungi (Persson et al., 2011; Strid et al., 2014; Skelton et al., 2019). However, the relationship between diversity of fungi in wood and decay rate is not always positive and can sometimes be negative as a consequence of increased antagonistic interactions among fungi (Boddy, 2000; Fukami et al., 2010; Dickie et al., 2012; Hiscox et al., 2015). Therefore, by introducing diverse fungal communities into wood, bark and ambrosia beetle could have a counter-intuitive negative effect on decomposition rates, even when they carry some wood-decaying taxa. Indeed, a recent beetle exclosure field experiment demonstrated that while beetles increased fungal diversity several-fold in recently killed pines, high densities of ambrosia beetles actually caused a significant reduction in wood decomposition (Skelton et al., 2019).

Our results may help explain the counter-intuitive results of previous insect exclosure experiments. Ulyshen et al. (2014) found that logs exposed to insects in the southeastern USA lost significantly more wood volume than insect-excluded logs. However, the remaining wood was significantly denser when insects were present. They speculated that the insects could have employed antibiotic that slowed microbial decomposition, resulting in denser wood. Alternatively, our results suggest that fungi carried into the wood by insects may have slowed decomposition by competing with wood-decay fungi.

Unlike the ascomycete beetle-associates, the introduced Asian basidiomycete *F. ambrosius* caused significant decay of earlywood tracheid cells of pine sapwood, resulting in mass loss similar to the common free-living pine decay fungus *G. curtisii*. It also caused visible decomposition of wood structure at microscopic and macroscopic levels. The amount of mass loss attributable to *F. ambrosius* was nearly identical to previous work that used similar methods to measure decay of loblolly pine sapwood from four species in the well-known wood-decaying polypore genus *Ganoderma* (Loyd et al., 2018b). Our results are also consistent with previous work that assessed decay of a hardwood (*Ailanthus*) in terms of both mass loss and loss of wood hardness from two ascomycete symbionts of ambrosia beetles (*Raffaelea subfusca* and *Fusarium* sp. AF-4) and *F. ambrosius* isolated from another introduced Asian beetle, *Ambrosiophilus atratus* (Kasson et al., 2016). Our

study had similar results showing minimal decay from ascomycete beetle-associates, but considerable mass loss from *F. ambrosius*. Thus, unlike any other ambrosia symbiosis known, this unusual ambrosia fungus and its introduced beetle vectors could contribute directly to wood decomposition during the early stages of saprotroph community development. Transcriptome and secretome analyses of other pioneering wood-decay fungi have elucidated mechanisms used to tolerate and metabolize resinous compounds in wood (Hori et al., 2014). Having the ability to colonize freshly cut wood and capture resources appears to be a very effective way for pioneer species of white-rot fungi such as *P. gigantea* or *F. ambrosius* to exclude other fungi. A better understanding of the genes involved with the transformation and detoxification of wood extractives by *F. ambrosius* is needed to better understand the ecology of this fungus and its beetle vectors.

Flavodon ambrosius illustrates the importance of considering symbiont functional traits to understand the impacts of their hosts. When *F. ambrosius* was combined with the ascomycete beetle associates, we observed decay rates that were not significantly different from controls which received only wood-decay fungi. This result indicates that inclusion of *Flavodon*-farming beetles into bark and ambrosia beetle assemblages could nullify the inhibitory effect of ascomycete associates on early decay in pine sapwood. Notably, *F. ambrosius* excluded *Ganoderma* from colonizing the cubes in the *F. ambrosius* treatment and the "all fungi" treatment, and thus the similar level of decay observed in cubes pre-inoculated with this fungus, either alone or in combination with other beetle associates, represent replacement and functional redundancy with the decayer, not a peaceful co-existence in the wood. Given that *Flavodon*-farming beetles have been widely introduced, are becoming widely established, and are rapidly increasing in some areas along with their exotic pioneer wood-decay fungus (Hulcr et al., 2018), these series of events could likely have a substantial impact on decomposition and the turnover of forest biomass, nutrients, and carbon.

5. Conclusions

The two common and widespread ophiostomatalean associates of bark and ambrosia beetles studied here may inhibit decay by competing with decay fungi. In contrast, the introduced basidiomycete ambrosia fungus *F. ambrosius*, causes decay similar to other well-known free-living white-rot fungi and nullifies the inhibitory effects of ascomycete beetle symbionts, suggesting that widespread introduction of this fungus and its vectors could significantly impact carbon turnover rates in forest ecosystems. An accurate understanding of the influence that wood-boring beetles have on forest ecosystems requires detailed knowledge of the functional traits of their phylogenetically diverse symbionts, and the ecological interaction between symbionts and non-symbiotic microorganisms.

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Supplementary data

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