Bark beetles locate fungal symbionts by detecting volatile fungal metabolites of host tree resin monoterpenes

Dineshkumar Kandasamy[1*], Rashaduz Zaman[1], Yoko Nakamura[2,3], Tao Zhao[4], Henrik Hartmann[5], Martin N Andersson[6], Almuth Hammerbacher[7] and Jonathan Gershenzon[1*]

[1] Department of Biochemistry, Max Planck Institute for Chemical Ecology, Jena, Germany

[2] Department of Natural Product Biosynthesis, Max Planck Institute for Chemical Ecology, Jena, Germany

[3]Research Group Biosynthesis/NMR, Max Planck Institute for Chemical Ecology, Jena, Germany

[4]School of Science and Technology, Örebro University, 701 82, Örebro, Sweden

[5] Department of Biogeochemical Processes, Max Planck Institute for Biogeochemistry, Jena, Germany

[6] Department of Biology, Lund University, Lund, Sweden

[7] Department of Zoology and Entomology, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa

*Corresponding authors

E-mail: dkandasamy@ice.mpg.de and gershenzon@ice.mpg.de

Abstract

Outbreaks of bark beetles have decimated millions of hectares of conifer forest worldwide in recent years. The ability of these tiny 3-6 mm long insects to kill mature trees over a short period has been ascribed to two factors: (1) mass attacks on the host tree to overcome tree defenses and (2) the presence of fungal symbionts that support successful beetle development in the tree. While the role of pheromones in coordinating mass attacks has been well studied, the role of chemical communication in maintaining the fungal symbiosis is poorly understood. We previously demonstrated that Eurasian spruce bark beetles (*Ips typographus*) can recognize beneficial fungal symbionts of the genera *Grosmannia*, *Endoconidiophora* and *Ophiostoma* by their *de novo* synthesized volatile compounds. We hypothesized that the fungal symbionts of the bark beetles might metabolize spruce resin monoterpenes of the beetle's host tree, Norway spruce (*Picea abies*), and that the volatile products could be used as cues by beetles for locating breeding sites with beneficial symbionts. *Grosmannia penicillata* and other fungal symbionts altered the profile of spruce bark volatiles by converting the major monoterpenes to oxygenated derivatives. Bornyl acetate was metabolized to camphor, and α- and β-pinene to *trans*-4-thujanol and other oxygenated products. Extensive electrophysiological measurements showed that bark beetles possess olfactory sensory neurons that are selective for these oxygenated symbiont metabolites. Compounds such as camphor and *trans*-4-thujanol attracted beetles at specific doses in olfactory experiments and stimulated the response of female beetles to a mixture of pheromones. Finally, the fungal symbiont was found to stimulate bark beetle tunneling on diets. Collectively, our results show that oxygenated metabolites of conifer monoterpenes produced by fungal symbionts are used by bark beetles as cues to find these essential

43 microbial symbionts. The oxygenated metabolites may aid beetles in assessing the
44 presence of the fungus, the defense status of the host tree and the density of
45 conspecifics at potential feeding and breeding sites.

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61 ## Introduction

Herbivorous insects respond readily to airborne chemical cues, and many interactions between insects and their host plants are known to be mediated by volatile organic compounds [1–4]. In contrast, volatile signals between herbivorous insects and their symbiotic microbes have been less studied, aside from a few well-known examples including ambrosia beetles, termites, and the vinegar fly *Drosophila melanogaster* [5–8]. Yet, such signals could be as critical for insect success as their response to host plant cues. In some insect-microbe symbioses, microbes transform host plant metabolites creating volatile signals that are used by insects for food or brood site selection [7–10]. For example, yeasts vectored by *D. melanogaster* metabolize dietary phenolic antioxidants and release volatile phenolics that attract both larvae and adults to feed on antioxidant-rich foods [11]. Nevertheless, there is still comparatively little information about how microbial transformation of host plant chemicals influences insect-microbe symbioses, and whether the resulting metabolites represent honest signals of partner benefits.

Microbial symbioses are especially characteristic of wood-boring insects such as bark and ambrosia beetles. Bark beetles have captured much attention recently because of their large-scale outbreaks in many parts of the world. In Europe, for example, the Eurasian spruce bark beetle (*Ips typographus*) has killed millions of hectares of spruce stands as a result of global warming and management practices that increase forest vulnerability to epidemic outbreaks [12–16]. *Ips typographus* feeds and raises broods in the phloem tissues of trees, which contain high levels of terpene and phenolic defense chemicals [12,17]. This insect overcomes its unfavorable environment by mass attacks and by introducing a suite of microbes into the host, including the ectosymbiotic

ophiostomatoid fungi- *Grosmannia penicillata*, *G. europhioides*, *Endoconidiophora polonica* and *Ophiostoma bicolor* that cause blue staining of infected wood [18–22]. Although the exact benefit of fungal symbionts to *I. typographus* is not yet well understood, these necrotrophic fungi may exhaust host tree defenses, metabolize host defense compounds, and provide nutritional benefits to larvae and adults [23–26].

Conifer oleoresins are a formidable defense against insects and pathogens, as they can poison and physically entrap invaders [27–30]. However, the volatile fraction of the resin, especially the monoterpenes, also plays a central role in the colonization of host trees by bark beetles [28,31,32]. After locating a suitable tree, pioneer male *I. typographus* oxidize the dominant host monoterpene α-pinene to *cis*-verbenol, which is used as an aggregation pheromone in combination with the *de novo* produced 2-methyl-3-buten-2-ol to attract conspecifics for a mass attack [33–35]. In addition to bark beetle pheromones, several other oxygenated monoterpenes such as terpinene-4-ol, camphor, *trans*-4-thujanol and borneol have also been detected at the entrance holes of *I. typographus* galleries [36–39]. Interestingly, the phloem colonized by ophiostomatoid fungi around these galleries also produces large amounts of oxygenated monoterpenes compared to galleries without evident fungal growth [37]. However, the ecological functions of these oxygenated monoterpenes remain poorly understood. In our previous work, we showed that *I. typographus* bark beetles utilize *de novo* synthesized fungal volatiles to maintain their association with specific beneficial symbionts and also to avoid saprophytes [40]. However, it is unknown which volatiles are produced by these fungi when they colonize their native substrate i.e., the phloem and sapwood of the tree.

In this study, we investigated the volatile compounds emitted when fungal symbionts of *I. typographus* infect the bark of their Norway spruce (*Picea abies*) host trees. We show that these fungi dramatically alter the volatile monoterpene composition of spruce bark and demonstrate, using single sensillum recordings, that adult *I. typographus* can 1) perceive the fungal-produced monoterpenes and 2) are attracted to these compounds in behavioral bioassays. Our results indicate that bark beetles respond to symbiont biotransformation products of host tree metabolites and employ them to identify suitable sites for feeding and breeding.

Results

Bark beetles are attracted to volatiles from their symbiotic fungi grown on a spruce bark medium

We first tested whether adult bark beetles were attracted to volatiles produced by the symbiotic fungus *G. penicillata* when grown on two different growth media, potato dextrose agar (PDA) and spruce bark agar (SBA). In laboratory trap bioassays, adult beetles were strongly attracted to volatiles emitted by *G. penicillata* grown on both PDA or SBA compared to their respective fungus-free agar controls (Fig 1) (PDA, $z = 3.34$, $p = 0.001$; SBA, $z = 2.83$, $p = 0.005$, Wilcoxon's test). However, bark beetles showed a much stronger attraction towards *G. penicillata* grown on SBA over the same fungus grown on PDA (Fig 1D) ($z = 4.28$, $p < 0.001$, Wilcoxon's test). This indicates that the volatile profile of this fungus grown on spruce bark agar is distinct and highly preferred by adult beetles compared to the volatile profile when grown on agar without spruce bark. Volatiles from several other bark beetle primary and secondary fungal symbionts,

129 such as *E. polonica* and *G. europhioides*, grown on SBA were also highly attractive to
130 adult beetles, although not all bark beetle-associated fungi tested in this way emitted
131 attractive volatile blends (S1 Fig).

132 **Symbiotic fungi alter the volatile profile of the bark**

133 To identify the differences between the volatile profiles of fungus-inoculated and fungus
134 free bark, headspace volatiles were analyzed using gas chromatography-flame
135 ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS).
136 Principal component analysis was performed using the total volatile profile from each
137 treatment 4 d after the inoculations. PCA analysis revealed that the volatile profile of *G*.
138 *penicillata*-inoculated bark is distinct from the uninoculated control and nearly 68 % of
139 the variation in the volatile profiles was explained by the first two principal components
140 (Fig 2A).

141 To identify which compound groups were altered significantly due to fungal
142 infection, we analyzed volatiles over a time course of 4 d, 8 d, 12 d and 18 d post
143 inoculation with *G. penicillata* (Fig 2B, S3 Fig, S2, S3, and S5 Tables). In total, 79
144 compounds comprising host tree and fungal volatiles were detected in all treatments
145 and classified into different groups, namely aliphatic hydrocarbons (17 compounds),
146 aromatics (2 compounds), monoterpene hydrocarbons (15 compounds), oxygenated
147 monoterpenes) (26 compounds), sesquiterpenes (17 compounds) and spiroketals (3
148 compounds). The proportion of total oxygenated monoterpenes gradually increased to
149 dominate the volatile profile of *G. penicillata*-infected bark reaching a maximum at 18 d
150 post inoculation ($F_{(3,14)}$ = 3.54, p = 0.04, ANOVA), while the proportion of total

oxygenated monoterpenes was unchanged in mock-inoculated controls (Fig 2B). Camphor was the major contributor to the overall increase of oxygenated monoterpenes, with the highest relative abundance after 18 d and a significant difference between time points ($F_{(1,16)}$ = 13.06, p = 0.002, ANOVA, Tukey's test) (S5 Table). The proportion of monoterpene hydrocarbons gradually decreased over the time course in both treatments (mock, $F_{(3,7)}$ = 11.6 , p = 0.004; *G. penicillata*, $F_{(3,15)}$ = 21.2, p < 0.001, ANOVA). The proportion of total sesquiterpenes also decreased significantly over time in *G. penicillata* infected bark ($F_{(3,15)}$ = 4.4, p = 0.02, ANOVA), but not in the mock-inoculated bark. When measuring emission rate (ng mg DW^{-1} h^{-1}), the emission of total monoterpene hydrocarbons in the control and the *G. penicillata* treated bark plugs was not significantly different at 4 d post inoculation (Fig 2C, S2 Table). However, we found a dramatic increase in the emission rate of total oxygenated monoterpenes at this time point in spruce bark inoculated with *G*. *penicillata* compared to the fungus-free control (Fig 2D) (9-fold increase, t = 7.38, p = 0.004, Welch's t-test). Out of 19 identified oxygenated monoterpenes in the bark inoculated with *G. penicillata*, a total of 15 compounds significantly increased between control and fungus infected bark including camphor (Fig 2E) (S2 Table) (51-fold increase, t = 7.7, p = 0.004, Welch's t-test), *endo*-borneol (18 fold increase, t = 6.7, p = 0.001), isopinocamphone (3-fold increase, t = 6.8, p < 0.001), verbenone (2-fold increase, t = 4.3, p = 0.005) and bornyl acetate (3-fold increase, t = 3.7, p = 0.01). Measurements conducted on other *I. typographus* fungal symbionts also showed differences in the volatile composition of fungal-inoculated versus control bark (S2 Fig) with increases in the proportion of oxygenated monoterpenes over time (except for *E. polonica*) (S3 Fig, S4, S6 and S7 Tables).

Symbiotic fungi produce oxygenated monoterpenes from spruce monoterpene hydrocarbons and bornyl acetate

To investigate whether a symbiotic fungus can metabolize the major spruce monoterpenes, PDA was enriched with 0.5 mg g^{-1} (-)-β-pinene, (-)-α-pinene or (-)-bornyl acetate. The amount of monoterpenes remaining in the medium after 4 d was estimated using GC/FID measurements from hexane extracts of agar plugs containing *G. penicillata* and a fungus-free control. Only the amount of (-)-bornyl acetate decreased significantly in *G. penicillata*-inoculated agar compared to fungus-free agar (Fig 2F) (t = -3.38, p = 0.003). The amounts of (-)-β-pinene and (-)-α-pinene did not differ between *G. penicillata* and fungus-free agar.

Next, we quantified the *G. penicillata* metabolites of (-)-α-pinene, (-)-β-pinene and (-)-bornyl acetate after adding these three major spruce monoterpenes separately to PDA. (Fig 2G). The metabolic profiles of the fungus grown on either (-)-α-pinene- or (-)-β-pinene-enriched agar were similar, expect that verbenone was produced by the fungus from (-)-α-pinene but not from (-)-β-pinene. The oxygenated monoterpene, terpinen-4-ol, was the major biotransformation product and (+)-isopinocamphone and (+)-*trans*-4-thujanol were the minor products produced from (-)-α-pinene and (-)-β-pinene (S5, S6 Figs). The dominant oxygenated monoterpenes, camphor and *endo*-borneol, were produced by *G. penicillata* from (-)-bornyl acetate (Fig 2G, bottom panel; S8 Fig), and their production coincided with the decrease of the precursor (Fig 2F). Similar results were obtained for the other fungal symbionts tested (S4-S8 Figs). These results collectively show that symbiotic fungi can dramatically alter the volatile profile of spruce bark by increasing the emission of oxygenated monoterpenes.

Bark beetles detect oxygenated monoterpenes through specialized olfactory sensory neurons (OSN) in their antennae

To test if bark beetle antennal olfactory sensilla contain OSNs that detect the biotransformation products of monoterpenes, we challenged 231 olfactory sensilla with a test panel comprising 92 ecologically relevant compounds diluted in paraffin oil (1 µg μl^{-1}) using single cell recordings (S1 Table). Only 23 (~10%) of the sensilla housed neurons that did not respond to any of the compounds from the odor panel although their OSNs showed spontaneous firing. We obtained odor-evoked responses with strong excitation (>120 Hz) from 198 OSNs and weak excitation (<50 Hz) from 10 OSNs, allowing the grouping of these neurons into different classes based on their response profile. From initial screening experiments at a 10 µg dose on filter paper (to determine the maximum receptive range of OSNs), we identified and classified 20 classes of OSNs. Three OSN classes responded primarily to fungal-produced oxygenated monoterpenes. We also identified neurons belonging to previously described OSN classes tuned to pheromones, host tree volatiles and non-host odorants ([41]; S3 Fig) that are not further considered here.

OSN classes tuned to fungal-produced oxygenated monoterpenes were identified in both the A_m and B_m regions on the antennae (Fig 3A). One of these OSN classes responded most strongly to (+)-isopinocamphone, and this class was highly specific to oxygenated monoterpenes, especially ketones (Fig 3C, left panel). Apart from (+)-isopinocamphone, relatively strong responses were also elicited by (+)-pinocamphone, (-)-isopinocamphone, (±)-pinocarvone, (±)-camphor, and (-)-pinocamphone. (Fig 3C, left panel). Dose-response tests showed that this OSN class

was the most sensitive to (+)-isopinocamphone of all the compounds tested with responses evident at a dose of 100 pg. The responses to (+)-pinocamphone, (-)-isopinocamphone, (±)-pinocarvone and (±)-camphor all appeared between 1 ng and 10 ng doses (Fig 3D, left panel). Another OSN class with specific responses to fungal-derived compounds responded most strongly to (+)-*trans*-4-thujanol and weakly to (±)-3-octanol, (±)-1-octen-3-ol, (+)- and (-)-terpinen-4-ol, and (+)- and (-)-α-terpineol (Fig 3C, middle panel). This OSN showed a 1000-fold lower response threshold to (+)-*trans*-4-thujanol compared to the next best ligands, the C_8 alcohols (Fig 3D, middle panel). Finally, an OSN class responding strongly to verbenone, α-isophorone and β-isophorone, followed by weaker responses to (-)- and (+)-*trans*-verbenol, pinocarvone, and (-)-*cis*-verbenol (Fig 3C, right panel) was also found. Dose- response tests revealed that this neuron class responded the strongest to α-isophorone across most tested doses, followed by slightly weaker and similarly strong responses to both verbenone and β-isophorone (Fig 3D, right panel).

A few previously characterized OSN classes for host tree monoterpenes, including the classes with primary responses to α-pinene, *p*-cymene, and Δ3-carene, respectively [41], showed varying secondary responses to some of the fungal-derived compounds tested here for the first time. For example, the α-pinene OSN class responded also to (+)-isopinocamphone, (-)-isopinocamphone and (±)-pinocarvone and weakly to (±)-camphor, (-)-myrtenol, *trans*-pinocarveol, carvone, borneol and (-)-fenchone (S9A Fig). The *p*-cymene OSN class showed intermediate responses to (+)-*trans*-4-thujanol and carvone (S9B Fig). Although the Δ3-carene OSN class showed high specificity towards Δ3-carene, ligands such as camphor and (-)-isopinocamphone

also elicited weak responses from this neuron class (S9C Fig). Collectively, single sensillum recordings indicated that bark beetles possess OSNs specific for detecting the oxygenated monoterpenes produced by their associated fungi, and these neurons do not respond to the respective hydrocarbon precursors. On the other hand, some oxygenated monoterpenes elicited variable responses in OSN classes responding primarily to monoterpene hydrocarbons

Oxygenated monoterpenes produced by fungal symbionts attract bark beetles

Our previous trap bioassays showed that volatiles from *G*. *penicillata* grown on spruce bark agar were strongly attractive to adult beetles (Fig 1C). Therefore, we asked how the addition of pure monoterpenes to the spruce bark agar could influence the behavior of beetles towards *G. penicillata*. Our bioassay revealed that addition of 0.1 mg g^{-1} and 0.5 mg g^{-1} (-)-β-pinene to the fungal growth medium did not affect the attraction of beetles towards *G. penicillata* when tested against a fungus-free control (Fig 4A, left) (0.1 mg g^{-1}, $z = 2.22$, $p = 0.02$; 0.5 mg g^{-1}, $z = 2.54$, $p = 0.01$, Wilcoxon's test). However, addition of 1 mg g^{-1} (-)-β-pinene completely abolished the attraction to *G*. *penicillata* and adult beetles were unresponsive in the binary choice test (Fig 4A, left). To further understand the different responses of beetles towards *G*. *penicillata* grown on varying concentrations of (-)-β-pinene, individual electrophysiologically-active (-)-β-pinene biotransformation products were used in trap bioassays against a mineral oil control. At the highest dose tested (1 mg), both *trans*-4-thujanol and terpinen-4-ol were avoided by adult beetles (Fig 4B *trans*-4-thujanol, left panel; terpinene-4-ol, right panel) (1 mg *trans*-4-thujanol, $z = -1.9$, $p = 0.05$; 1 mg terpinen-4-ol, $z = -1.7$, $p = 0.08$, Wilcoxon's test). On the other hand, 100 µg trans-4-thujanol significantly attracted adult beetles (Fig 4B, left

266 panel) (z = 2.78, p = 0.005, Wilcoxon's test). Adult beetles did not discriminate between
267 *G*. *penicillata* grown on (-)-β-pinene enriched medium and *G*. *penicillata* grown on non-
268 enriched medium (Fig 4E). Based on these results, we concluded that beetles show
269 concentration-specific responses to some (-)-β-pinene biotransformation products, but
270 not to (-)-β-pinene itself.

271 Addition of another major host tree monoterpene, (-)-bornyl acetate, to fungal
272 growth medium at 0.05 mg g^{-1} and 0.5 mg g^{-1} resulted in strong attraction of *I.*
273 *typographus* adults towards *G*. *penicillata* when tested against a fungus-free control
274 after 4 d incubation (Fig 4C, left panel) (0.05 mg g^{-1}, z = 3.31, p = 0.001; 0.5 mg g^{-1}, z =
275 3.21, p = 0.001, Wilcoxon's test). The major biotransformation product of (-)-bornyl
276 acetate that formed in this period (Fig 4C), camphor, was significantly more attractive to
277 adult beetles at a 100 µg dose than the mineral oil control (Fig 4D) (z = 2.58, p = 0.01,
278 Wilcoxon's test). However, adult beetles preferred *G*. *penicillata* grown on unenriched
279 medium against *G. penicillata* grown on high amount of (-)-bornyl acetate (0.5 mg g^{-1})
280 enriched medium (Fig 4F) (z = 2.12, p = 0.03, Wilcoxon's test) consistent with their
281 preference for lower amounts of camphor. By contrast, in the absence of fungus beetles
282 did not discriminate between diet enriched with monoterpenes and diet without
283 monoterpenes (S10 Fig). Collectively, these results show that fungal biotransformation
284 products of host tree monoterpenes can be perceived as attractive cues by adult
285 beetles, but this attraction varies with the concentration.

286 **Volatiles of symbiotic fungi increase bark beetle attraction to pheromones**

Previous studies have detected the emission of several oxygenated monoterpenes from bark beetle entrance holes on attacked trees during the colonization phase coincident with the emission of male-produced pheromones [36–38]. Therefore, we tested the role of fungal symbiont volatiles in the behavioral response of adult beetles towards their pheromones. *Ips typographus* has been shown to display sex-specific responses to its pheromone components [42,43], so individual sexes were tested separately. Female beetles were significantly more attracted towards the individual aggregation pheromone components *cis*-verbenol and 2-methyl-3-buten-2-ol (*cis*-verbenol, $z = 2.98$, $p = 0.003$; 2-methyl-3-buten-2-ol, $z = 2$, $p = 0.046$, Wilcoxon's test), and towards a mixture of the two pheromone components ($z = 5.19$, $p < 0.001$, Wilcoxon's test) compared to the mineral oil control (Fig 5A). By contrast, adult males did not make a significant choice between these options in accordance with previous studies [42, 61]. However, when beetles had to choose between the pheromone mixture with or without *G. penicillata* volatiles, females significantly preferred the pheromone mixture together with *G. penicillata* volatiles (Fig 5B) ($z = 3.41$, $p = 0.001$, Wilcoxon's test). These results indicate that, in addition to male-produced pheromones, female beetles might also utilize fungus-produced oxygenated monoterpenes as cues to select suitable breeding sites that include a beneficial symbiotic fungus.

Symbiotic fungi increase the tunneling of adult beetles

We previously showed that the symbiotic fungi of *I. typographus* stimulate tunneling of adult bark beetles in fungus-colonized medium [40,44,45]. Here we investigated if the addition of specific spruce monoterpenes to medium colonized by *G. penicillata* had an effect on tunneling. Overall, the presence of a symbiotic fungus increased the tunneling

success of adult beetles after 48 h. Multiple logistic regression analysis revealed that successful tunneling odds in (-)-bornyl acetate-amended medium were significantly influenced by the fungus when the beetle sex and the monoterpene treatment remained constant (Table 1) (β = 4.98, χ^2 = 20.99, p < 0.001). The presence of the fungus increased the tunneling odds by 145 units (percentage probability increased by 99%) compared to in the absence of the fungus. Additionally, males had significantly lower tunneling success compared to females (Table 1) (β = -1.72, χ^2 = 8.78, p = 0.003) with the tunneling odds for males being 0.18 units lower (percentage probability decreased by 15.2%) than for females. In (-)-β-pinene-amended medium, only the presence of the fungus significantly influenced the tunneling odds of beetles (Table 1) (β = 2.65, χ^2 = 20.45, p < 0.001), with an increase of 14.06 units (percentage probability increased by 93%) compared to in the absence of the fungus. Similarly, in (-)-α-pinene-amended medium only the presence of the fungus significantly influenced the tunneling odds of beetles (Table 1) (β = 2.02, χ^2 = 9.39, p = 0.002), with an increase of 7.51 units (percentage probability increased by 88%) compared to the absence of the fungus. Addition of the three monoterpenes without fungus did not have any effect on the tunneling behavior of adult beetles.

The growth of *G. penicillata* on monoterpene-enriched media resulted in significantly longer beetle tunnels than in the other treatments (Fig 6B-D) ((-)-β-pinene, $F_{(3,99)}$ = 4.95, p = 0.003; (-)-α-pinene, $F_{(3,92)}$ = 14.8, p < 0.001; (-)-bornyl acetate, $F_{(3,106)}$ = 6.6, p < 0.001, ANOVA, Tukey's test). However, there was no significant difference in tunnel lengths in treatments with fungus alone versus fungus plus monoterpenes except for the treatment with (-)-β-pinene (Fig 6B-D, Tukey's test). The sex of the beetle and

the interaction of sex with other treatments had no effect on the tunnel length. Altogether, these results show that adult *I. typographus* recognize fungal volatiles produced by symbionts growing on spruce bark as positive cues that stimulate tunneling in the presence of fungus.

Discussion

The successful attack of bark beetles on their host trees is invariably associated with free-living fungal symbionts. These ascomycete symbionts may detoxify the terpene-rich defensive resin of the host tree, hasten host tree death, provide nutritional benefits, and increase resistance to pathogens, [20,21,26]. Here, we documented the ability of *I. typographus* fungal symbionts to metabolize host tree monoterpenes to oxygenated derivatives that may assist adult beetles in locating suitable breeding and feeding sites [38,46]. Several oxygenated monoterpenes have been previously identified as volatiles released from trees that were attacked by *I. typographus* [36,37,39]. We showed that these compounds were likely fungal metabolites that become dominant components of

353 the volatile profile of fungus-infected bark within 12 days following infection. Bark beetle
354 olfactory sensory neurons could detect various oxygenated monoterpenes produced by
355 symbiotic fungi, and we identified three neuron classes that primarily respond to these
356 compounds. Major fungal-derived oxygenated monoterpenes attracted adult beetles
357 and stimulated tunneling of beetles on diets inoculated with monoterpene-metabolizing
358 fungal symbionts. The presence of the symbiont *G. penicillata* also increased the
359 attraction of female beetles to the aggregation pheromone.

360 **Oxygenated monoterpenes are widespread volatile cues in tree-feeding insects**

361 Various forest insects that are associated with fungal symbionts, such as bark beetles,
362 ambrosia beetles and wood wasps live in host trees producing large quantities of
363 monoterpene volatiles. These insects are often attracted to their fungal symbionts
364 through volatiles [6,40,47] and hence, fungal-produced monoterpene metabolites could
365 be critical components of the attractive volatile blends. For bark beetles, oxygenated
366 monoterpenes derived from either the beetles, fungi or host trees play many important
367 roles in their life history [48]. During mass attacks, the host-derived oxygenated
368 monoterpene *cis*-verbenol acts together with 2-methyl-3-buten-2-ol as an aggregating
369 signal for *I. typographus* to promote mass attack on individual trees [32,49]. Beetles
370 also utilize oxygenated monoterpenes to restrict the density of attack. Microbes lining
371 the gallery walls or living in the beetle gut oxidize *cis* and *trans*-verbenol into verbenone,
372 which inhibits the attraction of both sexes to fully colonized trees [50–52]. Furthermore,
373 mated male beetles produce ipsenol and ipsdienol, of which ipsenol acts as an anti-
374 attractant [52] and, an oxygenated monoterpene from host trees, 1,8-cineole, which is
375 produced in higher amounts in resistant or MeJA-primed trees, inhibits attraction of

beetles to their pheromones [53–55]. Oxygenated monoterpenes are also used as reliable cues by parasitoids of bark beetles to locate their prey [39,56].

Here we discovered that oxygenated monoterpenes emitted by bark beetle-associated fungi growing on agar amended with spruce bark attracted adult *I. typographus*. Previously, we demonstrated that fungi grown on potato dextrose agar without any spruce bark *de novo* produced other volatiles, mixtures of aliphatic and aromatic compounds, that attract newly emerged (callow) adult beetles [40]. These compounds were also detected here as major components of the volatile blend at later phases of fungal growth (S3-7 Table). Although we focused principally on the symbiont *G. penicillata* in this study, volatiles from other fungal symbionts were also investigated. The congener *G. europhioides* and *E. polonica* were also found to emit volatile blends attractive to adult *I. typographus* when growing on spruce bark agar (Fig. S1), but the volatiles of *O. bicolor* and the saprophyte *O. piceae* were not attractive. Since all of these species produce oxygenated derivatives of spruce bark monoterpenes (Tables S2-7), it is not the simple presence of oxygenated monoterpenes, but the entire volatile profile that determines its attractiveness.

Oxygenated monoterpenes signal the presence of fungi to bark beetles and so may modulate beetle colonization

The first chemical signals reported to mediate bark beetle colonization of their hosts were aggregation pheromones. Yet even in the presence of these pheromones, a large proportion of aggregating beetles that land on trees leave without tunneling into the bark [46,57,58]. This suggests that other cues may be needed to induce beetles to stay and

bore into the bark. Indeed, bark beetles have been shown to respond to signals from host and non-host species when selecting trees for colonization [32,40,59–62]. Based on our results, fungus-produced oxygenated monoterpenes might also belong to the list of colonization cues. For bark beetles, fungal metabolites can serve as indicators of host tree sectors where their fungal symbionts are already established. These compounds also provide evidence for the ongoing metabolism of host tree defenses, which could improve the success of bark beetle colonization.

Fungal volatiles also enhance the attraction of bark beetles to aggregation pheromones. Female *I. typographus* are known to use the aggregation pheromone (2-methyl-3-buten-2-ol and *cis*-verbenol) to locate trees suitable for mating and oviposition [46,63]. Here we showed that female *I. typographus* were more attracted at short range to a combination of pheromones plus fungal volatiles than to pheromones alone. Oxygenated monoterpenes and other fungal volatiles provide information about the presence of fungal symbionts, which promote the successful development of their offspring. Similarly, the pheromone component *cis*-verbenol, itself an oxygenated monoterpene produced by *I. typographus* from the host tree precursor α-pinene [64], provides information about the presence of other beetles, especially mates. The lack of response of males to pheromones in our experiments is not unexpected, as male *I. typographus* have been reported to be less responsive than females to high doses of pheromones in walking bioassays [43,63]. This behavior may help them avoid dense colonies of male conspecifics within a tree to reduce competition for mates and food.

The oxygenated metabolites of host monoterpenes produced by fungal symbionts not only attracted bark beetles, but also stimulated them to tunnel in a fungal-colonized

421 medium. Interestingly, both sexes showed increased tunneling in contrast to their
422 response to pheromones. The lack of differences in sex-specific responses could be
423 due to the fact that the nutritional advantage of feeding on fungus colonized spruce bark
424 medium is beneficial to both sexes [21,22].

425 The proportion of oxygenated monoterpenes to total monoterpenes in the volatile blend
426 of *G. penicillata* increased over the time course studied to nearly 50% at 12 days and
427 nearly 70% at 18 days, a trend also observed for *G. europhioides* and *O. bicolor*. Thus,
428 higher proportions of these compounds may indicate older fungal infection sites and
429 hence older beetle invasion sites that may be less attractive to newly arriving beetles
430 due to crowding. The lack of attraction and even repellency of higher concentrations of
431 individual oxygenated monoterpenes seen in laboratory bioassays in our and in
432 previous studies is consistent with this interpretation [65]. In fact, one oxygenated
433 monoterpene derivative has been already reported to inhibit *I. typographus* attraction.
434 Verbenone, which is produced by microbial oxidation or auto-oxidation of the
435 pheromone *cis*-verbenol, repels *I. typographus* in later phases of the attack cycle
436 [52,66,67].

437 Oxygenated monoterpenes are signals not only for bark beetles, but also for their
438 enemies. Both beetle predators and parasitoids employ these compounds and other
439 volatiles to locate bark beetle larvae hidden under the bark [68]. Specifically, a three-
440 component blend comprising camphor, isopinocamphone and terpinen-4-ol, all fungal
441 metabolites of host tree monoterpene hydrocarbons, was reported to attract a
442 coleopteran predator and several hymenopteran parasitoids of *I. typographus* in the
443 presence of host tree background signals [56,69,70]. A similar mechanism is used by

444 parasitoids of the wood wasp, *Sirex noctilio* to locate their concealed host insect under
445 the bark via the volatiles from the wood wasp fungal symbiont *Amylostereum areolatum*
446 [71]. Furthermore, the bark beetle predator, *Thanasimus formicarius* contains OSNs to
447 detect oxygenated monoterpenes such as camphor and pinocamphone [72]. Thus, any
448 benefit to the beetle arising from oxygenated monoterpene production by its symbiotic
449 fungi may come at the cost of revealing its presence to natural enemies that employ
450 these same volatiles to locate bark beetles.

451 **Formation of oxygenated derivatives may reduce monoterpene toxicity for bark**
452 **beetles**

453 The conversion of host tree monoterpene defenses by symbiotic fungi to oxygenated
454 products may alleviate toxicity to bark beetles. Terpene-rich resins are a general
455 defense of *P. abies* and other conifers against herbivores and pathogens [4,73,74].
456 Thus, it is not surprising that monoterpenes have exhibited toxicity to bark beetles in
457 many studies [75–77]. Monoterpene hydrocarbons, such as α-pinene are typically more
458 toxic to beetles than host tree-produced oxygenated monoterpenes, such as bornyl
459 acetate [78]. Hence the oxidative transformations carried out by fungal symbionts
460 described in this study could reduce toxicity to *I. typographus* through conversion to less
461 poisonous derivatives. Such detoxification of host tree defenses could represent a
462 significant benefit of fungal symbionts [79].

463 By contrast, oxygenated monoterpenes may be more toxic for fungi than monoterpene
464 hydrocarbons [80,81]. Thus, the initial oxidation of monoterpene hydrocarbons may not
465 constitute a detoxification unless it is a step towards further metabolism. The potential

toxicity of oxygenated monoterpenes may explain why these substances are readily degraded by fungi specialized on conifers such as *G. penicillata*, *Heterobasidion parviporum* and *Seridium cardinale* [80,81]. The fungus *G. clavigera*, a symbiont of the bark beetle *Dendroctonus ponderosae,* possesses genes encoding cytochromes P450 and other oxidative enzymes that are up-regulated by dietary monoterpenes [82,83]

Other sources of oxygenated monoterpenes in spruce-bark beetle interactions

Oxygenated monoterpenes emitted from trees attacked by *I. typographus* may arise from sources other than fungal symbionts. The host tree *P. abies* synthesizes large amounts of bornyl acetate [84] and small amounts of 1,8-cineole [85]. In these compounds, the oxygen functions are incorporated during biosynthesis from basic precursors, whereas the products from fungal symbionts are formed by oxidative modification of a previously formed monoterpene hydrocarbon skeleton. The compound *trans*-4-thujanol belongs to the latter group. We identified it as a *G. penicillata* metabolite of α- and β-pinene, but *trans*-4-thujanol may also be synthesized by the tree, although at low levels in *P. abies* bark [65]. As another alternative, this and other oxygenated monoterpenes could be produced via autoxidation. The degradation of monoterpenes upon exposure to air could explain the release of low but readily detectible amounts of oxygenated monoterpenes from uninfected control bark plugs in our and other studies. In the field, oxygenated monoterpenes other than bornyl acetate and 1,8-cineole have been detected from damaged *P. abies* trees when monoterpenes were exposed to air [86,87]. However, in the present study the emission rate from

uninfected bark plugs was much lower than from fungus-infected plugs, suggesting that microbial metabolism is a much more significant source of oxygenated monoterpenes than autoxidation [88]. However, since *P. abies* cell suspension cultures have been reported to oxidize added monoterpenes [89,90] the tree itself cannot be ruled out as a source of any of the detected oxygenated monoterpenes.

Among microbial sources of oxygenated monoterpenes are several yeast species including *Hansenula holstii, H. capsulata* and *Candida nitratophila*, which were isolated from *I. typographus,* and produce terpinen-4-ol, α-terpineol, borneol and *trans*-pinocarveol when grown in phloem medium or in α-pinene-supplemented medium [67]. In addition, another bark beetle species, *Polygraphus poligraphus*, which is sometimes found together with *I. typographus*, was shown to emit large amounts of terpinene-4-ol [91,92]. Intermediate amounts of α-terpineol, *cis*- and *trans*-4-thujanol were also identified from the hindgut as well as the entrance holes of this beetle's gallery and could be formed by this beetle or its associated microorganisms from host tree monoterpenes.

High selectivity of bark beetle olfactory neurons to oxygenated monoterpenes suggest their role in detecting symbiotic fungi

The bark beetles of *Ips typographus* possess several classes of olfactory sensory neurons (OSNs) that were shown to detect the oxygenated monoterpenes produced by their fungal symbionts with notable specificity. For example, the isopinocamphone OSN showed high specificity towards several monoterpene ketones produced by fungal symbionts, including (+)- and (-)-isopinocamphone, (+)- and (-)-pinocamphone,

510 camphor, and pinocarvone, but not to monoterpene alcohols such as borneol and *trans*-
511 pinocarveol. The absence of any response to monoterpene hydrocarbons indicates that
512 this OSN is not tuned to detect the host tree itself, but rather organisms metabolizing
513 the major host monoterpenes. The isopinocamphone OSN is similar to one recently
514 reported OSN class from *I. typographus* that responded best to pinocarvone and
515 camphor (OSN class named Pcn; isopinocamphone and pinocamphone were not
516 tested) [87]. Our work shows that (+)-isopinocamphone is the primary ligand of this OSN
517 class, based on its greater activity than the other active compounds. In addition, the
518 response profile of this OSN class matches very well with that of the odorant receptor
519 (OR) ItypOR29, which recently was characterized in *Xenopus laevis* oocytes [93].

520 Likewise, we showed that a previously described verbenone-sensitive OSN class [41]
521 also responds to *cis*- and *trans*-verbenol and β-isophorone, compounds that are
522 believed to arise from bark beetle metabolism of host tree terpenes [94]. Verbenone is
523 produced from the verbenols by microbes that colonize gallery walls and beetle guts.
524 Therefore, this OSN appears to be tuned to signals from various ecological sources
525 providing information on bark beetle density as well as microbial establishment
526 [46,51,86].

527 Another OSN class responded most sensitively to the monoterpene alcohol *trans*-4-
528 thujanol, a fungal symbiont metabolite of α- and β-pinene. This OSN also responded to
529 the fungal metabolites terpinen-4-ol and α-terpineol, as well as C_8 alcohols, but only at
530 the highest doses tested, which extends prior results for this OSN [87] to other
531 compounds from our greatly expanded test odor panel. Strong electroantennographic
532 activity in *I. typographus* in response to these oxygenated monoterpenes has also been

reported [86,87], and the response spectrum of this OSN class matches well with that of the receptor ItypOR23, which is evolutionarily related to ItypOR29 detecting isopinocamphone [93]. In the present study, fungal symbiont-produced oxygenated monoterpenes dominated the volatile profile of fungus-infected spruce bark and might thus indicate the defense status and degree of host colonization by fungus and beetles. However, the ecological relevance of oxygenated monoterpenes is likely to be context dependent, and many factors including the concentration of volatiles, blend ratios, timing of emission (early or late attack phase), as well as the nutritional state, age and sex of beetles, may determine the valence of these compounds.

Conclusion

We show that free-living fungal symbionts vectored by spruce bark beetles metabolize host tree monoterpene hydrocarbons to oxygenated derivatives as they infect the bark. These oxygenated volatile compounds serve as signals to indicate the establishment of fungi in beetle galleries and ultimately attract bark beetles and stimulate their tunneling. However, the roles of such fungal volatiles are context-dependent and can either attract or repel beetles depending on their concentrations, which reflect the physiological status of the microbes, the density of beetles and the stage of attack [40,95]. Since the ecological roles we have proposed for these oxygenated monoterpenes are based on laboratory assays with walking beetles, studies under natural conditions are necessary to confirm our findings. These compounds may also be useful in integrated pest management strategies as attractants or repellents of bark beetles perhaps in combination with pheromones [96–98]. In this way, microbial volatiles provide a rich

source of untapped insect semiochemicals that can be exploited for protecting forests from devastating pest species such as *I. typographus*.

1 Materials and methods

1.1 Fungal strains and growth medium

The fungal strains used in this study have been previously described [40] (listed in *Table S1*). In order to obtain spores from fungi, freshly inoculated PDA plates were incubated at room temperature for 15-20 d until the mycelium was old and dark. After 20 d, plates were kept briefly at 4˚C to induce sporulation. Four to six 1 cm diameter mycelium plugs were removed from each plate and inoculated into 20 mL potato dextrose broth and incubated at 25˚C at 150 rpm for 4 days. Once the broth was turbid, the spores were filtered using a 40 µm EASYstrainer™ (Greiner Bio-One, Frickenhausen, Germany), and the filtrate was spun down at 4200 rcf for 10 min to precipitate the spores. The supernatant was discarded and the spore suspension was washed three times with autoclaved water and then stored at 4˚C until used. The spore suspension prepared using this method was viable for several months when stored at 4˚C.

1.2 Bark beetle rearing

Bark beetles were reared and stored in the laboratory as described [40]. The starting beetle culture was obtained from an infested tree in October 2017 near Jena, Thuringia, Germany. Beetles were reared throughout the year in the laboratory in freshly cut spruce logs (ca. 30 cm diameter x 50 cm height) placed in an environmental chamber set at 25°C throughout the day, 65% relative humidity and a photoperiod of 20 h per day. Beetles emerged from breeding logs after ca. 35 days and were collected manually. Emerged adults were sexed based on the bristle density on their pronotum [99] and stored separately in Falcon tubes lined with moist paper at 4°C at least for a week before using them in bioassays. Adult beetles were used only once in bioassays.

1.3 Spruce bark diet

Spruce bark agar was prepared as follows: the outer bark of a freshly cut mature tree was scraped off gently using a drawing knife and the inner bark (phloem) was carefully peeled off using a chisel. The bark was cut into small pieces and ground to a fine powder in vibratory micro mill (Pulverisette 0, Fritsch GmbH, Idar-Oberstein, Germany). The instrument was pre-cooled with liquid nitrogen and bark pieces were pulverized at an amplitude of 2.0 for ca. 10 minutes with addition of liquid nitrogen every two minutes to prevent thawing. The ground powder was stored in Falcon tubes at -80°C until used for diet preparation. For preparing spruce bark diet, 7% powdered inner spruce phloem (w/v) was added to 4% Bactoagar (Roth) and heat sterilized at 121°C for 20 minutes.

1.4 Identification and quantification of headspace volatiles of fungal symbionts

Norway spruce bark plugs of approximately 28 mm diameter were removed from a freshly felled tree in July, 2017 and a single bark plug was placed inside a 250 mL

volatile collection glass bottle. Before removing the bark plugs, the surface of the bark and the cork borer were sterilized by thorough spraying with 70% ethanol in a laminar hood. An 100 µL quantity of spore suspension ($1*10^6$ cells mL^{-1}), prepared as described above, was added to the exposed section of the bark, and autoclaved water was added to the control treatment. Each treatment was replicated four times including the control. The glass bottle was secured tightly and incubated at 25˚C for 4 days. After 4 d, activated charcoal-filtered air was passed into the bottle inlet at the rate of 50 mL min^{-1} and the outlet air was funneled through a SuperQ adsorbent filter (150 mg) for 4 hours. Afterwards, the filters were eluted with 200 µL dichloromethane spiked with 10 ng $µL^{-1}$ nonyl acetate (Sigma Aldrich) as an internal standard and stored at -20˚C. The spruce bark plugs were oven dried at 80˚C for 6 hours after the experiment and the dry weight was measured.

The eluted volatile samples were subjected to GC-MS and GC-FID analysis using an Agilent 6890 series GC (Agilent, Santa Clara, CA, USA) (injection, 1 µl splitless; flow, 2 ml min^{-1}; temperature, 45 to 180°C at 6°C min^{-1} and then to 300°C at 100°C min^{-1} for 10 min) coupled either to an Agilent 5973 quadrupole mass selective detector (interface temperature 270 °C, quadrupole temperature 150°C, source temperature 230 °C; electron energy 70 eV) or a flame ionization detector (FID, temp. 300 °C). The constituents were separated on a DB-5MS column (Agilent (30 m x 0.25 mm x 0.25 µm)), with He (MS) or H_2 (FID) as carrier gas. The identity of each peak was determined by comparing its mass spectra and retention times to those of reference libraries (NIST98 and Wiley275) and authentic standards. The amount of each compound was

619 calculated from the peak area obtained from the FID detector relative to the internal
620 standard and standardized to the spruce bark dry weight.

621 **1.5 Time series headspace volatile collection**

622 For time series volatile analysis, spruce bark plugs (10 mm diameter) were removed
623 using a cork borer from a freshly felled spruce tree in October, 2016. Each spruce bark
624 plug was placed in a 15 mL clear glass vial (Supelco-Sigma-Aldrich) and 50 µL spore
625 suspension ($1*10^6$ cells mL^{-1}), prepared as described above, was added to treatment
626 plugs while control plugs received sterile water. The headspace volatiles were captured
627 on three polydimethylsiloxane (PDMS) sorbent silicone tubes (0.5 cm), which were hung
628 in each glass vial using a manually crafted metal hook attached to the bottom of
629 PTFE/silicone septa in the screw cap [100]. The headspace volatiles were collected
630 from each treatment for 2 h at 4, 8, 12 and 18 d after inoculation. After sampling,
631 silicone tubes were placed in 1.5 mL brown glass vials and stored at -20°C until
632 analysis.

633 Volatiles collected on PDMS tubes were analyzed using a GC-2010 plus gas
634 chromatograph coupled to a MS-QP2010 quadrupole mass spectrometer equipped with
635 a TD-20 thermal desorption unit (Shimadzu, Japan) and a GC Cryo-Trap filled with
636 Tenax. A single tube was placed in a 89 mm glass thermal desorption tube and
637 desorbed at a flow rate of 60 mL min^{-1} for 8 min at 200 °C under a stream of N_2
638 gas. The desorbed substances were focused on a cryogenic trap at -20 °C. The Tenax
639 adsorbent was heated to 230 °C and the analytes were injected using split mode
640 (1:100) onto a Rtx-5MS GC column (30 m x 0.25 mm x 0.25 µm) with helium as

carrier gas. Compounds were identified as above (1.5) from authentic standards and libraries, and quantified from the area of each peak obtained using GC-MS post run analysis software from Shimadzu. The PLS-DA plot in Fig 2A was generated by using MetaboAnalyst 3.0 software with normalized GC-MS data (both log transformed, and range scaled) [101].

1.6 Biotransformation of host tree compounds

Experiments were conducted in 9 cm Petri dishes containing 2% potato dextrose agar (PDA) supplemented with test solutions. The tested compounds were (-)-α-pinene, (+)-α-pinene, (-)-β-pinene, myrcene, γ-terpinene, terpinolene, sabinene, camphene, *p*-cymene, and (-)-bornyl acetate. Sources of these compounds are given in Table S1. Compounds were added after dissolving in dimethyl sulfoxide (DMSO). These were then added into PDA to reach a final concentration of 0.5 mg mL^{-1} before pouring into Petri dishes. A 5 mm agar plug containing a fungal colony was placed in the center of each dish and incubated at 25˚C in darkness for 6 d. Each treatment was replicated four times and for the control the PDA contained only DMSO plus monoterpene. The headspace volatiles were collected after 4d using three PDMS tubes, which were mounted on sterile metal wires and imbedded in PDA for one hour and stored at -20˚C. The identification and quantification of compounds were conducted in the same way as reported for the time series (section 1.5). The headspace volatiles from fungus grown on PDA enriched with myrcene, γ-terpinene, terpinolene, camphene and *p*-cymene did not yield detectable amounts of monoterpene transformation products on analysis.

To identify if symbiotic fungi can reduce the amount of monoterpenes in their substrate, fungi were grown on PDA enriched with 0.5 mg g^{-1} (-)-α-pinene, (+)-α-pinene, (-)-β-pinene, and (-)-bornyl acetate as described above. Control plates contained only DMSO and the tested monoterpene. After 4 d, three plugs of 6 mm diameter were removed, weighed and transferred to 1.5 ml sterile glass vials. Agar plugs were homogenized using sterile plastic pestles and 1 ml hexane (extraction solvent) spiked with 10 ng μL^{-1} nonyl acetate was added and samples were vortexed for 30 s. Supernatants were transferred to new vials and stored at -20°C until identification and quantification by GC-MS and GC-FID (1.4). Data analysis was identical to that reported above.

1.7 Chemical synthesis of (+)-isopinocamphone and β-isophorone

(+)-isopinocamphone ((1*R*,2*R*,5*S*)-2,6,6-Trimethylbicyclo[3.1.1]heptan-3-one). A mixture of (1*R*,2*R*,3*R*,5*S*)-(-)-isopinocampheol (200 mg, 1.30 mmol, Sigma-Aldrich) and Dess-Martin-periodinane (825 mg, 1.95 mmol) in anhydrous CH_2Cl_2 (15 mL) was stirred at room temperature for 1 hour, followed by the addition of water and sat. aq. $NaHCO_3$ solution. The mixture was extracted twice with methyl *t*-butyl ether. The organic phase was washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by short-path chromatography using an SPE cartridge (Chromabond SiOH, 6 mL, 500 mg, Macherey-Nagel, *n*-hexane:EtOAc = 10:1) to yield (+)-isopinocamphone (159 mg, 1.04 mmol, 80%). NMR measurements were carried out on a Bruker Avance AV-500HD spectrometer, equipped with a TCI cryoprobe using standard pulse sequences as implemented in Bruker Topspin ver. 3.6.1. (Bruker Biospin GmbH, Rheinstetten, Germany). Chemical shifts were referenced to the residual solvent signals of acetone-d_6 (δ_H 2.05/ δ_C 29.84) or $CDCl_3$ (δ_H 7.26/ δ_C 77.16), respectively.

686 ^{1}H-NMR (500 MHz, $CDCl_3$) δ ppm: 2.64 (*ddd*, *J*=18.6/3.0/3.0 Hz, 1H), 2.62 (*dddd,*
687 *J*=10.1/6.2/6.1/3.0 Hz, 1H), 2.52 (*bd*, *J*=18.6 Hz, 1H), 2.46 (*dq*, *J*=7.3/1.9 Hz, 1H), 2.12
688 (*ddd*, *J*=9.1/6.1/3.0 Hz, 1H), 2.06 (*ddd*, *J*=6.2/6.2/1.9 Hz, 1H), 1.31 (*s*, 3H), 1.21 (*d*,
689 *J*=7.3 Hz, 3H), 1.19 (*bd*, *J*=10.1 Hz, 1H), 0.88 (*s*, 3H). ^{13}C-NMR (125 MHz, $CDCl_3$) δ
690 ppm: 215.9, 51.7, 45.3, 45.1, 39.4, 39.1, 34.8, 27.4, 22.2, 17.3. GC-MS t_R: 13.6 min. EI-
691 MS (70 eV): *m/z* (%) 152 (14), 110 (15), 95 (44), 83 (89), 69 (97), 55 (100), 41(64).

692 **β-isophorone** (3,5,5-Trimethyl-3-cyclohexen-1-one). β-Isophorone was synthesized
693 from α-isophorone (Acros Organics, Fair Lawn, NJ, USA) following published methods
694 [102]. Since β-isophorone was very unstable during column chromatography, the
695 compound was used for the bioassay without purification. The purity of β-Isophorone
696 was 91% with 6% of α-isophorone as assessed by NMR analysis. ^{1}H-NMR (500 MHz,
697 acetone-d_6) δ ppm: 5.44 (*s*, 1H), 2.69 (*bs*, 2H), 2.27 (*s*, 2H), 1.70 (*bs*, 3H), 1.00 (*s*, 6H).
698 ^{13}C-NMR (125 MHz, acetone-d_6) δ ppm: 209.3, 133.0, 130.6, 53.6, 44.2, 36.9, 29.8,
699 22.8. GC-MS t_R: 10.3 min. EI-MS (70 eV): *m/z* (%) 138 (69), 123 (68), 96 (99), 95 (84),
700 81 (100).

701 Purity of the synthesized compounds was also determined using the following GC-MS
702 program: injection, 1 µl splitless; flow, 2 ml min^{-1}; temperature, 45 °C (held for 2 min) to
703 250 °C with 6 °C min^{-1}.

704 **1.7 Electrophysiology**

705 Laboratory reared adult beetles from the same German culture that were used in
706 bioassays were used for electrophysiological single sensillum recordings (SSR) using
707 tungsten microelectrodes according to established methodology [40,41], using the SSR

set-up (Syntech, Kirchzarten, Germany) and odor delivery system previously described [103]. The odor panel comprising 92 compounds consisted of beetle pheromones, host tree, non-host tree and fungal compounds ([40], Table S2). Both major and minor fungal volatiles identified during the chemical analysis were included in the odor panel. All odors were dissolved in odorless paraffin oil (w/v). SSR traces were analyzed as described [97] using Autospike 3.0 (Syntech). Males and females were initially screened for responses to the odor panel using a high stimulus dose (10 µg on filter paper placed inside capped standard Pasteur pipette odor cartridges [103]. OSN classes shown to primarily respond to fungus-derived oxygenated monoterpenes were subsequently studied in dose-response experiments with active stimuli diluted in ten-fold steps and tested from lowest to highest dose with the least active ligands tested first at each dose. To reduce variation due to odor depletion, stimulus cartridges were used for a maximum of 8 stimulations during screening and 2 stimulations during dose-response tests [104].

1.8 Trap bioassay

The trap bioassay used in this study has been described previously [40]. The setup was designed so that adult beetles had to make their choice through olfaction and not by contact cues. Fungi were inoculated on spruce bark agar-based diet and incubated at 25˚C for 4 days. With the help of a cork borer (10 mm diameter), bark plugs with or without fungus were inserted into circular cups (1.8 cm height * 1.8 cm diameter) facing each other. Two beetles were placed inside each arena and the olfactometer was placed inside a laminar flow cabinet in darkness. Each experiment was replicated at least 25 times with 2 beetles per replicate. The choice of beetles was determined periodically for up to six hours by counting the number of beetles trapped inside the

cups and represented as percentage choice (percentage of insects responding to either control traps or treatment traps or no response). Preliminary experiments showed that the sex of the beetle did not influence the olfactory response towards fungus grown either alone or in the diet enriched with monoterpenes. Therefore, two beetles were randomly chosen for trap bioassays.

For bioassays using terpenes, stock solutions were prepared by dissolving the compounds in DMSO, which were then added to 7% spruce bark agar to a final concentration of 0.05 to 1 mg g^{-1}. To determine the response of adult beetles to (-)-β-pinene and (-)-bornyl acetate amended diet containing the fungus, *G. penicillata* was used as this species emitted higher amounts of biotransformation products compared to other fungi. Controls were treated with DMSO plus monoterpene (no fungus) or DMSO plus *G. penicillata* (no monoterpene). 7% spruce bark agar plugs (10 mm) supplemented with monoterpenes or plugs containing *G. penicillata* were placed in the control cups, and *G. penicillata* colonized plugs from monoterpene-enriched medium were placed in the treatment cups. The volatile emission from each control and treatment plug used in the bioassays was determined using PDMS tubes as adsorbents and analyzed as described previously (section 1.5). For bioassays with synthetic compounds, stock solutions of authentic standards were prepared by dissolving them in mineral oil (w/v) and further diluted in $\log_{10}$ steps by dissolving in mineral oil. 10 µL was applied to 10 mm Whatmann filter paper laid on the top of spruce bark agar plugs placed inside the cups. Control traps were treated with 10 µL paraffin oil. For the experiment with pheromone blend in the presence of *G. penicillata* volatiles, *G. penicillata* colonized spruce bark plugs were placed in treatment cups and 10 µL of a

pheromone mixture (*cis*-verbenol:2-methyl-3-buten-2-ol in the ratio of 1:50 diluted 1:100 in paraffin oil) was applied to filter paper as described above. Control cups were treated with 10 µL of the pheromone mixture.

1.10 Tunneling behavior bioassay

Bark beetle tunneling behavior was assayed in 35 ×10 mm Petri dishes (Greiner Bio-one, Frickenhausen, Germany) filled with ca. 3 ml of spruce bark diet. The spruce bark diet was prepared as before with some modifications: 7% (w/v) spruce inner bark powder was mixed with 1% fibrous cellulose (Sigma), 2% glucose (Roth), and 4% Bactoagar (Roth) in water and autoclaved for 20 minutes at 121 ºC. Before pouring the medium into the Petri dishes, the medium was mixed with 2% solvent (DMSO: ethanol, 1:1) with 1 mg g^{-1} of various monoterpenes ((-)-α-pinene, (-)-β-pinene and (-)-bornyl acetate) and solvent only as a control. For treatment with fungus, 5 µl spore suspension of *G. penicillata* (1 x 10^6 cells mL^{-1}) was added to the center of Petri dishes containing monoterpene-enriched media or solvent controls and incubated at 25°C for 4 days. A single beetle was introduced per plate, and the plates were sealed with Parafilm and kept in the environmental chamber for 48 h under conditions described above (section 1.2). The beetles were monitored for their tunneling activity after 2, 4, 6, 24 and 48 h with tunneling recorded as a binary event. If beetles were inside the media, it was noted as 1 and if outside, noted as 0. After 48 h, tunnel lengths made by beetles in each plate were measured using Image J software. Each treatment was replicated with 15 male and female beetles.

1.11 Data analysis

IBM SPSS Statistics V25.0 was used to analyze the volatile differences between treatments (*E. polonica*-, *G. penicillata*-, *G. europhioides*-, *O. bicolor*-, and *O. piceae* treated bark samples and untreated control). Data were log-transformed to meet the assumptions of normal distribution, as needed. All individual compounds assigned to monoterpenes (MTs) or oxygenated monoterpenes were combined and their concentrations (in dry weight (ng h^{-1} mg^{-1})) were subjected to a t-test for estimating differences between control and *G. penicillata* (Fig. 2). Additionally, separate ANOVAs for all individual compounds in each group were also performed (Table S2). For volatile time course samples, a separate ANOVA test was performed for all individual compounds and compound groups from each fungus with time intervals as an independent factor (Table S3-7). All ANOVA tests were followed by Tukey's *post-hoc* tests to test for differences among treatment combinations. For behavioral bioassays, the CI values from each experimental group were analyzed by Wilcoxon's singed ranked test to compare the differences between control and treatment samples. Binary data from bark beetle tunneling assays were subjected to multiple logistic regression to analyze independent variables such as monoterpene, sex, and fungus that influence the tunneling activity of beetles (dependent variable) in the medium. During data analysis, the male was coded as 1 and female as 0, the presence of fungus coded as 1 and absence of fungus as 0, tunneling inside the medium coded as 1 and not tunneling or staying outside the medium as 0. After testing all possible independent variables and their interactions among them, the following best-fitted logistic regression model was created to predict the odds of beetles tunneling in the different media.

799 Ln [odds] (tunneling odds) = β0 + β1*compound+ β2*sex + β3*fungus

800 Here, β0 is constant whereas β1, β2, and β3 are logistic coefficients or estimates for the
801 parameters for compound, sex and fungus respectively. The strength of association
802 between beetle tunneling odds and effect of monoterpenes or sex or fungus is
803 expressed as odds ratios ($OR=exp^{\beta \cdot}$) where OR<1 indicates a negative relationship
804 between the two events, i.e., the tunneling event is less likely to happen in response to
805 a selected independent variable (coded as 1) in comparison with its base group (coded
806 as 0), OR=1 indicates no relationship between two events, OR>1 shows positive
807 relationship between two events.

808 ## Acknowledgements

809 We thank Bettina Raguschke, Emily Puckett and Daniel Veit for their assistance in the
810 laboratory. We are grateful for the support of Stefan Engeter from ThüringenForst for
811 permitting us to harvest spruce trees for the purpose of maintaining beetle culture in
812 laboratory. We also thank Jothi Kumar Yuvaraj for his assistance in single sensillum
813 recordings, and Christian Schiebe, Rikard Unelius, and Fredrik Schlyter for providing
814 some of the stimuli used in SSR.

815 ## Funding Disclosure

816 Financial support was provided by the Max Planck society to DK, RZ, YN, HH, AH and
817 JG. MNA acknowledges funding from the Swedish Research Council FORMAS (grants
818 #217-2014-689 and #2018-01444) the Crafoord foundation, and the Royal

Physiographic Society in Lund. The funders had no role in study design, data collection and analysis, decision to publish and writing.

Competing Interests

I have read the journal's policy and I declare that the authors of this manuscript have no competing interests.

Author Contributions

Conceptualization: Dineshkumar Kandasamy, Martin N Andersson, Almuth Hammerbacher, Jonathan Gershenzon

Data Curation: Dineshkumar Kandasamy

Formal Analysis: Dineshkumar Kandasamy, Rashaduz Zaman

Funding Acquisition: Jonathan Gershenzon, Martin N Andersson

Investigation: Dineshkumar Kandasamy, Rashaduz Zaman, Yoko Nakamura, Tao Zhao

Methodology: Dineshkumar Kandasamy, Martin N Andersson

Project Administration: Dineshkumar Kandasamy, Martin N Andersson, Almuth Hammerbacher, Jonathan Gershenzon

Resources: Henrik Hartmann, Martin N Andersson, Jonathan Gershenzon

Supervision: Dineshkumar Kandasamy, Martin N Andersson, Almuth Hammerbacher, Jonathan Gershenzon

Validation: Dineshkumar Kandasamy

Visualization: Dineshkumar Kandasamy

Writing – Original Draft Preparation: Dineshkumar Kandasamy

Writing – Review & Editing:, Jonathan Gershenzon, Almuth Hammerbacher, Martin N Andersson, Henrik Hartmann

References

1. Turlings TCJ, Erb M. Tritrophic interactions mediated by herbivore-induced plant volatiles: mechanisms, ecological relevance, and application potential. Annual Review of Entomology. 2018;63: 433–452. doi:10.1146/annurev-ento-020117-043507

2. Paré PW, Tumlinson JH. Plant volatiles as a defense against insect herbivores. Plant Physiology. 1999;121: 325–331. doi:10.1104/pp.121.2.325

3. Bruce TJA, Wadhams LJ, Woodcock CM. Insect host location: A volatile situation. Trends in Plant Science. 2005;10: 269–274. doi:10.1016/j.tplants.2005.04.003

4. Gershenzon J, Dudareva N. The function of terpene natural products in the natural world. Nature Chemical Biology. 2007;3: 408–414. doi:10.1038/nchembio.2007.5

5. Becher PG, Flick G, Rozpedowska E, Schmidt A, Hagman A, Lebreton S, et al. Yeast, not fruit volatiles mediate *Drosophila melanogaster* attraction, oviposition and development. Thompson K, editor. Functional Ecology. 2012;26: 822–828. doi:10.1111/j.1365-2435.2012.02006.x

6. Hulcr J, Mann R, Stelinski LL. The scent of a partner: ambrosia beetles are attracted to volatiles from their fungal symbionts. Journal of Chemical Ecology. 2011;37: 1374–1377. doi:10.1007/s10886-011-0046-x

7. Buser CC, Newcomb RD, Gaskett AC, Goddard MR. Niche construction initiates the evolution of mutualistic interactions. Bonsall M, editor. Ecology Letters. 2014;17: 1257–1264. doi:10.1111/ele.12331

8. Biedermann PHW, Kaltenpoth M. New Synthesis: The chemistry of partner choice in insect-microbe mutualisms. Journal of Chemical Ecology. 5 Feb 201440: 99. doi:10.1007/s10886-014-0382-8

9. Archetti M, Scheuring I, Hoffman M, Frederickson ME, Pierce NE, Yu DW. Economic game theory for mutualism and cooperation. Ecology Letters. Wiley/Blackwell (10.1111); 2011. 1300–1312. doi:10.1111/j.1461-0248.2011.01697.x

10. Six DL. Niche construction theory can link bark beetle-fungus symbiosis type and colonization behavior to large scale causal chain-effects. Current Opinion in Insect Science. 2020;39: 27–34. doi:10.1016/j.cois.2019.12.005

11. Dweck HKM, Ebrahim SAM, Farhan A, Hansson BS, Stensmyr MC. Olfactory proxy detection of dietary antioxidants in *Drosophil*a. Current Biology. 2015;25: 455–466. doi:10.1016/j.cub.2014.11.062

12. Wermelinger B. Ecology and management of the spruce bark beetle *Ips typographus*—a review of recent research. Forest Ecology and Management. 2004;202: 67–82. doi:10.1016/j.foreco.2004.07.018

13. Raffa KF, Aukema BH, Bentz BJ, Carroll AL, Hicke JA, Turner MG, et al. Cross-scale drivers of natural disturbances prone to anthropogenic amplification: the dynamics of bark beetle eruptions. BioScience. 2008;58: 501–517. doi:10.1641/B580607

14. Huang J, Kautz M, Trowbridge AM, Hammerbacher A, Raffa KF, Adams HD, et al. Tree defence and bark beetles in a drying world: carbon partitioning, functioning and modelling. New Phytologist. 2020;225: 26–36. doi:10.1111/nph.16173

15. Biedermann PHW, Müller J, Grégoire JC, Gruppe A, Hagge J, Hammerbacher A, et al. Bark beetle population dynamics in the anthropocene: challenges and solutions. Trends in Ecology and Evolution. Elsevier Current Trends; 2019. 914–924. doi:10.1016/j.tree.2019.06.002

16. Netherer S, Kandasamy D, Jirosová A, Kalinová B, Schebeck M, Schlyter F. Interactions among Norway spruce, the bark beetle *Ips typographus* and its fungal symbionts in times of drought. Journal of Pest Science. 2021. doi:10.1007/s10340-021-01341-y

17. Raffa KF, Grégoire JC, Lindgren BS. Natural history and ecology of bark beetles. bark beetles: biology and ecology of native and invasive species. Academic Press; 2015. 1–40. doi:10.1016/B978-0-12-417156-5.00001-0

18. Kirisits T. Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi. Bark and wood boring insects in living trees in Europe, a synthesis. Springer; 2007. 181–236.

19. Linnakoski R, Wilhelm de Beer ZB, Niemelä P, Wingfield MJ. Associations of conifer-infesting bark beetles and fungi in Fennoscandia. Insects. 2012. 200–227. doi:10.3390/insects3010200

20. Six DL, Wingfield MJ. The role of phytopathogenicity in bark beetle–fungus symbioses: A challenge to the classic paradigm. Annual Review of Entomology. 2011;56: 255–272. doi:10.1146/annurev-ento-120709-144839

21. Six DL. Ecological and evolutionary determinants of bark beetle - Fungus symbioses. Insects. 2012. doi:10.3390/insects3010339

22. Six DL. The bark beetle holobiont: Why microbes matter. Journal of Chemical Ecology. 2013;39: 989–1002. doi:10.1007/s10886-013-0318-8

23. Wadke N, Kandasamy D, Vogel H, Lah L, Wingfield BD, Paetz C, et al. Catechol dioxygenases catalyzing the first step in Norway spruce phenolic degradation are key virulence factors in the bark beetle-vectored fungus *Endoconidiophora polonica*. Plant Physiology. 2016;171: pp.01916.2015. doi:10.1104/pp.15.01916

24. Boone CK, Keefover-Ring K, Mapes AC, Adams AS, Bohlmann J, Raffa KF. Bacteria associated with a tree-killing insect reduce concentrations of plant defense compounds. Journal of Chemical Ecology. 2013;39: 1003–1006. doi:10.1007/s10886-013-0313-0

25. Six DL, Elser JJ. Extreme ecological stoichiometry of a bark beetle–fungus mutualism. Ecological Entomology. 2019;44: 543–551. doi:10.1111/een.12731

26. François L, Annie Y, Aurélien S. Stimulation of defenses by Ophiostomatoid fungi can explain attack success of bark beetles on conifers. Annals of Forest Science. 2009;66: 1–22. doi:10.1051/forest/2009066

27. Krokene P. Conifer defense and resistance to bark beetles. Bark beetles. Elsevier; 2015. 177–207. doi:10.1016/B978-0-12-417156-5.00005-8

28. Raffa KF, Berryman AA. The role of host plant resistance in the colonization behavior and ecology of bark beetles (Coleoptera: Scolytidae). Ecological Monographs. 1983;53: 27–49. doi:10.2307/1942586

29. Phillips MA, Croteau RB. Resin-based defenses in conifers. Trends in Plant Science. 1999;4: 184–190. doi:10.1016/S1360-1385(99)01401-6

30. Klepzig KD, Smalley EB, Raffa KF. Combined chemical defenses against an insect-fungal complex. Journal of Chemical Ecology. 1996;22: 1367–1388. doi:10.1007/BF02027719

935 31. Raffa KF. Terpenes tell different tales at different scales: Glimpses into the
936 chemical ecology of conifer - bark beetle - microbial interactions. Journal of
937 Chemical Ecology. 2014;40: 1–20. doi:10.1007/s10886-013-0368-y

938 32. Raffa KF, Andersson MN, Schlyter F. Host selection by bark beetles: Playing the
939 odds in a high-stakes game. Advances in Insect Physiology. 2016. 1–74.
940 doi:10.1016/bs.aiip.2016.02.001

941 33. Klimetzek D, Francke W. Relationship between the enantiomeric composition of
942 α-pinene in host trees and the production of verbenols in *Ips* species. Experientia.
943 1980;36: 1343–1345. doi:10.1007/BF01960087

944 34. Lanne BS, Ivarsson P, Johnsson P, Bergström G, Wassgren AB. Biosynthesis of
945 2-methyl-3-buten-2-ol, a pheromone component of *Ips typographus* (Coleoptera:
946 Scolytidae). Insect Biochemistry. 1989;19: 163–167. doi:10.1016/0020-
947 1790(89)90087-5

948 35. Bakke A, Frøyen P, Skattebøl L. Field response to a new pheromonal compound
949 isolated from *Ips typographus*. Naturwissenschaften. 1977;64: 98–99.
950 doi:10.1007/BF00437364

951 36. Leufvén A, Birgersson G. Quantitative variation of different monoterpenes around
952 galleries of *Ips typographus* (Colleoptera: Scolytidae) attacking Norway spruce.
953 Canadian Journal of Botany. 1987;65: 1038–1044. doi:10.1139/b87-144

954 37. Birgersson G, Leufvén A. The influence of host tree response to *Ips typographus*
955 and fungal attack on production of semiochemicals. Insect Biochemistry. 1988;18:
956 761–770. doi:10.1016/0020-1790(88)90098-4

957 38. Birgersson G, Bergström G. Volatiles released from individual spruce bark beetle
958 entrance holes: Quantitative variations during the first week of attack. Journal of
959 Chemical Ecology. 1989;15: 2465–2483. doi:10.1007/BF01020377

960 39. Pettersson EM, Boland W. Potential parasitoid attractants, volatile composition
961 throughout a bark beetle attack. Chemoecology. 2003;13: 27–37.
962 doi:10.1007/s000490300003

963 40. Kandasamy D, Gershenzon J, Andersson MN, Hammerbacher A. Volatile organic
964 compounds influence the interaction of the Eurasian spruce bark beetle (*Ips*
965 *typographus*) with its fungal symbionts. ISME Journal. 2019;13: 1788–1800.
966 doi:10.1038/s41396-019-0390-3

967 41. Andersson MN, Larsson MC, Schlyter F. Specificity and redundancy in the
968 olfactory system of the bark beetle *Ips typographus*: Single-cell responses to
969 ecologically relevant odors. Journal of Insect Physiology. 2009;55: 556–567.
970 doi:10.1016/j.jinsphys.2009.01.018

42. Schlyter F, Byers JA, Löfqvist J. Attraction to pheromone sources of different quantity, quality, and spacing: Density-regulation mechanisms in bark beetle *Ips typographus*. Journal of Chemical Ecology. 1987;13: 1503–1523. doi:10.1007/BF01012294

43. Schlyter F, Löfqvist J. Response of walking spruce bark beetles *Ips typographus* to pheromone produced in different attack phases. Entomologia Experimentalis et Applicata. 1986;41: 219–230. doi:10.1111/j.1570-7458.1986.tb00532.x

44. Zhao T, Kandasamy D, Krokene P, Chen J, Gershenzon J, Hammerbacher A. Fungal associates of the tree-killing bark beetle, *Ips typographus*, vary in virulence, ability to degrade conifer phenolics and influence bark beetle tunneling behavior. Fungal Ecology. 2018; 1–9. doi:10.1016/j.funeco.2018.06.003

45. Tanin SM, Kandasamy D, Krokene P. Fungal interactions and host tree preferences in the spruce bark beetle *Ips typographus*. Frontiers in Microbiology. 2021;12: 695167. doi:10.3389/fmicb.2021.695167

46. Byers JA. Behavioral mechanisms involved in reducing competition in bark beetles. Ecography. 1989;12: 466–476. doi:10.1111/j.1600-0587.1989.tb00924.x

47. Fernández Ajó AA, Martínez AS, Villacide JM, Corley JC. Behavioural response of the woodwasp *Sirex noctilio* to volatile emissions of its fungal symbiont. Journal of Applied Entomology. 2015;139: 654–659. doi:10.1111/jen.12211

48. Francke W, P. Vité J. Oxygenated terpenes in pheromone systems of bark beetles. Zeitschrift für Angewandte Entomologie. 1983;96: 146–156. doi:10.1111/j.1439-0418.1983.tb03655.x

49. Schlyter F, Birgersson G, Byers JA, Löfqvist J, Bergström G. Field response of spruce bark beetle, *Ips typographus*, to aggregation pheromone candidates. Journal of Chemical Ecology. 1987;13: 701–716. doi:10.1007/BF01020153

50. Leufvén A, Bergström G, Falsen E. Interconversion of verbenols and verbenone by identified yeasts isolated from the spruce bark beetle *Ips typographus*. Journal of Chemical Ecology. 1984;10: 1349–1361. doi:10.1007/BF00988116

51. Cale JA, Ding R, Wang F, Rajabzadeh R, Erbilgin N. Ophiostomatoid fungi can emit the bark beetle pheromone verbenone and other semiochemicals in media amended with various pine chemicals and beetle-released compounds. Fungal Ecology. 2019;39: 285–295. doi:10.1016/j.funeco.2019.01.003

52. Schlyter F, Birgersson G, Leufvén A. Inhibition of attraction to aggregation pheromone by verbenone and ipsenol - Density regulation mechanisms in bark beetle *Ips typographus*. Journal of Chemical Ecology. 1989;15: 2263–2277. doi:10.1007/BF01014114

1007 53. Schiebe C, Hammerbacher A, Birgersson G, Witzell J, Brodelius PE, Gershenzon
1008 J, et al. Inducibility of chemical defenses in Norway spruce bark is correlated with
1009 unsuccessful mass attacks by the spruce bark beetle. Oecologia. 2012;170: 183–
1010 198. doi:10.1007/s00442-012-2298-8

1011 54. Andersson MN, Larsson MC, Blazenec M, Jakus R, Zhang Q-H, Schlyter F.
1012 Peripheral modulation of pheromone response by inhibitory host compound in a
1013 beetle. Journal of Experimental Biology. 2010;213: 3332–3339.
1014 doi:10.1242/jeb.044396

1015 55. Binyameen M, Jankuvová J, Blaženec M, Jakuš R, Song L, Schlyter F, et al. Co-
1016 localization of insect olfactory sensory cells improves the discrimination of closely
1017 separated odour sources. Fox C, editor. Functional Ecology. 2014;28: 1216–
1018 1223. doi:10.1111/1365-2435.12252

1019 56. Pettersson EM. Volatile attractants for three Pteromalid parasitoids attacking
1020 concealed spruce bark beetles. Chemoecology. 2001;11: 89–95.
1021 doi:10.1007/PL00001837

1022 57. Toffin E, Gabriel E, Louis M, Deneubourg JL, Grégoire JC. Colonization of
1023 weakened trees by mass-attacking bark beetles: No penalty for pioneers,
1024 scattered initial distributions and final regular patterns. Royal Society Open
1025 Science. 2018;5. doi:10.1098/rsos.170454

1026 58. Paynter QE, Anderbrant O, Schlyter F. Behavior of male and female spruce bark
1027 beetles, *Ips typographus*, on the bark of host trees during mass attack. Journal of
1028 Insect Behavior. 1990;3: 529–543. doi:10.1007/BF01052016

1029 59. Erbilgin N, Krokene P, Kvamme T, Christiansen E. A host monoterpene
1030 influences *Ips typographus* (Coleoptera: Curculionidae, Scolytinae) responses to
1031 its aggregation pheromone. Agricultural and Forest Entomology. 2007;9: 135–
1032 140. doi:10.1111/j.1461-9563.2007.00329.x

1033 60. Zhang Q. Interruption of aggregation pheromone in *Ips typographus* (L.)(Col.
1034 Scolytidae) by non□host bark volatiles. Agricultural and Forest Entomology.
1035 2003;5: 145–153.

1036 61. Andersson MN. Mechanisms of odor coding in coniferous bark beetles: From
1037 neuron to behavior and application. Psyche J Entomol. 2012.
1038 doi:10.1155/2012/149572

1039 62. Andersson MN, Löfstedt C, Newcomb RD. Insect olfaction and the evolution of
1040 receptor tuning. Frontiers in Ecology and Evolution. 2015;3: 53.
1041 doi:10.3389/fevo.2015.00053

1042 63. Byers JA. Chemical ecology of bark beetles. Experientia (Basel). 1989;45: 271–
1043 283.

64. Renwick JA, Hughes PR, Krull IS. Selective production of *cis*- and *trans*-verbenol from (-)-and (+)-alpha pinene by a bark beetle. Science. 1976;191: 199 LP – 201. doi:10.1126/science.1246609

65. Blažytė-Čereškienė L, Apšegaitė V, Radžiutė S, Mozūraitis R, Būda V, Pečiulytė D. Electrophysiological and behavioural responses of *Ips typographus* (L.) to *trans*-4-thujanol—a host tree volatile compound. Annals of Forest Science. 2016;73: 247–256. doi:10.1007/s13595-015-0494-5

66. Kopper BJ, Klepzig KD, Raffa KF. Components of antagonism and mutualism in *Ips pini*–fungal interactions: relationship to a life history of colonizing highly stressed and dead trees. Environmental Entomology. 2004;33: 28–34. doi:10.1603/0046-225X-33.1.28

67. Leufvén A, Bergström G, Falsen E. Oxygenated monoterpenes produced by yeasts, isolated from *Ips typographus* (Coleoptera: Scolytidae) and grown in phloem medium. Journal of Chemical Ecology. 1988;14: 353–362. doi:10.1007/BF01022551

68. Boone CK, Six DL, Zheng Y, Raffa KF. Parasitoids and dipteran predators exploit volatiles from microbial symbionts to locate bark beetles. Environmental Entomology. 2008;37: 150–161. doi:10.1603/0046-225X(2008)37[150:PADPEV]2.0.CO;2

69. Grégoire JC, Couillien D, Krebber R, König WA, Meyer H, Francke W. Orientation of *Rhizophagus grandis* (Coleoptera: Rhizophagidae) to oxygenated monoterpenes in a species-specific predator-prey relationship. Chemoecology. 1992;3: 14–18. doi:10.1007/BF01261451

70. Pettersson EM, Birgersson G, Witzgall P. Synthetic attractants for the bark beetle parasitoid *Coeloides bostrichorum* Giraud (Hymenoptera: Braconidae). Naturwissenschaften. 2001;88: 88–91. doi:10.1007/s001140100209

71. Martínez AS, Fernández-Arhex V, Corley JC. Chemical information from the fungus *Amylostereum areolatum* and host-foraging behaviour in the parasitoid *Ibalia leucospoides*. Physiological Entomology. 2006;31: 336–340. doi:10.1111/j.1365-3032.2006.00523.x

72. Tømmerås BÅ. Specialization of the olfactory receptor cells in the bark beetle *Ips typographus* and its predator T*hanasimus formicarius* to bark beetle pheromones and host tree volatiles. Journal of Comparative Physiology A. 1985;157: 335–341. doi:10.1007/BF00618123

73. Keeling CI, Bohlmann J. Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. New Phytologist. 2006;170: 657–675. doi:10.1111/j.1469-8137.2006.01716.x

74. Pichersky E, Raguso RA. Why do plants produce so many terpenoid compounds? New Phytologist. 2018;220: 692–702. doi:10.1111/nph.14178

75. Raffa KF, Smalley EB. Interaction of pre-attack and induced monoterpene concentrations in host conifer defense against bark beetle-fungal complexes. Oecologia. 1995;102: 285–295. doi:10.1007/BF00329795

76. Franceschi VR, Krokene P, Christiansen E, Krekling T. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. New Phytologist. 2005. 353–376. doi:10.1111/j.1469-8137.2005.01436.x

77. Erbilgin N, Krokene P, Christiansen E, Zeneli G, Gershenzon J. Exogenous application of methyl jasmonate elicits defenses in Norway spruce (*Picea abies*) and reduces host colonization by the bark beetle *Ips typographus*. Oecologia. 2006;148: 426–436. doi:10.1007/s00442-006-0394-3

78. Chiu CC, Keeling CI, Bohlmann J. Toxicity of pine monoterpenes to mountain pine beetle. Scientific Reports. 2017;7. doi:10.1038/s41598-017-08983-y

79. Hammerbacher A, Schmidt A, Wadke N, Wright LP, Schneider B, Bohlmann J, et al. A common fungal associate of the spruce bark beetle metabolizes the stilbene defenses of Norway spruce. Plant Physiology. 2013;162: 1324–1336. doi:10.1104/pp.113.218610

80. Achotegui-Castells A, della Rocca G, Llusià J, Danti R, Barberini S, Bouneb M, et al. Terpene arms race in the *Seiridium cardinale* - *Cupressus sempervirens* pathosystem. Scientific Reports. 2016;6: 1–13. doi:10.1038/srep18954

81. Kusumoto N, Zhao T, Swedjemark G, Ashitani T, Takahashi K, Borg-Karlson AK. Antifungal properties of terpenoids in *Picea abies* against *Heterobasidion parviporum*. Forest Pathology. 2014;44: 353–361. doi:10.1111/efp.12106

82. Wang Y, Lim L, Madilao L, Lah L, Bohlmann J, Breuil C. Gene discovery for enzymes involved in limonene modification or utilization by the mountain pine beetle-associated pathogen *Grosmannia clavigera*. Applied and Environmental Microbiology. 2014;80: 4566–4576. doi:10.1128/AEM.00670-14

83. Lah L, Haridas S, Bohlmann J, Breuil C. The cytochromes P450 of *Grosmannia clavigera*: Genome organization, phylogeny, and expression in response to pine host chemicals. Fungal Genetics and Biology. 2013;50: 72–81. doi:10.1016/j.fgb.2012.10.002

84. Martin DM, Tholl D, Gershenzon J, Bohlmann J. Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of norway spruce stems. Plant Physiology. 2002;129: 1003 – 1018. doi:10.1104/pp.011001

85. Keeling CI, Weisshaar S, Ralph SG, Jancsik S, Hamberger B, Dullat HK, et al. Transcriptome mining, functional characterization, and phylogeny of a large terpene synthase gene family in spruce (*Picea* spp.). BMC Plant Biology. 2011;11. doi:10.1186/1471-2229-11-43

86. Kalinová B, Břízová R, Knížek M, Turčáni M, Hoskovec M. Volatiles from spruce trap-trees detected by *Ips typographus* bark beetles: Chemical and electrophysiological analyses. Arthropod-Plant Interactions. 2014;8: 305–316. doi:10.1007/s11829-014-9310-7

87. Schiebe C, Unelius CR, Ganji S, Binyameen M, Birgersson G, Schlyter F. Styrene, (+)-*trans*-(1R,4S,5S)-4-thujanol and oxygenated monoterpenes related to host stress elicit strong electrophysiological responses in the bark beetle *Ips typographus*. Journal of Chemical Ecology. 2019; 1–16. doi:10.1007/s10886-019-01070-8

88. Hunt DWA, Borden JH, Lindgren BS, Gries G. The role of autoxidation of α-pinene in the production of pheromones of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). Canadian Journal of Forest Research. 1989;19: 1275–1282. doi:10.1139/x89-194

89. Lindmark-Henriksson M, Isaksson D, Sjödin K, Högberg HE, Vaněk T, Valterová I. Transformation of α-pinene using *Picea abies* suspension culture. Journal of Natural Products. 2003;66: 337–343. doi:10.1021/np020426m

90. Lindmark-Henriksson M, Isaksson D, Vaněk T, Valterová I, Högberg HE, Sjödin K. Transformation of terpenes using a *Picea abies* suspension culture. Journal of Biotechnology. 2004;107: 173–184. doi:10.1016/j.jbiotec.2003.10.009

91. Rahmani R, Hedenström E, Schroeder M. SPME collection and GC-MS analysis of volatiles emitted during the attack of male *Polygraphus poligraphus* (Coleoptera, Curcolionidae) on Norway spruce. Zeitschrift fur Naturforschung - Section C Journal of Biosciences. 2015;70: 265–273. doi:10.1515/znc-2015-5035

92. Rahmani R, Wallin EA, Viklund L, Schroeder M, Hedenström E. Identification and field assay of two aggregation pheromone components emitted by males of the bark beetle *Polygraphus punctifrons* (Coleoptera: Curculionidae). Journal of Chemical Ecology. 2019;45: 356–365. doi:10.1007/s10886-019-01056-6

93. Hou X-Q, Kumar Yuvaraj J, Roberts RE, Unelius CR, Löfstedt C, Andersson MN. Functional evolution of a bark beetle odorant receptor clade detecting monoterpenoids of different ecological origins. bioRxiv. 2020; 2020.12.28.424525. doi:10.1101/2020.12.28.424525

94. Birgersson G, Schlyter F, Löfqvist J, Bergström G. Quantitative variation of pheromone components in the spruce bark beetle *Ips typographus* from different attack phases. Journal of Chemical Ecology. 1984;10: 1029–1055. doi:10.1007/BF00987511

95. Zhao T, Ganji S, Schiebe C, Bohman B, Weinstein P, Krokene P, et al. Convergent evolution of semiochemicals across Kingdoms: bark beetles and their fungal symbionts. ISME Journal. 2019;13: 1535–1545. doi:10.1038/s41396-019-0370-7

96. Davis TS, Crippen TL, Hofstetter RW, Tomberlin JK. Microbial volatile emissions as insect semiochemicals. Journal of Chemical Ecology. 2013;39: 840–859. doi:10.1007/s10886-013-0306-z

97. Kandasamy D, Gershenzon J, Hammerbacher A. Volatile organic compounds emitted by fungal associates of conifer bark beetles and their potential in bark beetle control. Journal of Chemical Ecology. 2016;42: 952–969. doi:10.1007/s10886-016-0768-x

98. Beck JJ, Vannette RL. Harnessing insect-microbe chemical communications to control insect pests of agricultural systems. Journal of Agricultural and Food Chemistry. 2017;65: 23–28. doi:10.1021/acs.jafc.6b04298

99. Schlyter F, Cederholm I. Separation of the sexes of living spruce bark beetles, *Ips typographus* (L.), (Coleoptera: Scolytidae). Zeitschrift für Angewandte Entomologie. 1981;92: 42–47. doi:10.1111/j.1439-0418.1981.tb01650.x

100. Kallenbach M, Oh Y, Eilers EJ, Veit D, Baldwin IT, Schuman MC. A robust, simple, high-throughput technique for time-resolved plant volatile analysis in field experiments. Plant Journal. 2014;78: 1060–1072. doi:10.1111/tpj.12523

101. Chong J, Wishart DS, Xia J. Using MetaboAnalyst 4.0 for Comprehensive and integrative metabolomics data analysis. Current Protocols in Bioinformatics. 2019;68. doi:10.1002/cpbi.86

102. Babler JH, Malek NC, Coghlan MJ. Selective hydrolysis of α,β-and β,γ-unsaturated ketals: A method for deconjugation of β,β-disubstituted α,β-unsaturated ketones. Journal of Organic Chemistry. 1978;43: 1821–1823. doi:10.1021/jo00403a047

103. Andersson MN, Schlyter F, Hill SR, Dekker T. What reaches the antenna? How to calibrate odor flux and ligand-receptor affinities. Chemical Senses. 2012;37: 403–420. doi:10.1093/chemse/bjs009

104. Andersson MN, Larsson MC, Svensson GP, Birgersson G, Rundlöf M, Lundin O, et al. Characterization of olfactory sensory neurons in the white clover seed weevil, *Apion fulvipes* (Coleoptera: Apionidae). Journal of Insect Physiology. 2012;58: 1325–1333. doi:10.1016/j.jinsphys.2012.07.006

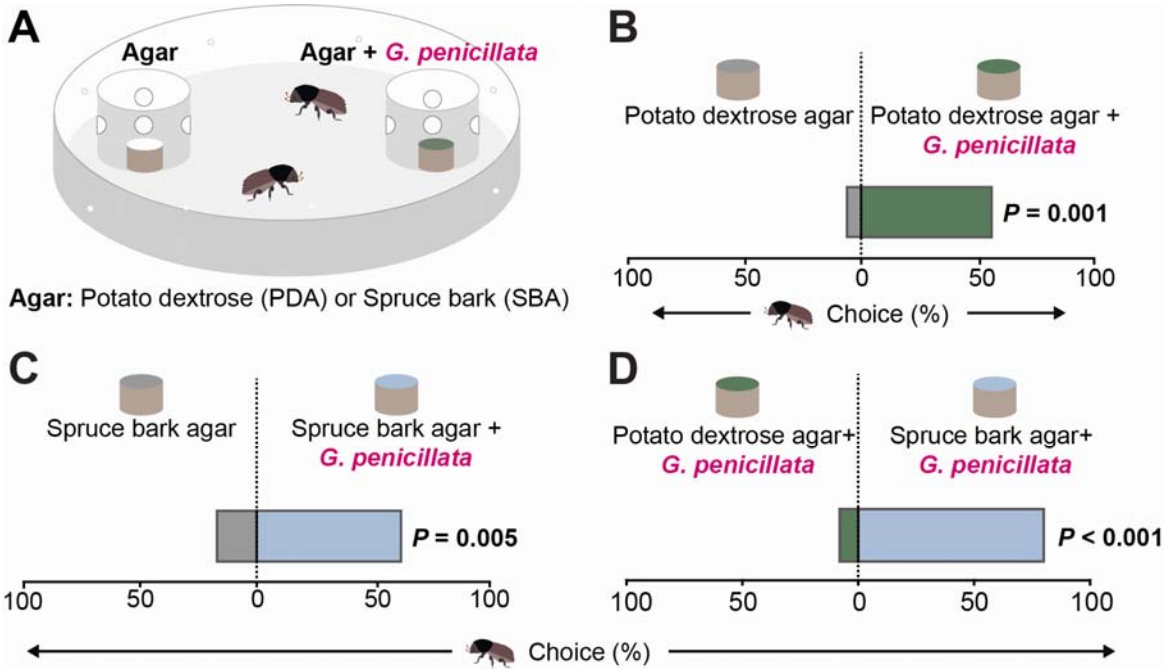

Figure 1: ***Ips typographus* uses volatile cues from spruce bark inoculated with *G. penicillata (Gp)* to detect this symbiotic fungus.**

(A) Arena used for trap bioassays to study the behavior of bark beetles to volatiles emitted by a symbiotic fungus. Cups containing agar (with and without fungus) were offered to beetles as odor samples. Holes on each side of the cup allowed the beetles to smell, but not touch the agar until they entered the cup and then could no longer escape. **(B), (C)** Adult beetles chose *Gp*-colonized agar medium over fungus-free medium (n = 25, with two beetles per replicate). **(D)** Adult beetles chose *Gp*-inoculated spruce bark agar over *Gp*-inoculated agar without spruce bark (n = 25). **(B), (C), (D)** Deviation of response indices against zero was tested using Wilcoxon's test. Asterisks denotes significant differences, *P < 0.01, ***P < 0.001.

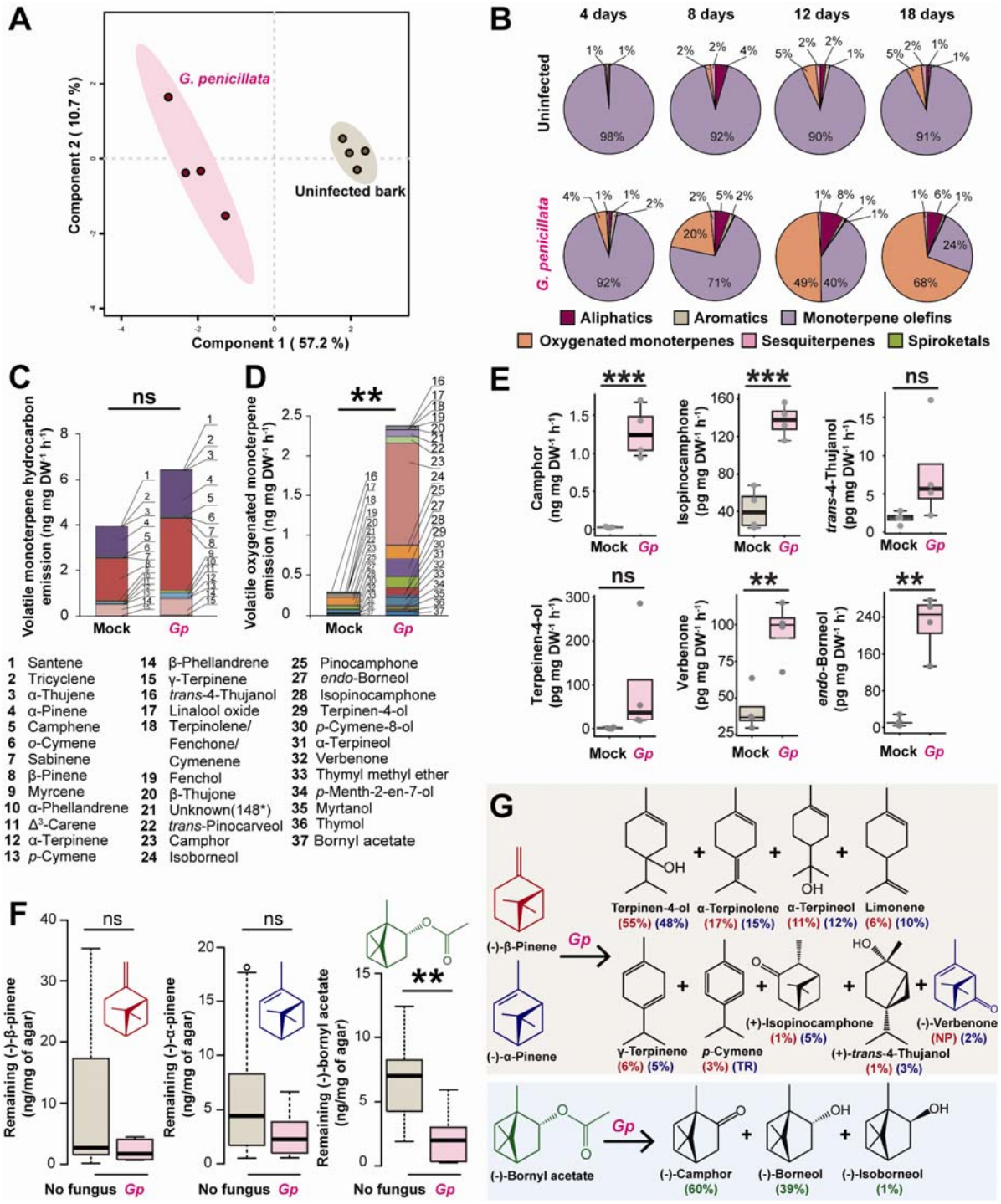

Figure 2: **Growth of *Ips typographus* symbiotic fungi on spruce bark induces increased emission of oxygenated monoterpenes.**

(A) Volatile emission pattern differed between spruce bark inoculated with *G. penicillata* (*Gp*) and uninfected bark 4 days after inoculation, as depicted in a partial least squares discriminant analysis (PLS-DA). Principal components (PC1 and PC2) explain 57.2% and 10.7% of total variation, respectively, and ellipses denote 95% confidence intervals around

each treatment. Complete volatile emission data by compound for *G. penicillata* and other *I. typographus* fungal symbionts are given in Table S2. **(B)** Changes in volatile emission profiles of spruce bark due to *G. penicillata* infection over an 18 d time course. Compounds are classified into six groups by their chemical structures (*n* = 5). Complete volatile emission data by compound and time point for *G. penicillata* and other symbionts are given in Tables S3-S6. (**C)**, (**D**) Emission of specific monoterpenes from fresh spruce bark inoculated with *G. penicillata* at four days post inoculation. Identified compounds were classified into monoterpene hydrocarbons **(C)** and oxygenated monoterpenes **(D).** The individual compounds are stacked within a single bar representing the total emission. Significant differences in the total emission levels induced by *G. penicillata* are denoted by asterisks above the bars (*n* = 4, Welch's t-test with ns= not significant *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The numbers denote the identities of the compounds in the stacked bars. Complete volatile emission data are given in Table S2**. (E)** Emission rate of major oxygenated monoterpenes from spruce bark inoculated with *G. penicillata* four days post inoculation. Asterisks indicate significant differences between the spruce bark-inoculated *G. penicillata* and the fungus-free control (Welch's t-test). **(F-G)** Metabolism of major spruce monoterpenes by *G. penicillata* after fungal-infected vs. uninfected PDA was supplemented with 0.5 mg/g of (-)-β-pinene, (-)-α-pinene and (-)-bornyl acetate. **(F)** The amounts of starting monoterpenes remaining after four days. Error bars represent SEM (n = 5). Asterisks indicate significant difference between the PDA-inoculated *G. penicillata* and the fungus-free control (Welch's t-test) with ns = not significant, **$P < 0.01$. **(G)** The most abundant metabolites of administered monoterpenes are depicted with their percentages relative to the total amounts of metabolites detected for each compound (derived from (-)-β-pinene in red; derived from (-)-α-pinene in purple; derived from (-)-bornyl acetate in green) (*n* = 4). Amounts were determined by headspace collection of volatiles from the agar (*n* = 3).

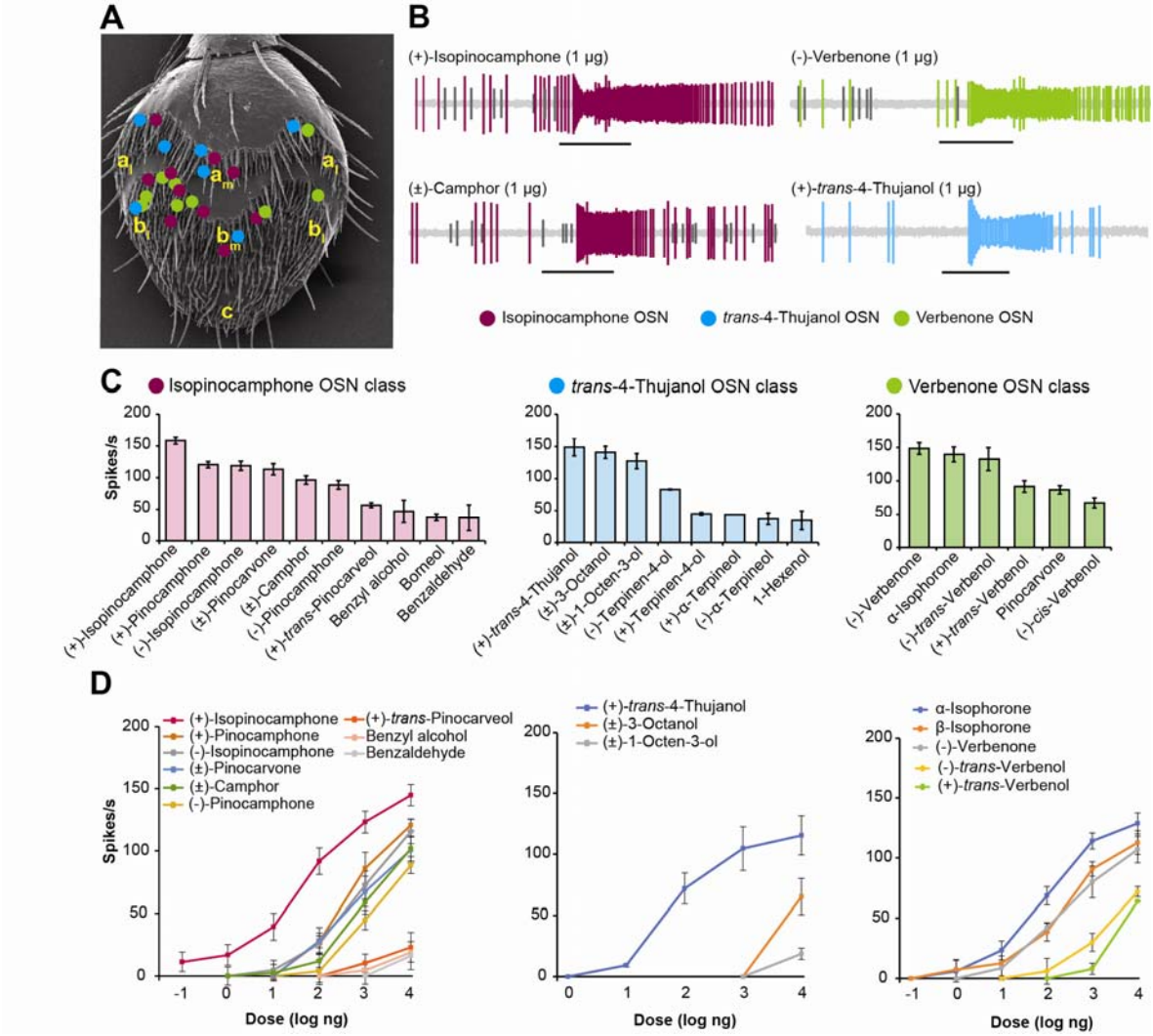

Figure 3: **Oxygenated monoterpenes derived from fungal metabolism of host tree monoterpene hydrocarbons are detected by specialized olfactory neurons in the *I. typographus* antenna**

(A) Mapping of three classes of olfactory sensory neurons (OSN) selective for oxygenated monoterpenes (isopinocamphone; verbenone; (-)-*trans*-4-thujanol) on the antenna. Olfactory sensilla housing these OSN classes are distributed in medial (subscript "m") and lateral (subscript "l") regions of sensillum areas a and b (but not in area c) of the antenna. **(B)** Representative spike trains from an isopinocamphone-responsive neuron stimulated with 1 µg of (+)-isopinocamphone (top left) and (±)-camphor (bottom left); a verbenone-responsive neuron stimulated with 1 µg (-)-verbenone (top right); a (+)-*trans*-4-thujanol-responsive neuron stimulated with 1 µg (+)-*trans*-4-thujanol (bottom right). Black horizontal bars indicate the 0.5 s odor puffs. **(C)** Response spectra of OSN classes responding predominantly to oxygenated monoterpenes produced by fungi at the 10 µg screening dose. The average number of spikes/second was recorded from the isopinocamphone-tuned OSN class (left) (n = 6 except (+) and (-)-pinocamphone (n = 3)), *trans*-4-thujanol-tuned OSN class (middle) (n = 5) and verbenone and isophorone-tuned OSN class (right) (n = 4) after neurons were stimulated with a panel of 97 odors. Error bars represent SEM. **(D)** Dose-response curves of the OSNs stimulated with their most active ligands: isopinocamphone-tuned OSN class (left)

((*n* = 9) except for (+) and (-)-pinocamphone (*n* = 3)), *trans*-4-thujanol-tuned OSN class (middle) (*n* = 3), and the OSN class tuned to isophorone and verbenone (right) (*n* = 3). Error bars represent SEM.

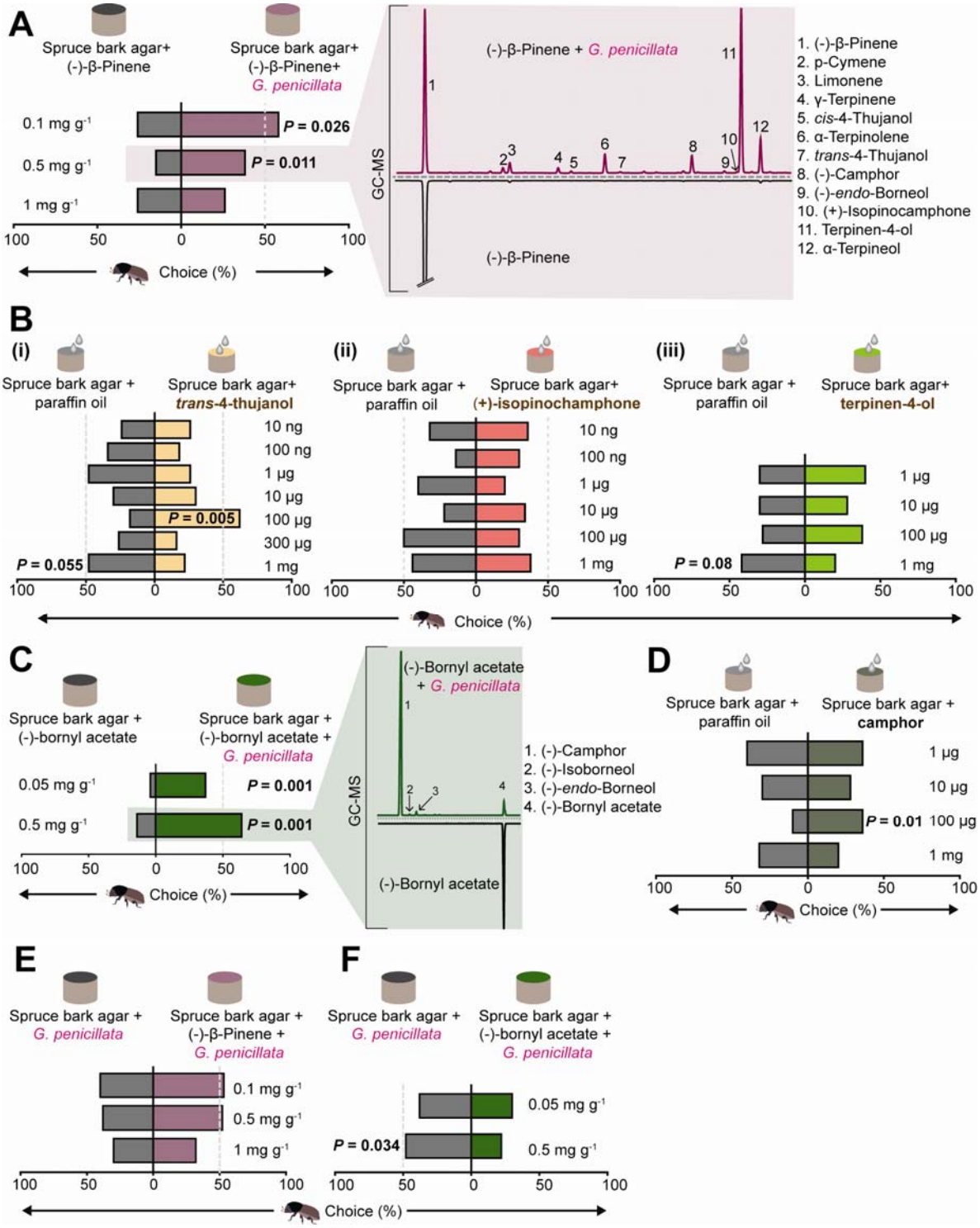

Figure 4: **Bark beetles are attracted to oxygenated monoterpenes produced by symbiotic fungi.**

(A) Adult beetles preferred spruce bark agar enriched with 0.1 mg/g, and 0.5 mg/g of (-)-β-pinene inoculated with *G. penicillata* for 4 days over spruce bark agar enriched with 0.1 mg/g, and 0.5 mg/g (-)-β-pinene but without fungus (left). GC-MS traces of the headspace volatiles of (-)-β-pinene-enriched agar with and without *G. penicillata* for 4 days (right) showing the oxygenated monoterpenes produced by the fungus from (-)-β-

pinene. Numbers refer to the identities of compounds. **(B)** Adult beetles chose *trans*-4-thujanol (left) at a 100 µg dose but avoided *trans*-4-thujanol (left) at a 1 mg dose diluted in mineral oil, when tested against a mineral oil control. Adult beetles showed indifferent responses to (+)-isopinocamphone (middle), and terpinen-4-ol (right), applied in various doses in mineral oil. **(C)** Adult beetles preferred spruce bark agar enriched with various amounts of (-)-bornyl acetate inoculated with *G. penicillata* for 4 days over spruce bark agar enriched with (-)-bornyl acetate but without fungus (left). GC-MS traces of the headspace volatiles of 0.5 mg/g (-)-bornyl acetate-enriched agar with and without *G. penicillata* (right) showing the oxygenated monoterpenes produced by the fungus from (-)-bornyl acetate. Numbers refer to the identities of compounds. **(D)** Adult beetles preferred (±)-camphor at a 100 µg dose against a mineral oil control, but not at other doses. **(E)** Adult beetles did not discriminate between *G. penicillata* on agar with three different concentrations of (-)-β-pinene and *G. penicillata* without (-)-β-pinene. **(F)** Adult beetles chose *G. penicillata* on agar without any administrated (-)-bornyl acetate vs. *G. penicillata* on agar with 0.5 mg/g (-)-bornyl acetate. **(A), (B), (C), (D), (E), (F)** Deviation of response indices against zero was tested using Wilcoxon's test. Asterisks denotes significant differences, *P<0.05, **P<0.01, ***P< 0.001. n = 25 for each trial.

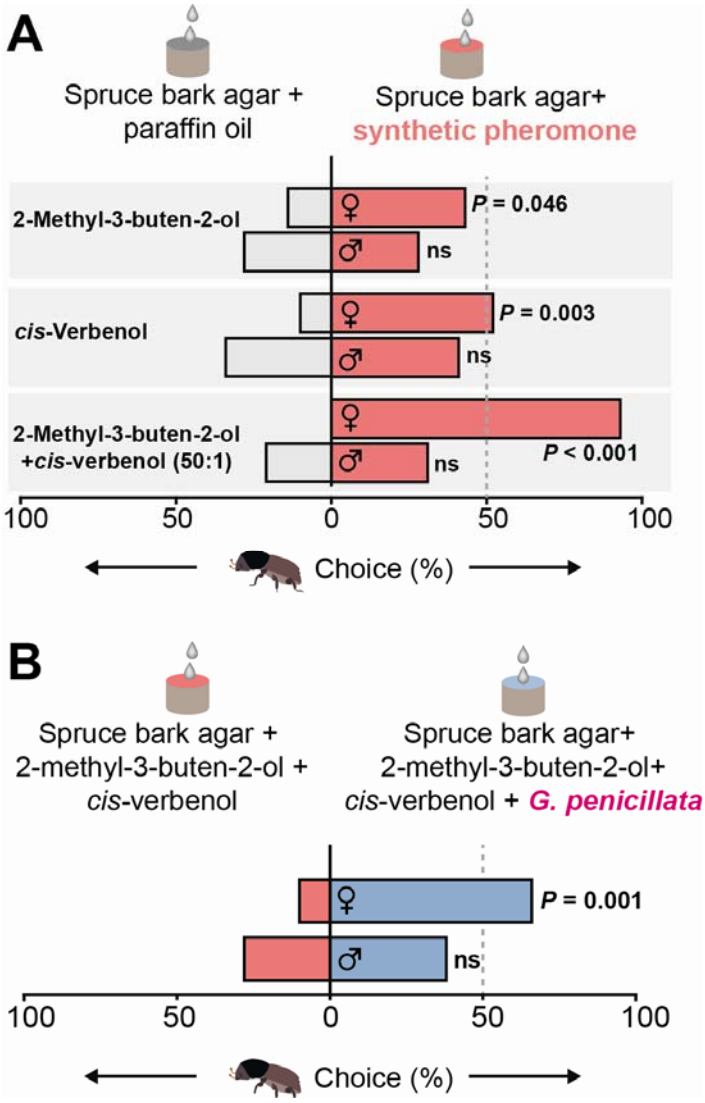

Figure 5: Female adult bark beetles are attracted towards a pheromone mixture in the presence of *G. penicillata* volatiles

(A) Adult females chose traps containing 2-methyl-3-buten-2-ol (MB) and *cis*-verbenol at 10^{-2} concentration diluted in mineral oil over control traps containing mineral oil. Females strongly preferred traps containing a binary pheromone blend (*cis*-verbenol: 2-methyl-3-buten-2-ol, 50:1) diluted in mineral oil over the mineral oil control (bottom). Adult males were unresponsive to these concentrations of individual pheromones and their blend. **(B)** Adult females preferred pheromone blend in the presence of *G. penicillata* volatiles over pheromone blend without fungus. **(A), (B)**. Deviation of response indices against zero was tested using Wilcoxon's test. Asterisks denotes significant differences, *P<0.05, **P<0.01, ***P< 0.001 (n = 28 for each experiment).

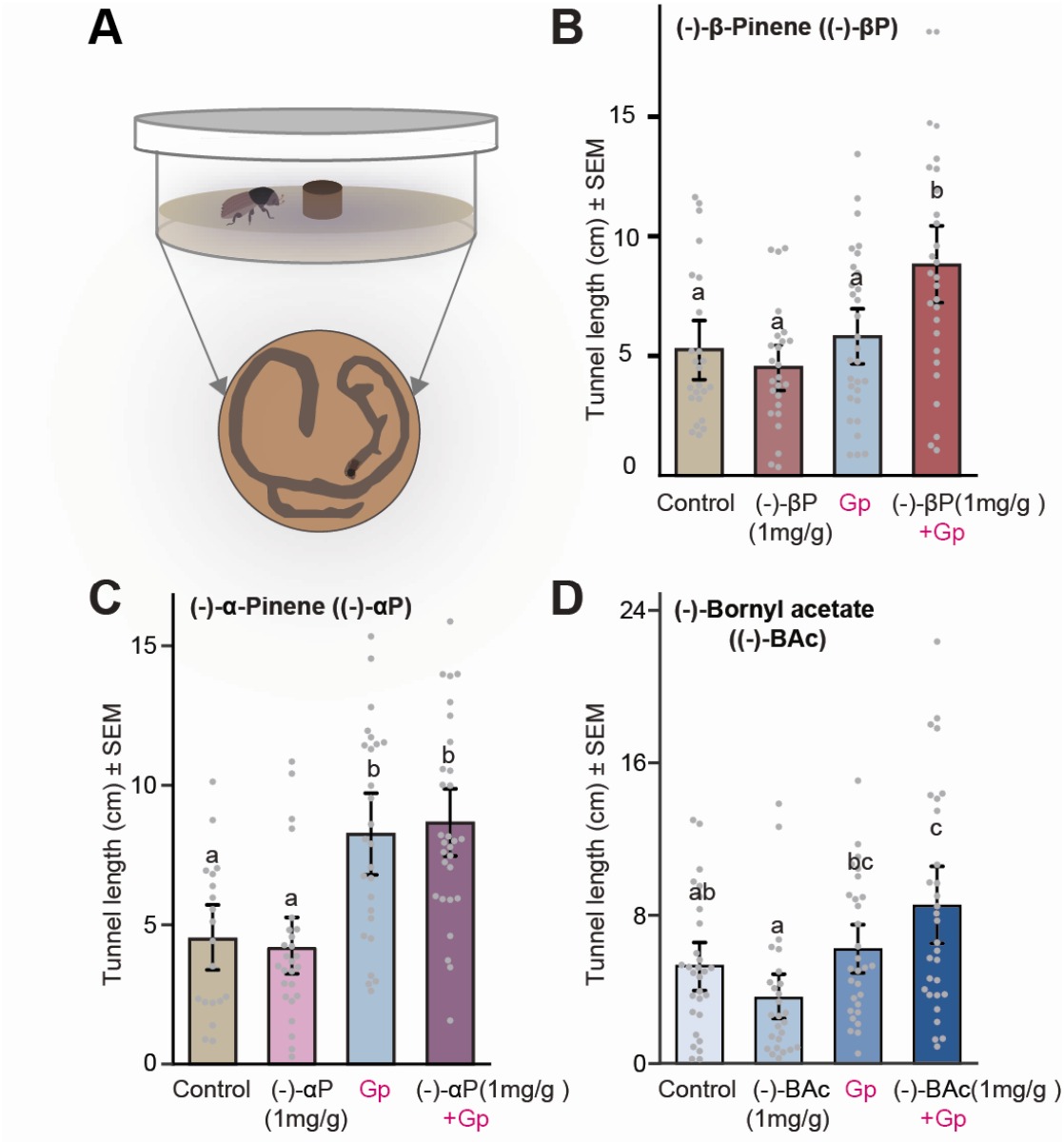

Figure 6: **Bark beetles tunnel more in monoterpene-enriched diet in the presence of a symbiotic fungus.**

(A) Schematic drawing of a Petri dish arena used for the no-choice tunneling assay. The dish was filled with monoterpene-enriched spruce bark agar and inoculated with *G. penicillata* (Gp) (top). Example of the tunneling pattern of an adult beetle within the fungus-colonized diet as pictured from the bottom side of Petri dish (bottom). **(B), (C), (D)** Tunnel lengths (cm) made by adult beetles after 48 hours in diet containing *G. penicillata* only, monoterpenes only, *G. penicillata* and monoterpenes, or controls with neither *G. penicillata* nor monoterpenes. Error bars represent SEM (n = 30 (15 ♂, 15 ♀) for each trial). Monoterpenes: (-)-β-pinene **(B)**, (-)-α-pinene **(C)**, (-)-bornyl acetate **(D)**. Different lowercase letters indicate significant differences between treatments (ANOVA, Tukey's test, P< 0.05).

Table 1: Multiple logistic regression analysis predicting the odds of adult bark beetles tunneling into media enriched in different monoterpenes with and without *G. penicillata* in a no-choice assay (see also Fig 6A).

Successful tunneling in diets with[a]	Predictors	β	SE[b]	Wald χ^2	*P* value	Exp(β)	95% CI for Exp(β)	
							Lower	Upper
(-)-Bornyl acetate	Monoterpene	-0.31	0.56	0.31	ns	0.73	0.25	2.19
	Sex	-1.72	0.58	8.78	**0.003**	0.18	0.06	0.56
	Fungus	4.98	1.09	20.99	**<0.001**	145.30	17.27	1222.52
(-)-β-Pinene	Monoterpene	-0.87	0.54	2.57	ns	0.42	0.14	1.22
	Sex	0.25	0.52	0.24	ns	1.29	0.47	3.55
	Fungus	2.65	0.58	20.45	**<0.001**	14.09	4.48	44.36
(-)-α-Pinene	Monoterpene	0.00	0.52	0.00	ns	1.00	0.36	2.75
	Sex	0.00	0.52	0.00	ns	1.00	0.36	2.75
	Fungus	2.02	0.66	9.39	**0.002**	7.51	2.07	27.28

[a]The reference category is unsuccessful tunneling
[b]Standard error of *β*
ns=not significant