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1 **FUNGAL SYMBIONTS OF THE SPRUCE BARK BEETLE**
2 **SYNTHESIZE THE BEETLE AGGREGATION PHEROMONE**
3 **2-METHYL-3-BUTEN-2-OL**

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38 **Abstract**

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

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58 Key words: Scolytinae, bluestain fungi, plant-insect-microbe interactions

INTRODUCTION

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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).

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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).

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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. 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..

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MATERIALS AND METHODS

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Fungal Symbionts

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Four common fungal associates of the spruce bark beetle were used in this study (Krokene and Solheim 1996; Jankowiak 2005). *Endoconidiophora polonica* (\equiv *Ceratocystis polonica*) is the most virulent of these species, 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

98 phloem necrosis (Krokene and Solheim 1996; Kirisits 2004). *Ophiostoma piceae* is a less
99 virulent secondary species that colonizes the sapwood during the later stages of beetle attack
100 (Kirisits 2004). All fungal isolates used in this study were obtained from the culture
101 collection of the Norwegian Forest and Landscape Institute in Ås, Norway. Isolates were
102 maintained on malt agar (2% malt, 1.5% agar) at 4 °C, and transferred to fresh malt agar at 25
103 °C 7-10 days before the start of the experiments.

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105 *Bioassays to Detect Pheromone Production by Fungal Symbionts*

106 The fungi's ability to produce beetle aggregation pheromones was assayed by incubating
107 fungi on Norway spruce bark in headspace vials and analyzing emitted volatiles. We prepared
108 bioassay units by taking 10-mm diameter bark plugs with a pre-drilled 4-mm diameter hole in
109 the center from a spruce log, placing the bark plugs individually in 20 ml screw top glass
110 headspace vials (Supelco, USA), and inserting a 4-mm diameter plug of sterile agar or agar
111 colonized by each of the four fungi into the hole in the center of the plug. We prepared a total
112 of 30 vials, with six replicates for each of the five treatments (agar with active growing
113 mycelium of *E. polonica*, *G. penicillata*, *G. europhioides* or *O. piceae* and sterile agar
114 control). Bark plugs were taken from a 0.5 m long log from a 48-year-old Norway spruce tree
115 felled in early May 2013 and kept at 4 °C at the chemical ecology laboratory at the Royal
116 Institute of Technology, Sweden until the bioassays started two days later. When the vials
117 had been loaded with agar/fungus plugs they were sealed by a stainless steel cap equipped
118 with a PTFE-faced butyl septum (Supelco, USA). Volatiles present in the headspace of each
119 vial were collected 1, 3, 5, and 7 days after incubation using an SPME autosampler. At each
120 sampling time the autosampler inserted a 65 µm polydimethylsiloxane/divinylbenzene
121 (PDMS/DVD) SPME fiber (Supecol, PA, USA) through the septum for 50 minutes before
122 injecting the collected volatiles into a GC-MS for separation, identification and quantification
123 (see below).

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125 To confirm that any MB detected in the bark plug incubation assay was *de novo* produced by
126 fungi, we did a labelled glucose experiment with *G. europhioides*, the fungus with the highest
127 MB production in the bark plug assay. We incubated *G. europhioides* on three different
128 growth media: (1) malt agar (2.0% malt, 1.5% agar), (2) malt agar with 0.5% ¹³C labeled
129 glucose (99%, Cambridge Isotope Laboratories, Inc.) or (3) malt agar with 0.5% unlabelled
130 glucose (99.5%, Sigma). For each type of medium six 20 ml headspace vials were filled with
131 3 ml medium at 50-60 °C using a sterilized plastic pipette. The vials were tilted about 30° to
132 increase the surface area of the malt agar medium. When the malt agar had cooled to room
133 temperature we placed a 4 mm malt agar plug colonized by *G. europhioides* at the centre of
134 three vials with each media type. The remaining vials were used as no-fungus controls. After
135 fungal inoculation, all vials were sealed as described above and kept at 25 °C for one week.
136 Volatiles present in the headspace of each vial were then collected by SPME fibre as
137 described above and analysed by GC-MS.

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139 *GC-MS Analysis*

140 All samples were analyzed using an Agilent 7890 A GC combined with a 5975C inert MSD
141 with triple-axis detector and a HP-5 capillary column (Agilent, 30 m, 0.25 mm id, 0.25 μ m
142 film thickness) (Agilent Technologies, CA, USA). Helium was used as the carrier gas at a
143 constant flow of 1 ml min⁻¹, the temperature of the ion source was 150 °C, the mass detector
144 was operated with a mass range of 30–400, and the electron impact ionization was 70 eV.
145 Immediately after SMPE collection, volatile samples were transferred to the injector to
146 desorb the volatiles at 225 °C for 5 min. MB and other volatiles in the samples were then
147 separated using a temperature program of 40 °C for 3 min, increasing to 160 °C at a rate of 4
148 °C min⁻¹, then to 230 °C at a rate of 20 °C min⁻¹ and then remaining constant for 5 min. To
149 verify the presence of MB, additional samples were collected by SPME fiber and analyzed
150 using an Agilent 7865 GC with a different type of column (DB-wax column, Supelco; 30 m,
151 0.25 mm id, 0.25 μ m film thickness) using the temperature program described above. MB
152 was identified by comparing retention times and mass spectra with available authenticated
153 standards in the HP-5 and DB-wax columns. The incorporation of ¹³C into MB by *G.*
154 *europioides* was confirmed by comparing the mass spectra of MB from fungi growing on
155 malt agar with 0.5% ¹³C labelled glucose versus unlabelled glucose.

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157 *Data analysis*

158 MB amounts emitted from the different treatments 1-7 days after incubation were subjected
159 to repeated measures one-way ANOVA (Statistica 6.0, Statsoft Inc., USA). Data were
160 log(X+1) transformed to correct for unequal variance and departures from normality, and
161 means were separated using Tukey HSD Post Hoc Test at p = 0.05.

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RESULTS

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166 We detected MB from most of the samples, including control bark incubated with sterile agar,
167 but there were large quantitative differences between treatments (F = 6.99, p < 0.01) (Figure
168 1A & 2). The highest levels of MB were detected in vials incubated with the two *Grosmannia*
169 species, with *G. europioides* emitting 35.5 \times more MB than the control (p < 0.01) and *G.*
170 *penicillata* emitting 10.1 \times more MB (p < 0.01) 7 days after incubation. Incubation with *E.*
171 *polonica* or *O. piceae* yielded low amounts of MB that did not differ significantly from the
172 sterile agar control (p = 0.92 for *E. polonica* and 0.06 for *O. piceae*).

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174 In addition to the quantitative differences there were also large temporal differences in MB
175 release between treatments: MB was detected a few hours after incubation from most samples
176 with *G.europioides* and *G. penicillata*, but not until three days after incubation with *E.*

177 *polonica*, *O. picea* or sterile agar. The highest levels of MB were detected 7 days after
178 incubation (Figure 2).

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180 To determine if MB was produced *de novo* by the fungus or if it was simply a byproduct of
181 fungal degradation of spruce tissues, we incubated *G.europhioides* on malt agar. MB was
182 detected in all three replicates with *G.europhioides* growing on malt agar, but not from the
183 sterile agar control, suggesting that MB was *de novo* produced by the fungus. To confirm the
184 biosynthetic origin of MB, we incubated *G. europhioides* in vials with malt agar containing
185 0.5% ¹³C labeled glucose. Labeled glucose was clearly incorporated into MB sampled in the
186 headspace above the fungus seven days after incubation (Figure 1B), showing that *G.*
187 *europhioides* can use glucose as a carbon source to produce MB.

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DISCUSSION

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

199

200 Bark beetle-associated microbes have previously been demonstrated to be involved in the
201 production of behaviour-regulating chemicals. Hulcr et al. (2011) observed that ambrosia
202 beetles are attracted to volatiles from their fungal symbionts under field conditions, but the
203 chemical substances involved were not identified. Other examples with a chemical
204 perspective include the *in vitro* conversion of the tree defense compound α -pinene to *cis*-
205 verbenol (an aggregation pheromone component of several bark beetles) by the bacterium
206 *Bacillus cereus* isolated from the gut of California fivespined ips *Ips paraconfusus* (Brand et.
207 al 1975), and the interconversion of verbenol and verbenone (an anti-aggregation pheromone)
208 by yeasts associated with the spruce bark beetle and the mountain pine beetle *Dentroctonus*
209 *ponderosae*, as well as a fungus associated with the southern pine beetle *D. frontalis* (Brand
210 et. al 1976; Hunt and Borden 1990; Leufven et. al 1984). Ingestion of an antibiotic inhibited
211 production of ipsenol and ipsdienol in male *I. paraconfusus*, suggesting that gut microbes
212 may be directly or indirectly involved in the production of these pheromone components
213 (Byers and Wood 1981). However, none of these previous studies have presented conclusive
214 evidence from e.g. labelling experiments showing that beetle-associated microbes produce
215 bark beetle pheromones *de novo*.

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217 MB is the most abundant aggregation pheromone component of the spruce bark beetle
218 (Lanne et. al 1989). It is also an aggregation pheromone component of several other *Ips* bark
219 beetles and an alarm pheromone in the European hornet *Vespa crabro* (Zhang et. al 2012).
220 MB is a five-carbon hemiterpenoid alcohol that was originally believed to be derived by the
221 spruce bark beetle from precursors in the host bark, but has since been demonstrated to be
222 produced *de novo* by the beetles (Lanne et. al 1989). Male beetles incorporate ¹⁴C into MB in
223 the gut following injection of ¹⁴C labelled glucose, acetate or mevalonate into the subcuticle.
224 The fact that mevalonate injection increase radioactivity of MB, suggests that MB is *de novo*
225 produced by the beetles in the gut via the mevalonate pathway. Still, it cannot be ruled out
226 that microorganisms in the beetle gut or elsewhere are responsible for, or contribute to, MB
227 production.

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229 Not only insects and microbes, but also plants seem able to produce MB. MB emission is
230 reported from the needles of several North American pine species (Harley et. al 1998) and
231 from the bark of several birch and aspen species (Zhang et. al 2012). In digger pine *Pinus*
232 *sabiniana* a bifunctional MB synthase has been identified that produces MB and isoprene in a
233 ~90:1 ratio via the dimethylallyl diphosphate (DMADP) pathway (Gray et. al 2011). We also
234 detected MB in small quantities in the headspace above our Norway spruce bark controls a
235 few days after the bark plugs were taken from the log. However, since spruce bark may carry
236 microbes capable of producing MB it is premature to conclude that Norway spruce tissues
237 were the source of the MB we detected.

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

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Figure legends

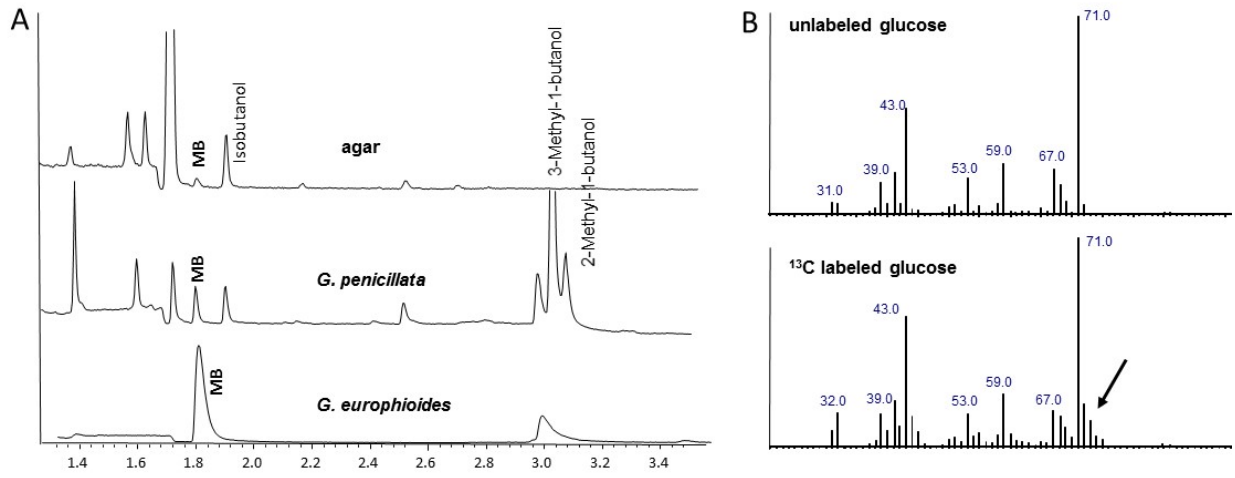
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310 **Figure 1.** (A). Representative chromatograms using a HP-5 column showing 2-methyl-3-buten-
311 2-ol (MB) released from bark with sterile agar and agar colonized by *Grosmannia penicillata*
312 and *G. europhioides*. (B). Representative mass spectra showing incorporation of ^{13}C into 2-
313 methyl-3-buten-ol (MB) produced by *Grosmannia europhioides* growing on malt agar with 0.5%
314 unlabeled glucose or ^{13}C labeled glucose.

315 **Figure 2.** 2-Methyl-3-buten-2-ol (MB) emission from Norway spruce bark incubated with
316 sterile malt agar or agar colonized by each of four fungal associates of the spruce bark beetle
317 *Ips typographus* 1-7 days after incubation. Data are expressed as means \pm 1 SE (n = 6). Stars
318 indicate significant differences from the control by repeated measures one-way ANOVA.

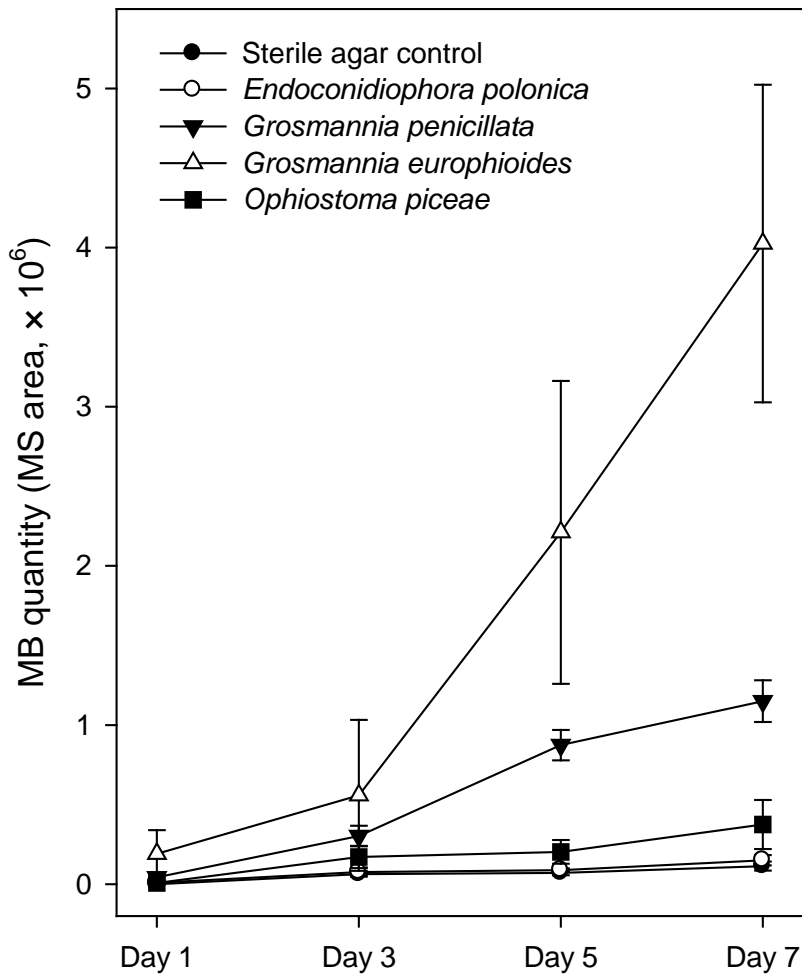
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Figure 1



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Figure 2



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