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Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi

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Abstract. 1. Colonisation of host trees by an endophytic herbivore, the spruce beetle, *Dendroctonus rufipennis*, is accompanied by invasion of its galleries by a number of fungal species. Four of these associated species were identified as *Leptographium abietinum*, *Aspergillus fumigatus*, *Aspergillus nomius*, and *Trichoderma harzianum*.

2. *Trichoderma* and *Aspergillus* significantly reduced spruce beetle survival and reproduction in controlled assays.

3. A previously undescribed behaviour was observed, in which spruce beetle adults exuded oral secretions, especially within fungus-pervaded galleries.

4. These oral secretions inhibited the growth of fungi except *A. nomius*, and disrupted the morphology of the latter. Administration of these secretions indicated a dose-dependent inhibitory effect.

5. Oral secretions cultured on microbiological media yielded substantial bacterial growth.

6. Filter-sterilised secretions failed to inhibit fungal growth, evidence that the bacteria are responsible for the antifungal activity.

7. Nine bacterial isolates belonging to the Actinobacteria, Firmicutes, Gammaproteobacteria, and Betaproteobacteria taxa were obtained from the secretions.

8. Bacterial isolates showed species-specific inhibitory activity against the four fungi antagonistic to spruce beetle. The bacterium with the strongest fungal inhibition activity was the actinomycete *Micrococcus luteus*.

9. The production of bark beetle secretions containing bacteria that inhibit fungal growth is a novel finding. This suggests an additional level of complexity to ecological associations among bark beetles, conifers, and microorganisms, and an important adaptation for colonising subcortical tissue.

Key words. Antagonism, *Aspergillus*, bark beetles, blue stain fungus, *Dendroctonus*, insect–fungal interactions, *Leptographium*, spruce beetle, symbiosis, *Trichoderma*.

Introduction

Many plant-feeding animals rely on symbiotic microorganisms to enhance food digestion, communicate, provide supplemental nutrition, and detoxify plant defence compounds (Frank, 1997). These mutualistic interactions may be of particular importance to herbivores whose diets are of relatively poor nutritional quality, and that exploit well-defended plants (Martin, 1987). Such conditions are especially prevalent for endophytic species in general and phloeophagous insects in particular.

Bark beetles (Curculionidae: Scolytinae) develop entirely beneath the bark of woody plants (Paine *et al.*, 1997). Most species are saprophytic, but some undergo landscape-level eruptions that cause nearly total mortality to millions of hectares of mature conifers under natural conditions (Holsten *et al.*, 1999; Raffa *et al.*, 2005). These eruptions affect ecosystem processes such as succession and fire cycles, pose important challenges to natural resource management, and cause substantial economic

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losses. Bark beetles colonise host trees by using aggregation pheromones to coordinate mass attacks that overwhelm host defences. They then construct a network of parental ovipositional and larval feeding galleries within the phloem tissue. Adults emerge from brood trees and colonise new hosts.

Each species of bark beetle is associated with several species of fungi. The tightness of these associations, the mechanisms of fungal transport, and the effects of these fungi on the beetle vary widely among systems (Paine *et al.*, 1997; Six, 2003b; Klepzig & Six, 2004). Some fungi may participate in pheromone synthesis (Brand *et al.*, 1976) or assist in various forms of nutrition such as by concentrating nitrogen (Barras, 1973; Ayres *et al.*, 2000; Six, 2003a). Others may be pathogenic, opportunistic, or antagonistic (Lombardero *et al.*, 2003; Six, 2003b).

The most widely described relationships involve Ascomycetes, commonly called blue stain or ophiostomatoid fungi, with which nearly all bark beetles species are associated (Wingfield *et al.*, 1993). Both beneficial and deleterious components to ophiostomatoid interactions with their beetle vectors have been described. For example, some *Ophiostoma* (then *Ceratocystis*) (Strobel & Sugawara, 1986), *Leptographium* (Solheim *et al.*, 1993), and *Ceratocystis* (Christiansen, 1985; Solheim & Safranyik, 1997; Lieutier, 2002) species may assist beetles in depleting tree resistance mechanisms. Conversely, some species have strong adverse effects on beetle development, both directly by competing for the food substrate, and indirectly by out-competing other beneficial species (Barras, 1970; Klepzig & Wilkens, 1997; Lombardero *et al.*, 2003; Six, 2003a,b). Further, some fungal associates may exert both positive and negative effects, and hence have been proposed to be conditional or context-dependent mutualists (Eckhardt *et al.*, 2004; Klepzig & Six, 2004; Kopper *et al.*, 2004).

The spruce beetle, *Dendroctonus rufipennis* Kirby, colonises trees in the genus *Picea* (Holsten *et al.*, 1999). Low density populations are mostly associated with dead or highly stressed trees, but eruptive populations can kill most of the mature vigorous trees over several million hectares (Wallin & Raffa, 2004). Spruce beetles show close associations with several ophiostomatoid fungi, of which *Leptographium abietinum* (Peck) Wingfield is predominant (Ohsawa *et al.*, 2000; Six & Bentz, 2003; Aukema *et al.*, 2005).

In laboratory assays, failure by spruce beetles to establish on white spruce, either in logs or excised phloem, is often associated with invasion of their galleries by green-spored hyaline hyphomycete fungi (Fig. 1a). These fungi frequently grow on the bodies of these dead beetles (Fig. 1b). However, information on whether these fungi are the cause of beetles' death, or if they are mere saprophytes that colonise post-mortem is currently lacking. Interactions of bark beetles with such fungi have been noted in other systems (Barras, 1969; Jassim *et al.*, 1990; Paine *et al.*, 1997; Six, 2003a).

The purpose of this research was to (a) determine if these hyphomycete and ophiostomatoid fungi associates have deleterious effects on the survival and reproduction of spruce beetles, (b) if so, what, if any, behavioural, associational, and/or physiological countermeasures might the insects employ to resist these invasions, and (c) whether beetle interactions with its various associated fungi differ.

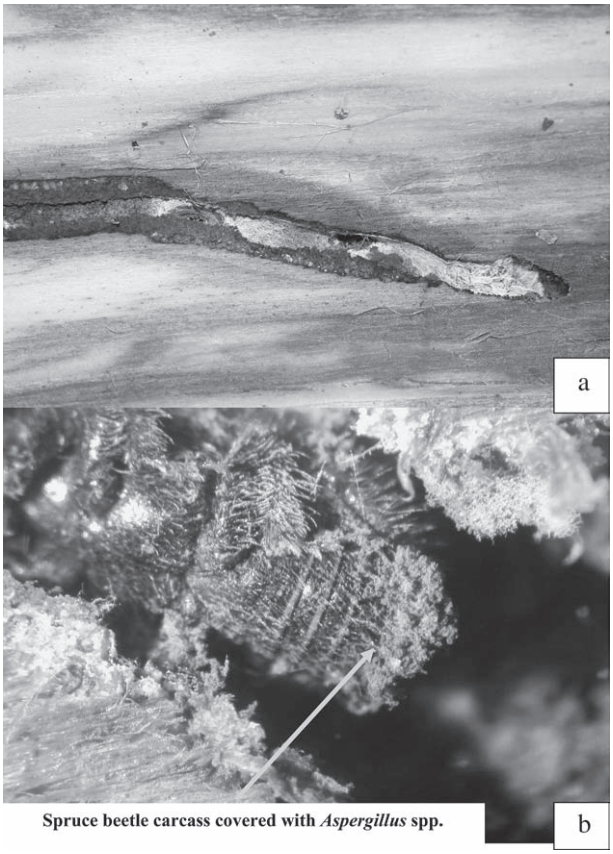

Fig. 1. Invasion of spruce beetle galleries by fungi in white spruce logs. (a) Beetle gallery invaded by *Trichoderma harzianum*. These conditions invariably led to the death of the adult beetles and brood. (b) The body of an adult spruce beetle covered by *Aspergillus* spp. These fungi resulted in higher adult mortality in the experimental arenas.

Materials and methods

Insects

Adult spruce beetles were collected from naturally infested trees on the Kenai Peninsula of Alaska, U.S.A., and shipped to the Forest Entomology Laboratory at the University of Wisconsin, Madison, Wisconsin, U.S.A. Upon arrival, the insects were segregated by gender, placed within 200 ml screw-cap glass jars, and provided with pieces of excised spruce phloem for food and crumpled Kimwipes® (Kimberly Clark, Roswell, Georgia) to absorb excess moisture, provide cold insulation and provide a surface for walking. The insects were then stored at 4 °C until needed for experiments.

Isolation and identification of fungi

Previous work in the authors' and other's laboratories (Six & Bentz, 2003; Aukema *et al.*, 2005) has demonstrated that the fungi associated with spruce beetle galleries can be easily obtained by streaking the insect's body, including mouthparts,

onto a suitable culture medium. Fungi were obtained by streaking the beetles' mouthparts three times across the centre of a disposable standard (10 × 1.5 cm) plate containing 10% Tryptic Soy Agar (TSA) (Difco™, Becton, Dickinson & Co., Sparks, Maryland). The four most common fungi were then maintained in pure culture. For the non-ophiostomatoid species, Malt Extract Agar (MEA) (Difco™) was used. Ophiostomatoid fungi (as determined morphologically) were cultured on MEA amended with 200 mg l^{-1} of cyclohexamide (MP Biomedicals, LLC, Aurora, Ohio) and 100 mg of streptomycin sulphate (Sigma–Aldrich Co., St. Louis, Missouri) (Jacobs & Wingfield, 2001). All cultures were maintained in darkness at 25 ± 2 °C.

The fungi were identified using a combination of morphological and molecular analyses. Morphological identification to genera and potential species was performed by examining slide mounts of the mycelium and conidiophore structures under a One-fifty Reichert compound microscope (Reichert Scientific Instruments, Warner–Lambert Technologies, Inc., Buffalo, New York) and comparing them to descriptions in Barnett and Hunter (1998) and Jacobs and Wingfield (2001). Identity of ophiostomatoid isolates was further verified by direct comparisons of fruiting structures with those of pure *L. abietinum* cultures provided by Thomas Harrington (Department of Plant Pathology, University of Iowa, Ames).

Molecular analyses consisted of sequencing the internally transcribed spacer (ITS) region of the ribosome (White *et al.*, 1990). Hyaline hyphomycete and ophiostomatoid fungi isolated from three different beetles were grown for 7 days on MEA plates under the conditions described above. The fungal tissue (200 mg FW) was harvested by scraping the surface of the media plates with flame-sterilised scalpels, and stored in autoclaved 1.5-ml microcentrifuge tubes in a freezer at –30 °C until needed for DNA extraction. Fungal DNA was extracted using Qiagen's (Qiagen Sciences, Valencia, California) mini plant kit following the manufacturer's instructions, with two exceptions: the cell lysing incubation time was increased to 2 h, and the final DNA elution was carried out with 50 μl of the elution buffer. Primers ITS1 and ITS4 (White *et al.*, 1990) were used to extend the desired region of the rDNA.

The thermocycler (PT-100, MJ Research, Inc., Watertown, Massachusetts) reaction conditions were: 3 min at 94 °C for initial denaturation, 1 min at 48 °C for annealing, 1 min at 72 °C for extension, 1 min at 94 °C for the second denaturation step, and 5 min at 72 °C for final extension. The steps were repeated for a total of 40 cycles.

The extended region (≈ 600 base pairs) was cleaned of any excess PCR reagents using Qiagen's QIAquick® PCR purification kit, as per manufacturer's instructions. Two microlitres of the clean PCR product was separately mixed with 1 μl of each of the ITS primers and 21 μl of water and sequenced by the University of Wisconsin, Madison Biotechnology Center. The sequences obtained with each of the two corresponding primers were assembled and cleaned using DNASTAR Seqman (DNASTAR, Inc., Madison, Wisconsin) and then subjected to a closest known sequence match in GenBank (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, Maryland) using the nucleotide–nucleotide BLAST search mode.

Effects of fungi on spruce beetle behaviour and performance

The experimental arena consisted of an 8 × 8 cm piece of spruce phloem with bark sandwiched between two 10 × 10 cm Plexiglas plates. The edges around the Plexiglas plates were sealed using 1.0 × 0.25 × 200 cm vinyl foam weatherseal strips (Thermwell, Home Depot, Madison, Wisconsin) cut to fit each side of the arena. The arenas were then bound together with two 2.54-cm binder clips on each side. These arenas were adapted from 'phloem sandwich' designs described by Taylor *et al.* (1992). The phloem pieces were obtained from white spruce logs ≈ 30 cm diameter cut 1–3 weeks in advance of set up. Logs were covered with molten paraffin wax at both ends immediately after tree felling to prevent desiccation, and stored at 4 °C until needed.

Initial experiments consisted of detailed observations of beetles during gallery construction in untreated phloem arenas. Subsequent experiments involved inoculation of the phloem with various fungal treatments. Fungal cultures were started by placing a 1-cm diameter mycelial plug in the centre of MEA media plates. The fungi were allowed to grow for 5–7 days under the conditions previously described. Phloem was surface sterilised by dipping into a 1:5:94 bleach : EtOH : H_2O solution for 1 min and then air dried within a laminar flow hood for approximately 15–20 min, after which no bleach or EtOH could be detected by smell. The sterile phloem pieces were then inoculated with five 1-cm diameter plugs of each of the test fungi by placing a plug at each of the corners and one at the centre of the phloem square so that the fungus side would be in direct contact with the phloem surface. Arenas mock-inoculated with clean MEA plugs served as controls. The inoculated pieces of phloem were then assembled into the arenas as described previously. Two spruce beetle male–female pairs were introduced per arena immediately after fungal inoculation. The beetles were allowed to feed and oviposit for 4 weeks. After this period, their performance was evaluated based on number of galleries, total gallery length (cm), number of eggs laid and per cent adult mortality. This experiment was replicated in duplicate and repeated over time using different groups of insects and phloem sources to obtain a total of 10 replicates for each of the four fungal species.

Inhibition of fungal growth by spruce beetle oral secretions

Oral secretions (referred to here as substances expelled from beetles via their mouthparts) from three male and three female beetles were collected by streaking each insect's mouthparts slowly and gently across the centre of a 10% TSA plate three times. To minimise contamination from other beetle body parts, insects were confined within a glass Pasteur pipette using two pieces of mesh material. A pipette bulb was then used to force autoclaved H_2O over the beetles' bodies 20 times. This procedure was repeated twice more using fresh sterile water. Clean spruce beetles were held perpendicular to the TSA plate with a pair of flame-sterilised stainless steel featherweight wide tip forceps so only the beetles' mouthparts were in direct contact with the media plates. The secretions were then washed off the

plate by rinsing with 1 ml of autoclaved double-distilled H_2O. Water was pipetted over the streaked region repeatedly to maximise collection of the oral secretions.

The above secretion suspension was transferred to a 1.5-ml autoclaved microcentrifuge tube and its volume brought up to 1 ml with sterile H_2O. This suspension (100%), along with 1/10 (10%) and 1/100 (1%) dilutions and sterile H_2O control (0%) were used to test antifungal activity. The bioassay arena consisted of a 10% TSA plate on which four 1-cm autoclaved filter paper discs (Schleicher & Schuell Microscience, Riviera Beach, Florida) were equidistantly placed. Each filter paper disc received 100 μl of (a) sterile H_2O (control), (b) 1% oral secretion, (c) 10% oral secretion, or (d) 100% oral secretion. Each plate was then point inoculated with one of the four fungal species. The cultures were allowed to grow in the dark for 8 days, at which time the diameter of the area free of fungus around each discs was measured and recorded. Six replicates of each fungal species/oral secretion dilution combination were set up at one time.

Presence and bioactivity of microorganisms in spruce beetle oral secretions

To determine if microbes were present in spruce beetle oral secretions, secretions were collected from males and females as described above. The original suspension volume was split in two. One 500-μl aliquot was kept intact (non-sterile), and the other was passed through a 0.2 μm sterile nylon syringe filter (Fisher Scientific, Hampton, NH, U.S.A.) to remove microbes (sterile). A new filter was used for each sample. A 100-μl aliquot of each oral secretion treatment was plated on 10% TSA, and the plates were examined after 5 days for the presence of microbial growth.

To determine if the microorganisms present in spruce beetles' oral secretions were bioactive against fungi, the same bioassay described above was used. Three paper discs were placed equidistantly on a 10% TSA plate. Each disc received 100 μl of (a) non-sterile secretions, (b) filter-sterilised secretions, or (c) sterile water control. Plates were either point inoculated in the centre with *T. harzianum* or received a 0.5-cm mycelial plug of *L. abietinum*. Mycelial plugs were used for *L. abietinum* because this fungus grows more slowly than *T. harzianum*. Fungal inhibition was measured when the edge of the fungal mycelia reached the control filter paper disc (5–7 days), as described previously.

Isolation, identification, and bioactivity of bacterial isolates from spruce beetle oral secretions

Individual pure isolates were obtained by scraping the bacterial growth ring around the filter papers from the fungal inhibition bioassay. The bacterial growth surrounding a filter paper disc was suspended in 10 ml of autoclaved water, the suspension was mixed well to ensure a homogeneous sample, and a 100-μl aliquot was diluted into 1 ml autoclaved water. Four 10-fold serial dilutions were then made of the second suspension and 100-μl aliquots representative of each dilution were plated on 10% TSA. This procedure was repeated for a total of five microbial growth rings, originating from five different plates. The plates were allowed to incubate in darkness at 25 ± 2 °C for 7–10 days, after which representatives of the microbial morphologies associated with all the plated samples were individually transferred to new media. The clean isolates obtained in this manner were used for identification following 16S rRNA gene sequencing and for evaluation of fungal inhibition ability.

For extraction of DNA, bacterial isolates were grown individually in 5 ml of tryptic soy broth at 25 °C for 24–48 h. DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol described previously (Broderick *et al.*, 2004). The 16S rRNA genes were amplified by PCR using primers 27f and 1492r (Broderick *et al.*, 2004). PCR products were purified using AMPure magnetic beads (Agencourt Bioscience Corp., Beverly, Massachusetts). Bacterial isolates were then sequenced using 16S rRNA gene primers 27F and 787R (Broderick *et al.*, 2004). Sequencing reactions were performed using BigDye (Perkin–Elmer Corp., Wellesley, Massachusetts). Sequenced products were purified with Sephadex G-50 columns (Pharmacia Biotech, Piscataway, New Jersey). Nucleotide sequences were determined on an ABI 377 DNA sequencer (Applied Biosystems, Foster City, California) at the University of Wisconsin, Madison Biotechnology Center.

All nucleotide sequences obtained in this study were deposited in GenBank under the accession numbers DQ370001, DQ370002, DQ370003, and DQ370004 for the fungal sequences, and DQ370005, DQ370006, DQ360486, DQ360487, DQ360488, DQ360489, DQ360490, and DQ360491 for bacterial sequences.

To determine the fungal inhibition ability of each bacterial isolate, an assay was performed on a standard Petri plate containing 50% TSA (15 g tryptic soy broth +15 g agar per litre) microbiological media. The bacterial isolate was streaked across the centre of the plate in a straight line, dividing the plate in two equal sections, and allowed to grow for 24 h. The next day, two plugs (0.5 cm) of a fungal species were placed approximately 2 cm from the bacterial streak and approximately 2 cm from each other. Another two plugs were placed on the other side of the streak in the same manner, to give a total of four plugs per plate. Controls received fungal plugs, but the bacteria were replaced by a sterile water streak. The fungi were allowed to grow for 6 days, at which time the diameter of mycelial growth of each plug was measured. The four plugs within each plate were averaged to estimate the mean fungal growth per replicate. The bacterial and fungal isolates were tested against each other in a pairwise manner. The experiment was performed so that all fungi were tested against all bacteria at one time. A total of eight replicates were obtained for each bacterium/fungus combination.

Statistical analyses

Data were found to conform to the assumptions of normality using a Box–Cox power of transformation test. The effects of fungal treatment on the number of spruce beetle galleries, total gallery length, number of eggs laid, and adult per cent survival

were evaluated using analyses of variance (ANOVA) (PROC GLM; SAS Institute, 1996). ANOVAs with significant *P*-values ($P = 0.05$) for treatment effects were then followed by Tukey's mean separation tests. The same approach was used to test for effects of beetle gender, fungal species, oral secretion dilution, and their interaction on the diameter of the zone free of fungal growth around the filter paper discs (clear zone) in fungal inhibition assays. Analyses of variance were also performed to test the inhibitory ability of the bacterial isolates on the growth diameter attained by each fungus. Significant effects ($P = 0.05$) were followed by Tukey's mean separation tests.

Results

Isolation and identification of fungi

One ophiostomatoid and three green spore-forming fungi were obtained by scraping spruce beetle mouthparts and oral secretions across the media. The genera of these four fungi were morphologically determined to be *Leptographium* (the ophiostomatoid fungus), *Aspergillus* (two of the green spore-forming fungi), and *Trichoderma* (one green spore-forming fungus). The ophiostomatoid species was identified as *L. abietinum* after direct morphological comparisons of conidiophores and conidia with those from pure cultures, and molecular analysis. Sequences for the ITS region of this fungus yielded 98–100% identity to *L. abietinum* for 213–214 base pairs. Also following sequencing of the ITS ribosomal region, the remaining fungi were determined to be (a) *Aspergillus fumigatus* Fresen with 96–97% identity for 212 base pairs, (b) *Aspergillus nomius* Kurtzman with 100% identity for 255 base pairs, and (c) *Trichoderma harzianum* Rifai (= *T. atroviride*) with 98% identity for 591 base pairs.

Effect of fungi on spruce beetle behaviour and performance

Upon invasion of their galleries by these fungi under non-amended conditions, adult spruce beetles exhibited two previously undescribed behaviours: alteration of their typical gallery construction and active secretion of oral substances. Some beetles either abandoned the infested galleries or confined themselves within enlarged chambers at the distal end of the parental gallery (Fig. 2), in contrast to typical linear galleries without such elaborations. Beetles within these chambers isolated themselves from the fungus-pervaded gallery by packing the tunnel leading to the chamber with their faecal pellets and other debris (Fig. 2). Insects remained in these chambers for substantial periods of time, ranging from a couple of weeks to several months, until they died.

Some adult beetles displayed a rather peculiar previously unknown behaviour. They moved all their appendages about in an inward–outward motion, and in so doing, spread liquid substances away from or around their bodies. Beetles were seen secreting relatively copious amounts of a substance orally (Fig. 3), producing faecal pellets, and then proceeding with the leg motion described above.

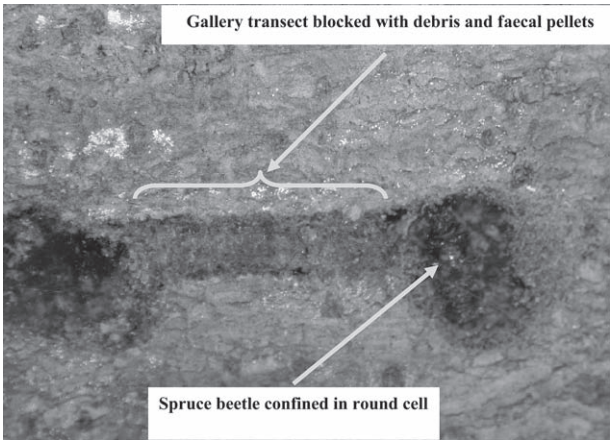

Fig. 2. Adult spruce beetles in galleries invaded by *Trichoderma* and *Aspergillus* spp. The beetle shown here has confined itself within enlarged chambers in the distal end of the gallery.

All of the fungal species that were inoculated into beetle galleries had significant negative effects on the number and total length of galleries compared with mock-inoculated controls ($F_{4,44} = 3.8$, $P = 0.0098$ and $F_{4,44} = 3.8$, $P = 0.0116$ respectively) (Fig. 4a,b). Oviposition was also significantly reduced by inoculation with *A. fumigatus*, *A. nomius*, or *T. harzianum* ($F_{4,44} = 5.42$, $P = 0.0012$). There was no significant reduction in oviposition due to the predominant spruce beetle ophiostomatoid associate *L. abietinum* compared with controls (Fig. 4c). Per cent adult survival was lower in all the fungal treatments than in the controls but, it was only significantly reduced for the two *Aspergillus* species (Fig. 4d) ($F_{4,44} = 3.63$, $P = 0.0122$).

Inhibition of fungal growth by spruce beetle oral secretions

Oral secretions inhibited the growth of all but one of the fungi, as evidenced by the zones clear of mycelial growth surrounding the filter paper discs receiving these treatments (Fig. 5). Fungal species ($F_{3,60} = 21.19$, $P = 0.0001$), oral secretion dilution ($F_{2,60} = 34.3$, $P = 0.0001$), and their interaction ($F_{6,60} = 4.8$, $P = 0.0004$) had significant effects on the diameter of the clear zone. No effects were found of beetle gender or its interaction with oral secretion dilution for any fungal species.

Growth of *L. abietinum* was significantly inhibited ($F_{2,15} = 18.9$, $P = 0.0001$) by all concentrations of oral secretions tested (Fig. 6). *A. fumigatus* and *T. harzianum* were also significantly inhibited by spruce beetle oral secretions, but only by the 10% and 100% suspensions ($F_{2,15} = 3.2$, $P = 0.0005$ and $F_{2,15} = 9.2$, $P = 0.0025$ respectively) (Fig. 6). The inhibitory effect of the oral secretion was concentration dependent for all three of these fungal species.

The only species in which no significant inhibition zones were observed from any oral secretion dilution was *A. nomius*. This may have been partly due to the tendency of this species to cast spores and start new colonies all over the Petri plate surface. Nonetheless, the morphology of these colonies was altered

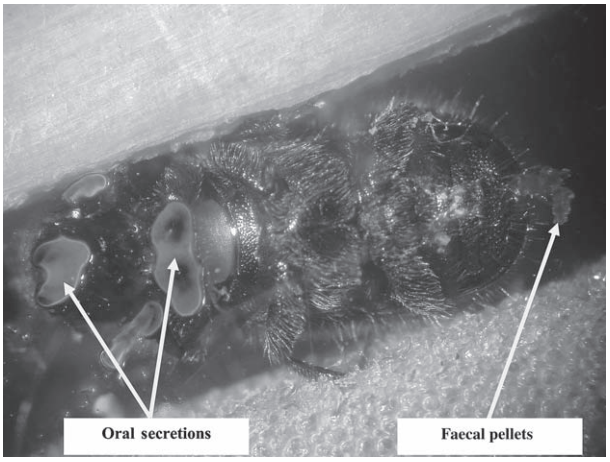

Fig. 3. Spruce beetle producing oral secretions and faecal pellets before boring into a new phloem arena.

by exposure to the secretions from beetle mouthparts. All of the colonies within plates containing the oral secretions showed very sparse mycelial growth and weakened, collapsed conidiophore stalks. In addition, the conidia were mustard yellow coloured rather than the typical pea green of normal *A. nomius* colonies. A similar effect on mycelial growth and discoloration of the conidiophores was evident in *A. fumigatus* and *T. harzianum*, but not *L. abietinum*. A bacterial growth ring was observed around the filter paper discs receiving the oral secretion treatments.

Isolation, identification, and bioactivity of bacterial isolates from spruce beetle oral secretions

All isolations from the non-sterile oral secretions yielded substantial growth of a number of morphologically different bacterial colonies. Bacterial colonies could be observed on the culture plates 24–48 h after spreading. Plates receiving the filter-sterilised oral secretions did not contain any microbial growth.

Growth of *L. abietinum* and *T. harzianum* was inhibited by the non-sterile, but not by the sterile, fraction of the oral secretions (Fig. 7). No inhibition was observed with the water controls. As before, a bacterial growth ring was evident only around the filter paper disc receiving the non-sterile fraction.

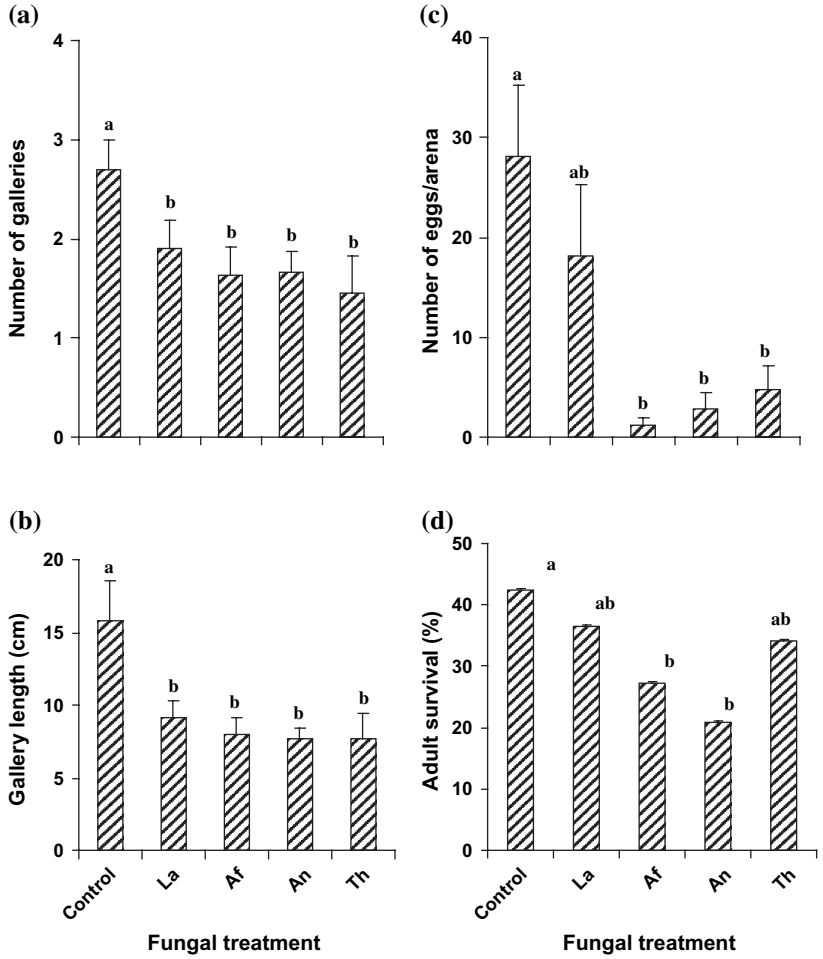

Fig. 4. Effect of four fungal species isolated from spruce beetle oral secretions on the insects' performance in laboratory arenas. (a) Gallery number, (b) total gallery length, (c) number of eggs laid, and (d) per cent adult survival. Bars headed by the same letter are not statistically different (Tukey's mean separation test; $P = 0.05$). Error bars denote 1 SE. La, *Leptographium abietinum*; Af, *Aspergillus fumigatus*; An, *Aspergillus nomius*; and Th, *Trichoderma harzianum*.

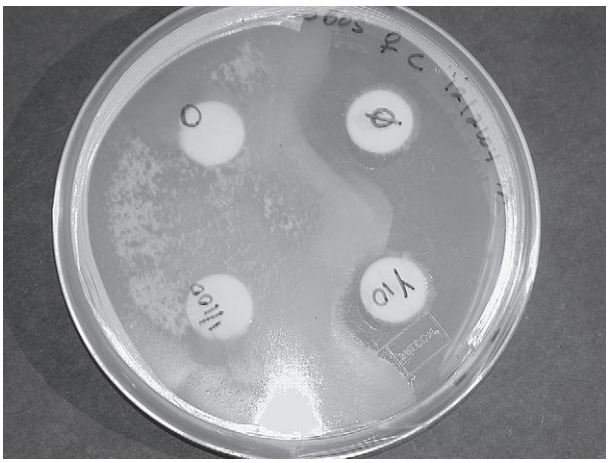

Fig. 5. Reduced fungal growth due to applications of spruce beetle oral secretion dilutions. 0, water control, no secretions; Ø, 100% secretion suspension; 1/10, 10% secretion suspension; 1/100, 1% secretion suspension.

Isolations yielded a total of nine bacterial isolates that were consistently associated with all the growth rings sampled. Identifications, based on BLAST search identity to closest known sequences are: *Pseudomonas viridiflava*, Gammaproteobacteria, 99% identity for 530 of 535 base pairs; *Streptomyces* sp., Actinobacteria, 99% identity for 1392 of 1398 base pairs (87F primer sequence); *Microbacterium* sp., Actinobacteria, 97% identity for 1045 of 1082 base pairs (87F primer sequence); *Bacillus pumilus*, Firmicutes, 99% identity for 752 of 755 base pairs; *Alcaligenes faecalis*, Betaproteobacteria, 98% identity for 889 of 897 base pairs (87F primer sequence); *Raolultella ornithinolytica*, Gammaproteobacteria, 99% identity for 725 of 732 base pairs; *Serratia grimesii*, Gammaproteobacteria, 99% identity for 546 of 549 base pairs; *Staphylococcus hominis*, Firmicutes, 99% identity for 731 of 733 base pairs, and *Micrococcus luteus*, Actinobacteria, 99% identity for 577 of 579 base pairs.

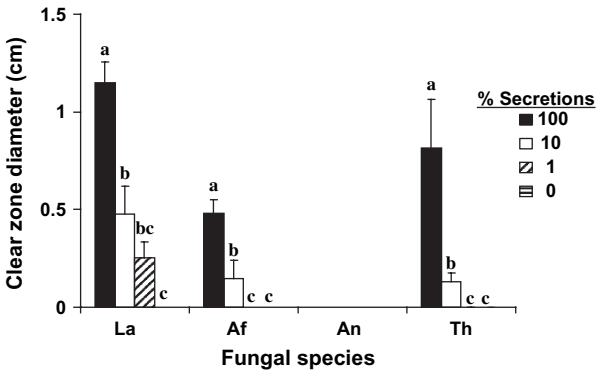

Fig. 6. Effects of oral secretion serial dilutions on growth of *Leptographium abietinum* (La), *Aspergillus fumigatus* (Af), *Aspergillus nomius* (An), and *Trichoderma harzianum* (Th) are shown. Bars within fungal species headed by the same letter are not statistically different (Tukey's mean separation test; $P = 0.05$). Error bars denote 1 SE.

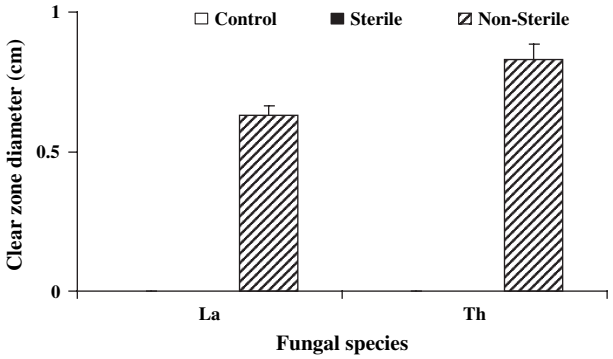

Fig. 7. Effects of non-sterile and filter-sterilised fractions on the growth of *Leptographium abietinum* and *Trichoderma harzianum*. As there was never any inhibition by filter-sterilised fractions, no statistical tests were performed.

Fungal growth was significantly affected by the bacterial isolate ($F_{8,84} = 33.4$, $P < 0.0001$) and the fungal species ($F_{3,29} = 30.1$, $P < 0.0001$) (Fig. 8) being tested. Therefore, data for each fungus are presented separately (Fig. 8). The growth of the ophiostomatoid, *L. abietinum*, was significantly inhibited by seven of the isolates, followed by *T. harzianum*/*atroviride* and *A. fumigatus*, each being significantly inhibited by six of the isolates. The fungus inhibited by the least number of bacteria was *A. nomius*, whose growth was significantly reduced by two isolates. The growth of *L. abietinum* was completely suppressed by *A. faecalis*, *Serratia grimesii*, *S. hominis*, and *M. luteus*, and significantly reduced by *P. viridiflava* and *B. pumilus* (Fig. 8a). *Trichoderma harzianum* was not completely suppressed by any of the bacteria, but was most inhibited by *S. grimesii* followed by *P. viridiflava*, *Streptomyces* sp., *B. pumilus*, *A. faecalis*, and *M. luteus* (Fig. 8b). The growth of the *Aspergillus* spp. was completely suppressed by *M. luteus*, but the inhibitory activity of the other bacteria varied between the two species. For example, the growth of *A. fumigatus* was significantly reduced by *P. viridiflava*, *Microbacterium* sp., *A. faecalis*, *S. grimesii*, and *S. hominis* (Fig. 8c); whereas that of *A. nomius* was significantly reduced only by *R. ornithinolytica* (Fig. 8d).

Discussion

Opportunistic green-spored hyaline hyphomycetes, e.g. *Trichoderma*, *Aspergillus*, and *Penicillium* species, are ubiquitous, and have airborne spores that can come in contact with plants and insects (Duchaine *et al.*, 2000). They are unable to colonise living trees, but can rapidly colonise dead trees. Hence, these fungi are commonly associated with decaying wood, including spruce (Garcia & Morrell, 1999; Lumley *et al.*, 2001), and with wood-feeding insects such as termites (Hendee, 1933) and bark beetles (Barras, 1969; Jassim *et al.*, 1990; Six, 2003b). Results presented here indicate that their associations with spruce beetles are antagonistic, with invasion of galleries by

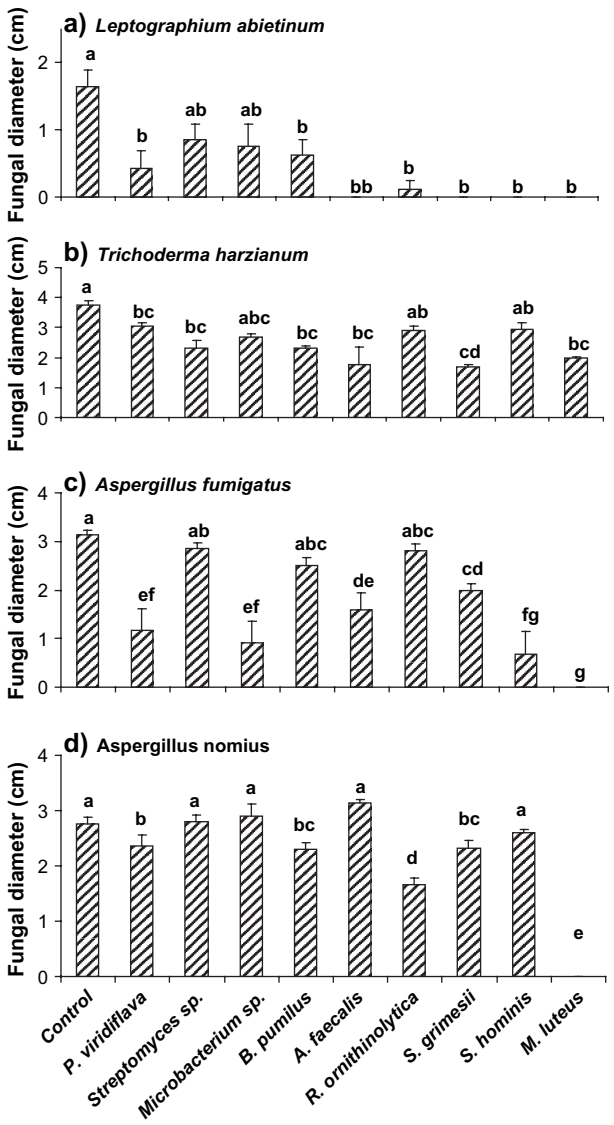

Fig. 8. Inhibitory activity of bacterial isolates obtained from *Dendroctonus rufipennis* oral secretions against species of fungi associated with these insects. Bars within fungal species headed by the same letter are not statistically different (Tukey's mean separation test; $P = 0.05$). Error bars denote 1 SE.

these fungi decreasing beetle survival and reproduction. Reports on the effects of *Aspergillus* and *Trichoderma* species on insects are scant, but they have also been shown to adversely affect West Indian wood termites *Cryptotermes brevis* (Moein 1992), *Oncopeltus fasciatus*, and elm beetles, *Scolytus* spp. (Jassim *et al.*, 1990).

Leptographium abietinum is the ophiostomatoid fungus most frequently associated with spruce beetles, being found on 80–100% of beetles (Six & Bentz, 2003; Aukema *et al.*, 2005). Despite this high incidence, the cost/benefit to the beetle remains to be determined. The negative impact of *L. abietinum* on spruce beetle gallery construction raise the possibility that it is a partial competitor, phoretically transported between hosts by, and then competing for food with, its vector. Another possibility, perhaps supported by the constancy of this association and the reported pathogenicity of related fungi (Solheim et al., 1993; Strobel & Sugawara, 1986), is that this fungus provides some benefit to the beetles, but only under a narrow range of conditions such as during the host colonisation phase. Thus, the spruce beetles' oral secretions could provide a means to restrict fungal growth to levels where there is no threat of competition. Conceivably, spruce beetle secretions might even digest the fungal tissue to be used as nutritional supplement. Mycetophagy is common among beetles, including species within the Scolytinae (Harrington, 2005; Six, 2003b).

The presence of bacteria in the beetles' oral secretions, and the inhibition of fungal growth by the non-filtered but not filter-sterilised fractions, suggest these microorganisms are responsible for the observed antifungal activity. This hypothesis is confirmed with data obtained from fungal inhibition assays, which showed species-specific differential inhibition activity by the nine bacterial isolates from spruce beetle oral secretions. The bacterium found to have the most inhibitory activity against three of the four fungi tested was an actinomycete, *M. luteus*. A similar phenomenon has been described for fungus-growing ants, where actinomycete bacteria inhibit the growth of an opportunistic fungal parasite (Currie *et al.*, 1999). Also, Santos *et al.* (2004) found that bacteria in the genus *Burkholderia*, associated with nests of fungus-growing ants, inhibited the same opportunistic fungal parasite in addition to a number of entomopathogenic fungi. *Pseudomonas viridiflava* has been reported to produce a broadly acting class of antimycotic substances called ecomycins (Miller *et al.*, 1998), but because these are not active against *Aspergillus* or *Trichoderma* (Martin, 1987), the high inhibitory activity of this species against *A. fumigatus* is probably due to metabolites other than ecomycins. *Bacillus pumilus* produces antifungal metabolites that inhibit a number of *Aspergillus* and other species (Moein & Rust, 1992; Munimbazi & Bullerman, 1998; Bottone & Peluso, 2003). Actinomycetes and *Serratia* species isolated from lettuce rhizosphere possess antifungal activity against the plant pathogen *Sclerotinia minor* (El-Tarabily *et al.*, 2000). *Pseudomonas* and *Raoultella* spp. have been isolated from guts of the wood-boring longhorn beetle, *Saperda vestita* Say, in the authors' laboratory (Delalibera *et al.*, 2005). Species in the genera *Pseudomonas*, *Serratia*, *Bacillus*, *Staphylococcus*, *Microbacterium* and *Micrococus* have been recovered from the midguts of third-instar gypsy moth larvae, *Lymantria dispar* L., using culture-dependent methods (Broderick *et al.*, 2004). Some species of *Pseudomonas* and *Microbacterium* also show antibiotic activity against nematodes (Gravato-Nobre *et al.*, 2005; Hodgkin *et al.*, 2000; Sturz & Kimpinski, 2004). Interestingly, various nematode species are consistently associated with spruce beetles (Cardoza *et al.*, 2006).

In summary, spruce beetles can at least partially defend against gallery invaders by integrating behavioural responses, i.e. production and subsequent spreading with appendages of oral secretions, with symbiotic bacteria having antifungal properties. Although, this behaviour was most evident in dry phloem infected with opportunistic fungi, the authors have also seen spruce

beetles egesting and smearing oral secretions while entering fresh phloem. Thus, this may represent an important preventative step during the gallery-constructing behaviour of some bark beetles.

These results support the view that although symbiosis is most commonly studied as a two-species system, the involvement of other organisms can facilitate or perturb such relationships (Currie *et al.*, 1999; Currie, 2001; Klepzig *et al.*, 2001; Lombardero *et al.*, 2003). In the case of these endophytic insects, some opportunistic fungi exploit a habitat made available by the herbivores and exert strong antagonistic effects, as do the hyphomycete fungi identified in this study. More closely associated fungi, such as *L. abietinum*, have partly antagonistic and, perhaps, context-dependent relationships with their vector. Other associates benefit the herbivores by restricting the growth of competing fungi, as do the bacteria in the spruce beetles' oral secretions. In addition, behavioural adaptations, such as leg spreading and chamber confinement, by the herbivore may enhance the protective role of these bacteria. The extent of beetle reproduction and population change is therefore at least in part an outcome of these complex interactions.

A number of questions remain to be answered for a more complete ecological understanding of this system. Among these are: the origin of these secretions, whether bacteria are harboured in specialised structures, the relative prevalence of bacteria in mouthparts vs. guts, and the incidence of such relationships in other insects. These aspects are concurrently being investigated by the authors.

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