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Research

Incidental Fungi in Host Trees Disrupt the Development of *Sirex noctilio* (Hymenoptera: Siricidae) Symbiotic Fungus and Larvae

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Abstract

The woodwasp, *Sirex noctilio* Fabricius, is an exotic pest of *Pinus* L. in the southern hemisphere and North America, and it is an emerging threat in northeastern China. Adult woodwasps have numerous insect competitors for oviposition substrates, and developing larvae must compete for resources with other subcortical invaders. Its mutualistic fungus *Amylostereum areolatum* (Fr.) Boidon, is less competitive than many other fungal colonists present in pine ecosystems. This study investigated the effects of incidental, host-colonizing fungi on the growth and development of woodwasp larvae and *A. areolatum*. Fungi were isolated from dead *S. noctilio* larvae within galleries (primarily *Trichoderma* Pers, *Ophiostoma* Sydow, and *Sphaeropsis* Sacc.), and effects of these fungi on woodwasp brood survival were investigated via inoculations of *S. noctilio*-infested logs. Larval mortality was significantly increased in sample logs inoculated with *Ophiostoma minus* (Hedgc.) Sydow *Phlebiopsis gigantea* (Fr.) Jülich, *Trichoderma atroviride* Bissett, *Trichoderma viride* Pers, and *Trichoderma harzianum* Rifai. Inoculation of logs with *O. minus* resulted in the highest mortality and greatest reductions in wood moisture content. When grown on artificial media, these fungi grew faster than and inhibited growth of *A. areolatum* mycelium. We propose that the adverse effects of incidental fungi on the survival of *S. noctilio* larvae may be caused at least in part by an indirect mechanism involving inhibition of the fungal symbiont. The findings provide potentially valuable information for suppressing *S. noctilio* populations using microbial control agents.

Key words: incidental fungi, *Sirex noctilio* larvae, *Amylostereum areolatum*, mortality, moisture content

Sirex noctilio Fabricius is native to Eurasia and North Africa, but has been accidentally introduced into several countries in the southern hemisphere, North America, and most recently China (Spradbery and Kirk 1978, Li et al. 2015a). Although considered a secondary pest of pine species in its native range, the wasp has attracted considerable attention due to its ability to kill a variety of pine species in regions where it has been introduced (Hurley et al. 2007, 2015; Batista et al. 2018). It was first detected in China in 2013, and by 2018, it was found that attacking Mongolian pine (*Pinus sylvestris* var. *mongolica* Litvinov) plantations spanning 22 cities in northeastern China and causing enormous economic and ecological losses (Li et al. 2015, Sun et al. 2016).

During oviposition, *S. noctilio* females inoculate the host tree with an obligate symbiotic fungus, *Amylostereum areolatum* (Fr.) Boidon (Basidiomycotina: Corticiaceae) that contributes to tree death and acts as an external gut for digestion of recalcitrant lignocellulosic compounds (Thompson et al. 2013, 2014). Along with the fungus, the wasp also injects a toxic mucus that affects tree defenses and assists the fungus in colonizing the host (Coutts and Dolezal 1969). Thus, insects, toxins, and fungi act together to damage host trees.

The management of *S. noctilio* is based on two components: 1) baited traps to monitor population levels and 2) biological control. Baited traps are important tools for population monitoring in helping to detect the woodwasps and time control measures (Hurley et al. 2015, Poisson et al. 2016). The parasitic nematode *Beddingia* (=*Deladenus*) *siricidicola* Bedding (Neotylenchidae) has provided the most successful control strategy for *S. noctilio*, attaining almost 100%

parasitism and sterilization of female *S. noctilio* emerging from inoculated trap trees (Bedding 2009, 1972). Additionally, several parasitic wasps were introduced to Australia in the late 1980s to manage this pest, including *Schlettererius cinctipes* Cresson (Stephanidae) as well as species belonging to the genera *Ibalia* (Ibaliidae), *Rhyssa* (Ichneumonidae), and *Megarhyssa* (Ichneumonidae) (Taylor 1976). *Ibalia leucospoides* (Hochenwarth) is by far the most successful parasitoid and can significantly reduce *Sirex* populations (Collett and Elms 2009). Furthermore, Vega et al. (2009) found that application of environmentally friendly insecticides can play an important role in regulating pest populations. However, the level of control achieved generally is insufficient.

In Australia, effectiveness of biological control by *Beddingia siricidicola* was reduced by another exotic pest, the bark beetle, *Ips grandicollis* Eichhoff (Coleoptera: Scolytidea) (Yousuf et al. 2014a; Slippers et al. 2015). *Ophiostoma ips*, a blue stain fungus transmitted by the bark beetle, alters the tree moisture content, thereby influencing the survival, growth, and spread of *B. siricidicola* in the wood (Carnegie et al. 2006, Yousuf et al. 2014a). In China, infestations of the native bark beetles *Ips sexdentatus* Boerner, 1776 and *I. acuminatus* Gyllenhal, 1827 (Coleoptera: Curculionidae) are common during the flight season of *S. noctilio* (authors' personal observation) and are thought to make trees more susceptible to attack. Conversely, infestation of host trees by some weak plant pathogens, such as beetle-vectored blue stain fungi (*Leptographium* and *Ophiostoma*), can inhibit the growth of *A. areolatum*, and affect the feeding of nematodes on *A. areolatum*, thereby adversely impacting the nematode biocontrol program for the woodwasp (Wahl 2017; Wang et al. 2019).

Sirex noctilio larvae and their fungal symbiont have competitors for resources while developing in trees. Past research has shown that some fungal species are antagonistic toward *A. areolatum* but cannot completely replace colonies in the host tree (Coutts and Dolezal 1965, Ryan et al. 2011). Other studies have demonstrated that some species of wood-colonizing fungi completely overgrow the mycelium of *A. areolatum*, leading to its death (Wang et al. 2019). We hypothesized that some fungi may inhibit the growth of *A. areolatum* in the host tree, which could impede the development and survival of *S. noctilio* larvae in the natural environment.

No direct research has been conducted on these interactions or their implications. The objectives of the present study were 1) to identify fungi isolated from the surface of dead *S. noctilio* larvae in host trees (fungi infecting *S. noctilio* larvae in the host are herein referred to as incidental fungi), 2) to evaluate the ability of those fungi to inhibit the growth and development of *S. noctilio* larvae and influence the moisture content of the host tissue, and (3) to investigate interactions between *A. areolatum* and incidental fungi in artificial cultures.

Materials and Methods

Collection of *S. noctilio* Larvae Infected with Fungi

Larvae infected with fungi were obtained from trees in *S. noctilio*-infested plantations near the city of Yushu, China (44°43′52″ N, 126°55′7″ E). Mongolian pines naturally infested with *S. noctilio* were selected by observing typical oviposition symptoms (i.e., resin beads at each ovipositor insertion) and observation of larvae in split logs cut from these trees. Five trees were selected and cut into six to eight 1-m-long bolts and transferred to the sterile insect and quarantine room at Beijing Forestry University. *Sirex noctilio* larvae infected with fungi were collected from split bolts and preserved in sterilized centrifuge tubes and kept at 4–5°C until further study.

Isolation of Incidental Fungi from the Surface of *S. noctilio* Larvae

Fungal hyphae were removed from the surface of apparently colonized *S. noctilio* larvae with a sterile blade (on an ultra-clean bench) and cultured in potato dextrose agar (PDA) media amended with antibiotics streptomycin sulfate (100 mg/L), and tetracycline (50 mg/L) added to suppress bacterial growth. All isolates were incubated at 25 ± 1°C and 70 ± 5% relative humidity (RH) for 1–4 wk or until growth of mycelia. Agar cubes (~1 mm^3) were removed aseptically from the edge of colonies and transferred to fresh PDA plates. Each colony was transferred at least three more times until a visually uniform culture was obtained. Mycelia and spores were transferred to 20% glycerol in purified, distilled water (v/v) and stored at −80°C. Additionally, fungal cultures were generated on PDA slants in centrifuge tubes and stored under sterile mineral oil at 4°C.

Morphological and Molecular Identification of Incidental Fungi

Fungi were identified based on both morphology and internal transcribed spacer (ITS) sequencing. For initial identification using ITS sequencing, DNA was extracted from fungal mycelia on fresh cultures using an Extract-N-Amp tissue PCR kit (Sigma–Aldrich Corporation.St. Louis, MO) following the manufacturer's instructions. Fungal ribosomal ITS1, 5.8S (where present), and ITS2 regions were amplified using fungus-specific ITS1 and ITS4 primers (Gardes and Bruns 1993) in reactions (25 µl) containing 23 µl of Master Mix (Tsingke, China), 1 µl of each primer, and 1 µl of template DNA. Polymerase chain reaction (PCR) amplification was done with an initial denaturation step at 98°C for 2 min, followed by 30 cycles with denaturation at 98°C for 10 s, annealing at 50°C for 15 s, extension at 72°C for 15 s, and a final extension step of 5 min at 72°C.

PCR amplification products were separated by electrophoresis on 1% (w/v) agarose gels and stained with ethidium bromide for visual examination. PCR products were purified by using an agarose gel DNA extraction kit (Takara, Japan) and sequenced at Qinke Biotech (Beijing, China). The resulting sequences were used in BLAST searches of GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and sequences sharing ≥99% similarity with a partial 28S rDNA sequence (~600 bp) were considered to represent identical species (Wu et al. 2013). When sequences shared <99% similarity with known species, morphological features were used to identify the fungi. Specifically, mycelium morphology, mycelium surface texture, colony color, production of pigments and their diffusion in the medium, spore production, and mycelium growth rate on PDA plates were evaluated. Fungi that did not sporulate on this medium were transferred onto a different artificial medium (water 400 ml, phloem 15 g, agar 15 g) to activate sporulation (Wang et al. 2012). Morphology (size, color, shape, and ornamentation) of conidiomata, conidiogenous cells, conidiophores, and conidia were evaluated for the anamorph; sporomata and their associated structures as well as spore morphology were evaluated for the teleomorph (Saucedo-García et al. 2014).

Effects of Fungal Inoculation on *S. noctilio* Larval Development in Naturally Infested Bolts

Ten Mongolian pine trees (with >30 clear resin beads observed on each tree) were obtained from the *S. noctilio*-infested *P. sylvestris* var. *mongolica* plantation in Yushu (44°43′52″ N, 126°55′7″ E), in April 2017. These trees were cut into 1.2-m-long bolts (>15 cm diam). After sealing the cut ends with wax, bolts were transferred to the insect quarantine room at Beijing Forestry University. The diameter was

measured and recorded to ensure that growth conditions in sample logs inoculated with each fungus were similar. Lengthwise lines of six, 6-mm diameter holes (~20 cm apart) were drilled to a depth of ~4 cm with a sterile increment borer (Haglof, Sweden; Hartshorn et al. 2017). Three parallel lines of holes (at 120° intervals around the circumference) were drilled per log (18 holes in total; Supp Fig. 1 [online only]; Carnegie and Bashford 2012). To ensure a sterile environment, surfaces of the quarantine room were exposed to ultraviolet light for 30 min before drilling inoculations. The six primary fungi isolated from *S. noctilio* (*Trichoderma harzianum* Rifai 1969, *Trichoderma atroviride* Bissett 1984, *Trichoderma viride* Pers 1794, *Sphaeropsis sapinea* (Fr.) Dyko 1980, *Ophiostoma minus* (Hedgc.) Sydow 1919, and *Phlebiopsis gigantea* (Fr.) Jülich 1978) and the symbiont *A. areolatum* (extracted from *S. noctilio* female mycangia; Boros 1968) were used as inoculum. Pairs of mycelial plugs (diameter = 5 mm) of actively growing fungi were then inoculated into each hole. Holes inoculated with two plugs of sterile PDA media served as negative controls. One fungal species was inoculated onto any single bolt. Three independent replicates (inoculation of three sample logs) were performed for each fungus and control. Holes inoculated with fungi or controls were then filled with sterilized sawdust from the xylem of Mongolian pine and sealed with foam rubber. All logs were wrapped in plastic film and incubated at 25 ± 3°C and 65 ± 5% RH.

We gravimetrically measured the moisture content of the sample logs before and after inoculation. For each bolt, six fresh wood cores (a single line) removed during creation of inoculation sites were weighed; then cores were placed in an aluminum dish inside a dehydrator and dried at 105°C for 48 h before being re-weighed. After 2 mo of incubation, 10 wood cores (6-mm diam.) were sampled throughout the length of each inoculated log (one sample every 12 cm), and their moisture content was measured as per the pre-inoculation samples (Yousuf et al. 2014b). Following collection of the second set of cores, *S. noctilio* larvae were dissected from logs and the numbers of individuals with or without visible fungal growth was determined. These fungi were isolated and identified according to the methods described previously.

Fungal Growth Rate

The six predominant fungal species isolated from dead *S. noctilio* larvae as well as *A. areolatum* were grown singly on PDA media in an incubator at 25 ± 1°C and 70 ± 5% RH. After 7 d, mycelium plugs from the edge of each fungal colony were removed with a sterile punch (4-mm diam.) and placed at the center of a fresh 9-cm diameter Petri plate containing PDA media. Mycelial growth was measured daily by using the crossing method (Li et al. 2015b). Growth generally reached the edge of the Petri plate after 7 d. Each experiment was replicated five times.

Effects of Incidental Fungi on Growth of *A. areolatum* in Artificial Media

The in vitro activity of fungi isolated from dead *S. noctilio* larvae against *A. areolatum* was determined using dual culture assays (Cottyn et al. 2001). Two mycelial plugs (4-mm diam.) of incidental fungi (prepared as above) were placed at opposite ends of a 9-cm diameter Petri plate and 10 mm from the edge of the plate. A plug of *A. areolatum* was placed at the center of the plate. Because the growth rate of *A. areolatum* was much slower than for the incidental fungi (Wang et al. 2019), *A. areolatum* was inoculated 3 d earlier. Plates inoculated with *A. areolatum* alone served as controls. Each treatment was repeated three times, and all plates were incubated at 25°C for 7 d. Radial growth of incidental fungi and *A. areolatum* was recorded daily, and inhibition was evaluated using the formula:

$$P(\%) = 100 \times [(C - d) - (T - d)] / (C - d)$$

where P is the percent growth inhibition, C is the *A. areolatum* colony radius in controls, T is the *A. areolatum* colony radius in dual cultures, and d is the radius of the mycelial plug.

Statistical Analysis

Isolation frequency (IR%) was calculated as the number of times a species was isolated divided by the total number of samples incubated (Fröhlich et al. 2000). Fungal prevalence was calculated as the isolation frequency of a given species divided by the sum of the isolation frequencies of all fungi (Wang and Guo 2007). The moisture content of the sample logs could not be transformed to meet assumptions, therefore, comparison of the moisture content of the sample logs before and after inoculation for all fungi was achieved with a nonparametric Kruskal–Wallis test. One-way analysis of variance was used to analyze parameters measured in fungal growth rate experiments and effects of incidental fungi on growth of *A. areolatum*. Differences between treatment means were evaluated using Tukey's honestly significant differences (HSD) tests. Pairwise comparisons between the mortality of *S. noctilio* larvae in logs treated with different fungi and those of the control group were analyzed with a normal approximation of the Pearson's χ^2-test.

Results

Morphological and Molecular Identification of Incidental Fungi

In total, 114 fungal isolates were obtained from the surfaces of 127 dead *S. noctilio* larvae in host trees. Fungi were assigned to 27 representative morphotypes based on cultural characteristics, and isolates were identified as 12 species within 9 genera based on rRNA gene ITS sequences or morphological features (Table 1). Among the nine genera, 8 (11 species) were within the phylum *Ascomycota*, and 1 was within *Basidiomycota* (1 species). The most abundant genera were *Trichoderma* (53.5%), *Sphaeropsis* (15.8%), and *Ophiostoma* (11.4%). The most frequently isolated fungal species were *Trichoderma harzianum*, *T. atroviride*, *T. viride, Sphaeropsis sapinea*, *Ophiostoma minus*, and *Phlebiopsis gigantea*.

Effects of Fungal Inoculation on *S. noctilio* Larval Development in Naturally Infested Bolts

Pairwise comparisons showed that the mortality of *S. noctilio* larvae was significantly lower in the control bolts than in bolts treated with *O. minus*, *T. atroviride*, *T. viride*, *P. gigantea*, or *T. harzianum*; but not bolts treated with *A. areolatum* or *S. sapinea* (Table 2). Fungi isolated from dead larvae were generally the same as in the inoculum used for the bolt, and these included *T. harzianum*, *T. atroviride*, *T. viride*, and *P. gigantea* (Table 2). *Trichoderma atroviride* had the highest frequency of re-isolation (87%). In contrast, in bolts inoculated with *O. minus*, this fungus was isolated from only 47% of 32 dead larvae, and the other dead larvae failed to show signs of *O. minus* infection. *Amylostereum areolatum* was not isolated from the surface of dead larvae. When *S. noctilio* larvae were colonized by *O. minus* or *S. sapinea*, their bodies turned black, but bodies were yellow/green when colonized by *Trichoderma* (see Supp Fig. 2 [online only]).

The moisture content of bolts before fungal inoculation did not differ significantly ($\chi^2 = 7.74$, df = 7, $P = 0.356$; Fig. 1). However, 2

Table 1. Isolation frequency (IR%) and dominance of fungi isolated from the surface of dead *Sirex noctilio* larvae in naturally colonized trees

Taxa	Identification method	Closest species (Accession No.)	Similarity (%)	No of isolates	Frequency of isolation (%)	Applications or ecological significance	Reference(s)
Trichoderma harzianum Rifai 1969	Molecular	*Trichoderma harzianum* (JN039048)	100	24	18.9	Inhibits tree pathogens	Yuan et al. (2019)
Trichoderma atroviride Bissett 1984	Molecular	*Trichoderma atroviride* (KR868397)	100	20	15.7	Inhibits tree pathogens	Romana et al. (2015)
Sphaeropsis sapinea (Fr.) Dyko 1980	Molecular	*Sphaeropsis sapinea* (KF766159)	99	18	14.2	Tip blight fungi of pine tree	Treena et al. (2001)
Trichoderma viride Pers 1794	Molecular	*Trichoderma viride* (HM037962)	99	15	11.8	Inhibits tree pathogens	Romana et al. (2015)
Ophiostoma minus (Hedgc.) Sydow 1919	Molecular	*Ophiostoma minus* (GU134172)	99	13	10.2	Blue-staining fungus of wood	Yousuf et al. (2014a)
Phlebiopsis gigantean (Fr.) Jülich 1978	Molecular	*Phlebiopsis gigantean* (KP135389)	100	8	6.3	Inhibits tree pathogens	Rishbeth (1963)
Penicillium sp. Link 1809	Morphological	*Penicillium* sp. (HM469409)	97	7	5.5	Produces insect-resistant alkaloids	Du et al. (2009)
Fusarium solani (Mart.) Saccardo 1881	Molecular	*Fusarium solani* (EU719658)	99	3	2.4	Saprophyte	Martinez et al. (1992)
Bionectria ochroleuca (Schwein.) Schroers 1997	Morphological	*Bionectria ochroleuca* (HM037945)	97	2	1.6	Biocontrol fungus	Chen et al. (2014)
Trichoderma koningii Oudem 1902	Molecular	*Trichoderma koningii* (HM037975)	99	2	1.6	Inhibits tree pathogens	Chirame et al. (2005)
Alternaria alternate (Fr.) Keissl 1912	Molecular	*Alternaria alternate* (KJ173524)	99	1	0.8	Pathogenic fungus of pine	Sankar et al. (2012)
Chaetomium globosum Kunze 1829	Molecular	*Chaetomium globosum* (KM268644)	99	1	0.8	Biocontrol fungus	Wang et al. (2019)
Total				114	89.8		

Similarity : ITS region sequence identity between the isolates in this study and closest species in GenBank.

mo after inoculation with fungi, the moisture content of bolts differed significantly among treatments (χ^2 = 31.46; df = 7; P < 0.01). Only *O. minus* bolts lost moisture at a significantly higher rate than controls (Fig. 1).

Growth rates on artificial media differed significantly among the seven fungi (F = 174.7, df = 6, P < 0.01; Table 2). *Sphaeropsis sapinea* exhibited the fastest growth rate (11.7 ± 0.3 mm/d), whereas *A. areolatum* displayed the slowest growth rate (3.1 ± 0.2 mm/d). No significant differences in growth rate were noted among the three *Trichoderma* species (F = 2.28, df = 2, P > 0.05).

Effects of Incidental Fungi on Growth of *A. areolatum* in Artificial Media

In dual culture assays, the six fungal isolates varied in their capacity to reduce *A. areolatum* growth (F = 860.1, df = 5, P < 0.01; Fig. 2). Four fungi, *P. gigantea*, *T. viride*, *T. harzianum*, and *T. atroviride* continued to grow after encountering *A. areolatum* mycelium (Fig. 3), and eventually completely covered the latter (Supp Fig. 3 [online only]). *Trichoderma atroviride* and *T. harzianum* exhibited the strongest inhibitory effect. However, *O. minus* and *S. sapinea* displayed low antagonistic ability against *A. areolatum*, and a confrontation situation was observed when the two fungi and *A. areolatum* met (see Supp Fig. 3 [online only]). Confrontational behavior was observed for 15 d, during which the size of fungal colonies remained unchanged, even though colonies appeared healthy.

Discussion

Twelve fungal species and nine genera were isolated from dead *S. noctilio* larvae in colonized Mongolian pine trees. *Trichoderma*, *Sphaeropsis*, and *Ophiostoma* were the most abundant genera among the isolates, and most fungal species were the same as those isolated in a previous study from Mongolian pine trees displaying woodwasp damage (Wang et al. 2016). The present study is the first report that fungal species which occur incidentally (i.e., that are non-symbionts) in *S. noctilio*-colonized trees can affect the survival of *S. noctilio* larvae.

Previous research demonstrated that some fungi affect the female adult woodwasp's selection of oviposition sites and inhibit the mycelial growth of *A. areolatum*. In previous surveys, we also found that some dead larvae were infected with fungi in host trees. Thus, we hypothesized that some fungi also may affect the development and survival of *S. noctilio* larvae in host tree. Our bioassays of the effects of certain incidental fungi present on cadavers of *S. noctilio* larvae showed that inoculation of these fungi into bolts of pine naturally colonized by *S. noctilio* reduced larval survival, and furthermore, these fungi could inhibit growth of the fungal symbiont of *S. noctilio* on artificial media.

In our study, test fungi included common saprophytes (*Trichoderma* sp. and *P. gigantea*) and opportunistic pathogens of *Pinus* spp. (*S. sapinea* and *O. minus*). The mortality rates of *S. noctilio* larvae varied among fungal species tested, and fungal species inhibited the mycelial growth of the woodwasp's symbiont *A. areolatum* to varying degrees.

The present study found that *S. noctilio*-colonized bolts inoculated with the blue stain fungus *O minus* exhibited higher larval mortality than bolts treated with other fungi. However, *O. minus* was re-isolated from only 47% of dead larvae in *O. minus*-treated bolts (Table 2), and other dead larvae had no signs of *O. minus* colonization. Previous research found that survival of *Dendroctonus frontalis* Zimmermann larvae (Coleoptera: Curculionidae: Scolytinae) in galleries is negatively impacted by growth of *O. minus*,

Table 2. Virulence of incidental fungal isolates against the survival of *Sirex noctilio* larvae

Fungal isolate	Growth rate (mm/day)[a]	Total[b]	Mortality (%)[c]	χ^2(df)	*P*-value[d]	Re-isolated from larva[e]	Another fungus in larvae[f]
Control	-	37	16.2	-	-	-	-
Trichoderma harzianum	9.85 ± 0.16 bc	24	41.6	4.87 (1)	*	7	3
Trichoderma atroviride	10.66 ± 0.34 b	27	55.5	10.95 (1)	**	13	2
Ophiostoma minus	9.38 ± 0.10 c	46	69.5	23.51 (1)	**	15	17
Trichoderma viride	10.21 ± 0.28 bc	33	57.5	12.90 (1)	**	15	4
Sphaeropsis sapinea	11.91 ± 0.25 a	29	20.6	0.22 (1)	ns	2	4
Phlebiopsis gigantea	7.48 ± 0.11 d	34	50.0	9.23 (1)	**	11	6
Amylostereum areolatum	3.09 ± 0.17 e	30	16.6	0.02 (1)	ns	5	0

[a]Growth rates followed by different letters were significantly different according to Tukey's HSD tests ($P < 0.05$).

[b]Total: total number of larvae obtained from sample logs treated with different fungi and control.

[c]Mortality: the number of dead larvae divided by the total number of larvae, these larvae were obtained from sample logs treated with different fungi and control.

[d]*p*-value: proportion of live to dead larvae differed significantly from controls; $*P < 0.05$; $**P < 0.01$; ns, $P \geq 0.05$.

[e]Re-isolated from larva: this indicates the number of wasp larvae for which the fungus re-isolated matched the incidental fungi that was originally used to inoculate sample logs.

[f]Another fungus in larvae: indicate other dead larvae for which the fungus re-isolated did not match the incidental fungi that was originally used to inoculate sample logs.

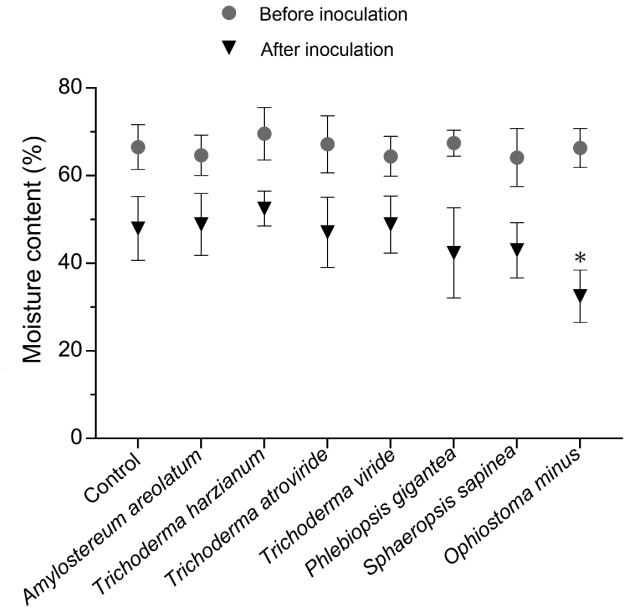

Fig. 1. Moisture levels in test logs at time of inoculation with fungi and 2 mo afterward. Asterisk denotes a significant change in moisture content occurred from inoculation with *O. minus* (Kruskal–Wallis test, $\alpha = 0.05$) but not other fungi. .

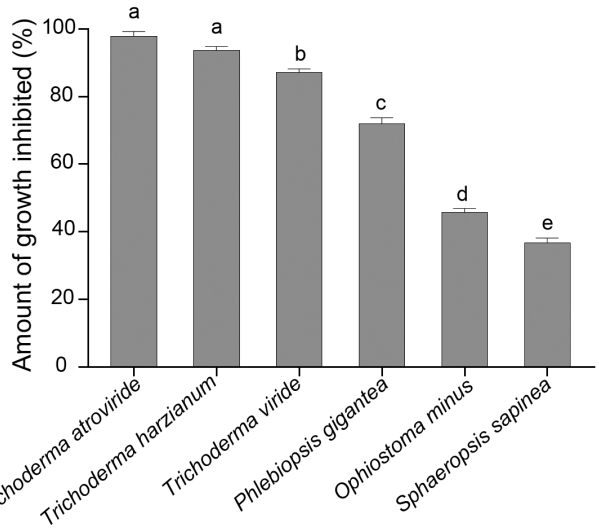

Fig. 2. Inhibition of growth of *A. areolatum* (see text for description of this measurement) when grown in culture with different incidental fungal associates of *S. noctilio*. Different letters indicate significant differences between treatments (Tukey's HSD test; $\alpha = 0.05$).

a fungal symbiont of the beetle's phoretic mites that competes with the beetle's fungal mutualist, *Entomocorticium* sp. (Scott et al. 2008). Similarly, death of *S. noctilio* larvae could have been due in part to interference by *O. minus* with growth and persistence of the woodwasp's fungal symbiont *A. areolatum*, which is essential for the woodwasp's capacity to derive nutrients from host wood (Thomson 2014). Consistent with this hypothesis, *O. minus* was antagonistic to growth of *A. areolatum* in petri plates (Fig. 2). However, it was a weaker inhibitor than four of the other tested fungi, and, unlike these other fungi, it did not overgrow *A. areolatum* colonies (Fig. 2).

Yousuf et al. (2014b) found that wood colonized by blue stain fungi dries more quickly. Wood moisture content is an important factor that directly affects *S. noctilio* survival, growth, and development (Madden and Coutts 1979), and thus, reduction of the wood moisture content by *O. minus* growth could have reduced larval survival. Therefore, we propose that *O. minus* growth negatively impacts *S. noctilio* larvae through at least two possible mechanisms: first, by directly inhibiting growth of the larvae's symbiont *A. areolatum*, and second, by reducing wood moisture content and thereby interfering indirectly with both larval and symbiont development (Supp Fig. 4 [online only]; Scott et al. 2008; Hurley et al. 2012). In addition, *S. noctilio* larvae have a fully enclosed growing environment in host trees and will die if they leave the gallery. Therefore, it is not certain that the *O. minus* is directly infecting and killing the larvae. The negative impacts of *O. minus* on *S. noctilio* are implied by the previously reported avoidance of fungus-colonized tissue by *S. noctilio*. Adult female *S. noctilio* rarely oviposit in areas previously colonized by blue stain fungi (Foelker 2016), and presence of *Ophiostoma* and *Trichoderma* can inhibit *Sirex* oviposition at localized sites on trees (Spradberry and Kirk 1978, Schubert et al. 2008). Galleries of *S. noctilio* larvae generally avoid wood colonized by blue stain fungi (Coutts 1969, Yousuf et al. 2014a). Furthermore,

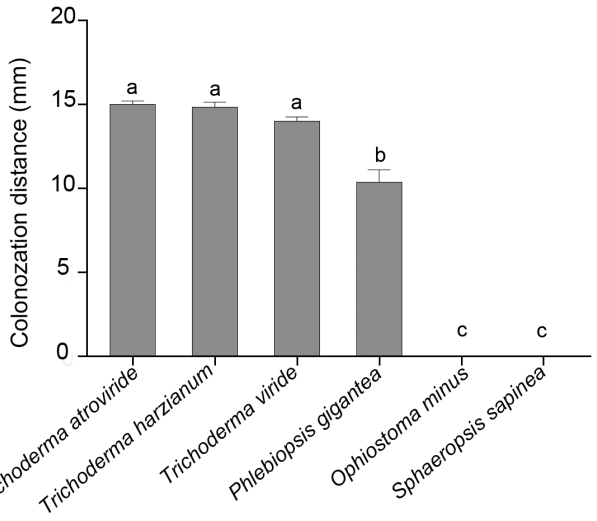

Fig. 3. Colony growth by test fungi during 5 d after encountering the *A. areolatum* colony. Mean growth distances (bars) associated with different letters were significantly different (Tukey's HSD test; $\alpha = 0.05$).

volatiles produced by *O. minus* have a repellent effect on wasps (Wang et al. 2019).

Likewise, the present study found that *S. noctilio* larvae in bolts inoculated with *Trichoderma* spp. or *P. gigantea* had significantly higher mortality (41.8–58%) than larvae in control bolts (16.2%), and these same fungi were re-isolated from the body surfaces of the majority of dead larvae in these respective fungal treatments. As with *P. gigantea*, *Trichoderma* species may increase larval mortality by inhibiting growth of *Amylosterium* or possibly even killing the fungus. Previous studies demonstrated that several enzymes produced by *Trichoderma* species possess antibiotic activity against fungal pathogens of plants (Howell 2003). Furthermore, *Trichoderma* spp. and *P. gigantea* have been successfully employed as biocontrol agents for the treatment of pruning wounds on urban trees to prevent colonization by wood decay fungi (Schubert et al. 2008). Additionally, *T. atroviride*, *T. viride*, *T. harzianum*, and *P. gigantea* completely covered the mycelia of *A. areolatum* in Petri plate assays. This suggests that these fungi can also cover substrates previously colonized by *A. areolatum* in host trees. We also found that *Trichoderma* spp. and *P. gigantea* exhibit faster growth rates than *A. areolatum*, and thus, these species might occupy and monopolize suitable host tissue before *A. aereolatum*.

No significant differences were noted between the control group and *A. areolatum* or *S. sapinea* groups in terms of larval mortality. This is not surprising since *S. sapinea* is a weak pathogen (Stanosz 1997). In previous studies, *S. sapinea* was found to be a less aggressive competitor of *A. aereolatum* than species of the genus *Trichoderma* (Stanosz 1997, Wang et al. 2019).

The influence of co-habiting pine insects on the survival of *S. noctilio* larvae is likely to be indirect (Ryan et al. 2012). Yousuf et al. (2014a,b) correlated the presence of *I. grandicollis* with smaller *Sirex* broods, higher larval mortality, reduced *Amylostereum* growth, and ineffective parasitism by *D. siricidicola*. This disruption of *Sirex* development is likely due to the *Ips*-associated fungus *Ophiostoma ips* (Rumb.) outcompeting *A. areolatum* and causing the host substrate to become otherwise unsuitable for *Sirex* development. In northeast China, a variety of insect species colonize Mongolian pine, creating competition for resources with *Sirex*. Native bark beetles *I. sexdentatus* and *I. acuminatus* can transmit their associated blue stain fungus *O. minus* and produced these effects in Mongolian pine. *Ophiostomatoid* (which includes blue stain) fungi are commonly vectored by bark beetles, and these fungi can kill resin canal cells and ray parenchyma of host trees (Edmonds et al. 2011). *Ophiostoma minus* can block water flow in the xylem, thereby reducing wood moisture and making the habitat inhospitable for *S. noctilio* larvae. The findings demonstrate the potential for incidental fungi as biological control agents for *S. noctilio* and highlight the need for more fundamental research for understanding the ecological relationships among incidental fungi, the symbiotic fungus *A. areolatum*, and the pest *S. noctilio*.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

Acknowledgments

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