

Fungal Symbionts of the Spruce Bark Beetle Synthesize the Beetle Aggregation Pheromone 2-Methyl-3-buten-2-ol

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Abstract Tree-killing bark beetles depend on aggregation pheromones to mass-attack their host trees and overwhelm their resistance. The beetles are always associated with phytopathogenic ophiostomatoid fungi that probably assist in breaking down tree resistance, but little is known about if or how much these fungal symbionts contribute to the beetles' aggregation behavior. In this study, we determined the ability of four major fungal symbionts of the spruce bark beetle *Ips typographus* to produce beetle aggregation pheromones. The fungi were incubated on Norway spruce *Picea abies* bark, malt agar, or malt agar amended with 0.5 % ¹³C glucose. Volatiles present in the headspace of each fungus were analyzed for 7 days after incubation using a SPME autosampler coupled to a GC/MS. Two *Grosmannia* species (*G. penicillata* and *G. europioides*) produced large amounts of 2-methyl-3-buten-2-ol (MB), the major component in the beetles' aggregation pheromone blend, when growing on spruce bark or malt agar. *Grosmannia europioides* also incorporated ¹³C glucose into MB, demonstrating that the fungi can synthesize MB *de novo* using glucose as a carbon source. This is the first clear evidence that fungal symbionts of bark beetles can produce components in the aggregation pheromone blend of their beetle vectors. This provides new insight into the possible ecological roles of fungal symbionts in bark beetle systems and may deepen our understanding of species interactions and coevolution in these important biological systems.

Keywords Scolytinae · Bluestain fungi · Plant-insect-microbe interactions

Introduction

Bark beetles are the most devastating tree-killers in conifer forests worldwide, and their impact appears to be increasing with global climate change (Raffa et al. 2008). Aggregation pheromones are central to the attack strategy of tree-killing bark beetles, since pheromones coordinate the rapid mass-attacks required to overwhelm tree defenses (Blomquist et al. 2010). Another key component in the beetles' attack strategy appears to be an association with ophiostomatoid fungal symbionts that assist the beetles in exhausting tree defenses (Krokene 2015). The powerful anatomical and chemical defenses of healthy conifers deter most insect and pathogen attacks (Franceschi et al. 2005), but tree-killing bark beetles have evolved the ability to convert some of the trees' defense chemicals into beetle aggregation pheromones (Renwick et al. 1976).

Bark beetle aggregation pheromones are usually a blend of 2–3 oxygenated monoterpenes, isoprenes, or other compounds (Blomquist et al. 2010). Many of these compounds are produced *de novo* in the beetles' midgut or fat body (Blomquist et al. 2010; Lanne et al. 1989), whereas some are derived from host monoterpenes, either by the beetles themselves (Renwick et al. 1976) or by symbionts in the beetle gut (Brand et al. 1975). We still know very little about if, or how much, the beetles' fungal symbionts contribute to beetle aggregation. Knowledge about fungal volatile emission and its function in bark beetle host finding and aggregation is useful to understand conifer-bark beetle interactions and coevolution and to develop novel pest management methods against these important forest pests.

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In this study, we quantified pheromone production by four fungal symbionts of the spruce bark beetle *Ips typographus*, the major tree-killing bark beetle attacking Norway spruce *Picea abies*. Two symbionts (*Grosmannia penicillata* and *G. europhioides*) produced large amounts of 2-methyl-3-buten-2-ol (MB), the major pheromone component of *I. typographus*, in both bark and malt agar, indicating that fungal symbionts can *de novo* produce the beetles' aggregation pheromone.

Methods and Materials

Fungal Symbionts Four common fungal associates of the spruce bark beetle were used in this study (Jankowiak 2005; Krokene and Solheim 1996). *Endoconidiophora polonica* (\equiv *Ceratocystis polonica*) is the most virulent, with a strong ability to colonize fresh sapwood and kill trees in experimental mass-inoculations (Krokene and Solheim 1996). *Grosmannia penicillata* (\equiv *Ophiostoma penicillatum*) and *G. europhioides* (\equiv *O. europhioides \equiv *O. piceaperdum*) are other primary invaders that grow well in the phloem, and contribute to phloem necrosis (Kirisits 2004; Krokene and Solheim 1996). *Ophiostoma piceae* is a less virulent secondary species that colonizes the sapwood during the later stages of beetle attack (Kirisits 2004). All fungal isolates used were obtained from the culture collection of the Norwegian Forest and Landscape Institute in Ås, Norway. Isolates were maintained on malt agar (2 % malt, 1.5 % agar) at 4 °C, and transferred to fresh malt agar at 25 °C 7–10 d before the start of the experiments.*

Bioassays to Detect Pheromone Production by Fungal Symbionts The fungi's ability to produce beetle aggregation pheromones was assayed by incubating fungi on Norway spruce bark in headspace vials and analyzing emitted volatiles. We prepared bioassay units by taking 10-mm diam bark plugs with a pre-drilled 4-mm diam hole in the center from a spruce log, placing the bark plugs individually in 20 ml screw top glass headspace vials (Supelco, USA), and inserting a 4-mm diam plug of sterile agar or agar colonized by each of the four fungi into the hole in the center of the plug. We prepared a total of 30 vials, with six replicates for each of the five treatments (agar with active growing mycelium of *E. polonica*, *G. penicillata*, *G. europhioides*, or *O. piceae* and sterile agar control). Bark plugs were taken from a 0.5 m long log from a 48-yr-old Norway spruce tree felled in early May 2013 and kept at 4 °C at the chemical ecology laboratory at the Royal Institute of Technology, Sweden until the bioassays started 2 d later. After the vials had been loaded with agar/fungus plugs, they were sealed by a stainless steel cap equipped with a polytetrafluoroethylene (PTFE)-faced butyl septum (Supelco, USA). Volatiles present in the headspace of each vial were collected 1, 3, 5, and 7 days after incubation using

an SPME autosampler. At each sampling time, the autosampler inserted a 65 μ m polydimethylsiloxane/divinylbenzene (PDMS/DVD) SPME fiber (Supelco, PA, USA) through the septum for 50 min before injecting the collected volatiles into a GC/MS for separation, identification, and quantification (see below).

To confirm that any MB detected in the bark plug incubation assay was *de novo* produced by fungi, we did a labeled glucose experiment with *G. europhioides*, the fungus with the highest MB production in the bark plug assay. We incubated *G. europhioides* on three different growth media: (1) malt agar (2.0 % malt, 1.5 % agar), (2) malt agar with 0.5 % ^{13}C labeled glucose (99 %, Cambridge Isotope Laboratories, Inc.), or (3) malt agar with 0.5 % unlabeled glucose (99.5 %, Sigma). For each type of medium, six 20 ml headspace vials were filled with 3 ml medium at 50–60 °C using a sterilized plastic pipette. The vials were tilted about 30° to increase the surface area of the malt agar medium. When the malt agar had cooled to room temperature, we placed a 4 mm malt agar plug colonized by *G. europhioides* at the center of three vials with each media type. The remaining vials were used as no-fungus controls. After fungal inoculation, all vials were sealed as described above and kept at 25 °C for 1 wk. Volatiles present in the headspace of each vial then were collected by SPME fiber as described above and analyzed by GC/MS.

GC/MS Analysis All samples were analyzed using an Agilent 7890 A GC combined with a 5975C inert MSD with triple-axis detector and an HP-5 capillary column (Agilent, 30 m, 0.25 mm id, 0.25 μ m film thickness) (Agilent Technologies, CA, USA). Helium was used as the carrier gas at a constant flow of 1 ml min $^{-1}$, the temperature of the ion source was 150 °C, the mass detector was operated with a mass range of 30–400, and the electron impact ionization was 70 eV. Immediately after SMPE collection, volatile samples were transferred to the injector to desorb the volatiles at 225 °C for 5 min. MB and other volatiles in the samples then were separated using a temperature program of 40 °C for 3 min, increasing to 160 °C at a rate of 4 °C min $^{-1}$, then to 230 °C at a rate of 20 °C min $^{-1}$, and then remaining constant for 5 min. To verify the presence of MB, additional samples were collected by SPME fiber and analyzed using an Agilent 7865 GC with a different type of column (DB-wax column, Supelco; 30 m, 0.25 mm id, 0.25 μ m film thickness) using the temperature program described above. MB was identified by comparing retention times and mass spectra with available authentic standards in the HP-5 and DB-wax columns. The incorporation of ^{13}C into MB by *G. europhioides* was confirmed by comparing the mass spectra of MB from fungi growing on malt agar with 0.5 % ^{13}C labeled glucose vs. unlabeled glucose.

Data Analysis 2-Methyl-3-buten-2-ol amounts emitted from the different treatments 1–7 d after incubation were subjected

to repeated measures one-way ANOVA (Statistica 6.0, Statsoft Inc., USA). Data were $\log(X + 1)$ transformed to correct for unequal variance and departures from normality, and means were separated using Tukey HSD *Post Hoc* Test at $P = 0.05$.

Results

We detected MB from most of the samples, including control bark incubated with sterile agar, but there were large quantitative differences among treatments ($F = 6.99$, $P < 0.01$) (Figs. 1a, 2). The highest levels of MB were detected in vials incubated with the two *Grosmannia* species, with *G. europhoides* emitting $35.5 \times$ more MB than the control ($P < 0.01$) and *G. penicillata* emitting $10.1 \times$ more MB ($P < 0.01$) 7 d after incubation. Incubation with *E. polonica* or *O. piceae* yielded low amounts of MB that did not differ significantly from the sterile agar control ($P = 0.92$ for *E. polonica* and 0.06 for *O. piceae*) (Fig. 2).

In addition to the quantitative differences, there also were large temporal differences in MB release among treatments: MB was detected one day after incubation from most samples with *G. europhoides* and *G. penicillata*, but not until three days after incubation with *E. polonica*, *O. picea* or sterile agar. The highest levels of MB were detected seven days after incubation (Fig. 2).

To determine if MB was produced *de novo* by the fungus or if it was simply a byproduct of fungal degradation of spruce tissues, we incubated *G. europhoides* on malt agar. MB was detected in all three replicates with *G. europhoides* growing on malt agar, but not from the sterile agar control, suggesting that MB was *de novo* produced by the fungus.

To confirm the biosynthetic origin of MB, we incubated *G. europhoides* in vials with malt agar containing 0.5 % ^{13}C labeled glucose. Labeled glucose clearly was incorporated into MB sampled in the headspace above the fungus seven days after incubation (Fig. 1b), showing that *G. europhoides* can use glucose as a carbon source to produce MB.

Discussion

In this study, we have demonstrated that the bark beetle symbiont *G. europhoides* and probably *G. penicillata* can produce MB *de novo*, using glucose as a carbon source. To our best knowledge, this is the first demonstration of *de novo* pheromone synthesis by a bark beetle-associated fungus. Fungi generally dissimilate organic compounds such as glucose through one or more glycolysis pathways and then go on to produce various alcohols, ketones, and benzenoids through fermentation or heterotrophic pathways (Davis et al. 2013). However, the biosynthesis pathway for MB production in fungi is completely unknown, as MB is rarely reported from *Grosmannia* or any other microbial source.

Bark beetle-associated microbes previously have been demonstrated to be involved in the production of behavior-regulating chemicals. Hulcr et al. (2011) observed that ambrosia beetles are attracted to volatiles from their fungal symbionts under field conditions, but the chemical substances involved were not identified. Other examples with a chemical perspective include the *in vitro* conversion of the tree defense compound α -pinene to *cis*-verbenol (an aggregation pheromone component of several bark beetles) by the bacterium *Bacillus cereus* isolated from the gut of California fivespined ips *Ips paraconfusus* (Brand et al. 1975), and the

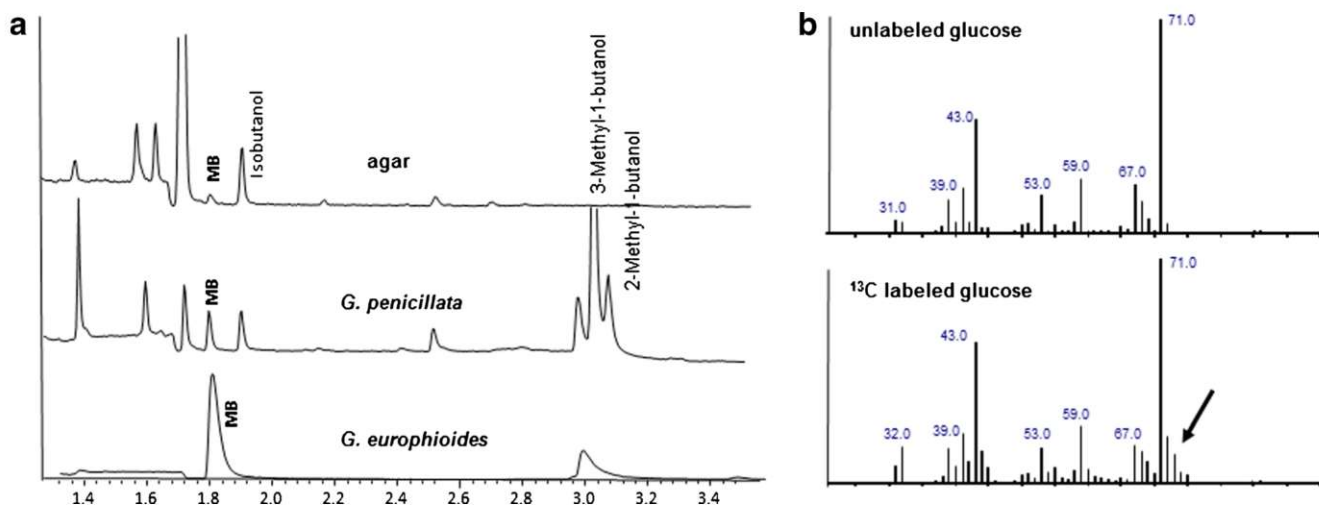


Fig. 1 **a** Representative chromatograms using an HP-5 column showing 2-methyl-3-buten-2-ol (MB) released from bark with sterile agar and agar colonized by *Grosmannia penicillata* and *G. europhoides*. **b** Representative mass spectra showing

incorporation of ^{13}C into 2-methyl-3-buten-ol (MB) produced by *Grosmannia europhoides* growing on malt agar with 0.5 % unlabeled glucose or ^{13}C labeled glucose

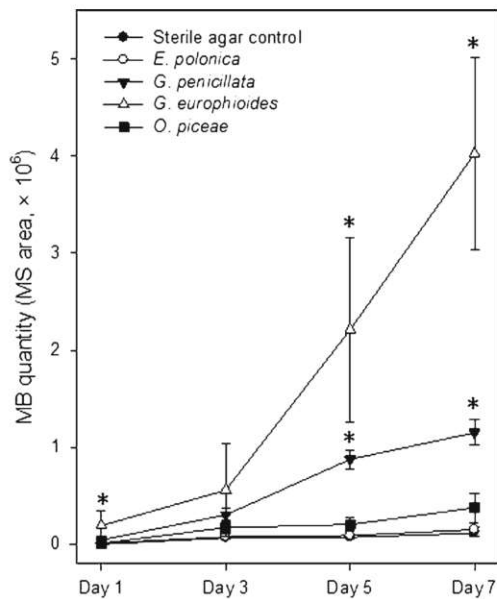


Fig. 2 2-Methyl-3-buten-2-ol (MB) emission from Norway spruce bark incubated with sterile malt agar or agar colonized by each of four fungal associates (*Endoconidiophora polonica*, *Grosmannia penicillata*, *Grosmannia europhoides*, *Ophiostoma piceae*) of the spruce bark beetle *Ips typographus* 1–7 days after incubation. Data are expressed as means \pm SE ($N = 6$). Stars indicate significant differences from the control by repeated measures one-way ANOVA

interconversion of verbenol and verbenone (an anti-aggregation pheromone) by yeasts associated with the spruce bark beetle and the mountain pine beetle *Dendroctonus ponderosae*, as well as a fungus associated with the southern pine beetle *D. frontalis* (Brand et al. 1976; Hunt and Borden 1990; Leufven et al. 1984). Ingestion of an antibiotic inhibited production of ipsenol and ipsdienol in male *I. paraconfusus*, indicating that gut microbes may be directly or indirectly involved in the production of these pheromone components (Byers and Wood 1981). However, none of these previous studies have presented conclusive evidence from e.g., labelling experiments showing that beetle-associated microbes produce bark beetle pheromones *de novo*.

2-Methyl-3-buten-2-ol is the most abundant aggregation pheromone component of the spruce bark beetle (Lanne et al. 1989). It also is an aggregation pheromone component of several other *Ips* bark beetles and an alarm pheromone in the European hornet *Vespa crabro* (Zhang et al. 2012). MB is a five-carbon hemiterpenoid alcohol that was originally believed to be derived by the spruce bark beetle from precursors in the host bark, but has since been demonstrated to be produced *de novo* by the beetles (Lanne et al. 1989). Male beetles incorporate ¹⁴C into MB in the gut following injection of ¹⁴C labeled glucose, acetate, or mevalonate into the subcuticle (Lanne et al. 1989). The fact that mevalonate injection increase radioactivity of MB, suggests that MB is *de novo* produced by the beetles in the gut via the mevalonate pathway.

Still, it cannot be ruled out that microorganisms in the beetle gut or elsewhere are responsible for, or contribute to, MB production.

Not only insects and microbes, but also plants seem able to produce MB. MB emission is reported from the needles of several North American pine species (Harley et al. 1998) and from the bark of several birch and aspen species (Zhang et al. 2012). In digger pine, *Pinus sabiniana*, a bifunctional MB synthase has been identified that produces MB and isoprene in a ~90:1 ratio via the dimethylallyl diphosphate (DMADP) pathway (Gray et al. 2011). We also detected MB in small quantities in the headspace above our Norway spruce bark controls a few days after the bark plugs were taken from the log. However, since spruce bark may carry microbes capable of producing MB, it is premature to conclude that Norway spruce tissues were the source of the MB we detected.

In summary, our finding that bark beetle-associated fungi produce substantial amounts of MB *de novo* suggests that these fungi may play a role in the aggregation of spruce bark beetles. It also provides new insight on the interaction and coevolution between insects and microbial symbionts. However, since fungal establishment and pheromone production may be slow relative to bark beetle mass-attacks, future studies are needed to determine how much the symbionts' chemical signaling contributes to bark beetle aggregation behavior in nature.

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