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Toxicity of Norway spruce (*Picea abies*) defense compounds to the spruce bark beetle (*Ips typographus)*

Master Thesis

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science (M.Sc.) in Molecular Life Sciences

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From Cox's bazar, Bangladesh

Jena, December 2019

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ABBREVIATIONS

µl/l	micro liter/liter
AFI	anti- feedant index
DMSO	dimethylsulfoxide
DMAPP	dimethylallyl diphosphate
ET	effective threshold
FID	flame ionization detection
FPP	fernesyl diphosphate
FPR	false positive rate
GABA	gamma amino butyric acid
GC- MS	gas chromatograpgy- mass spectrometry
GGPP	geranyl geranyl diphosphate
GPP	geranyl diphosphate
MEP	methyl- erythritol- phosphate
MEV	mevalonate
mg/g	milligram/gram
IPP	isopentanyl diphosphate
LC	lethal concentration
MT	monoterpene
OMT	oxygenated monoterpene
PDA	potato dextrose agar
ROC	receiver operating characteristic
TPR	true positive rate

ABSTRACT

Background: The Norway spruce bark beetle *Ips typographus* has become one of the most destructive forest pests in Europe. Due to climate change, bark beetles are expanding their range to non-native regions causing vast ecological and economic losses globally. Despite the known toxicity of host tree monoterpenes to other bark beetle species, little is known about the toxicity of individual monoterpenes to both sexes of *I. typographus*. Therefore, I investigated 1) the toxicity of the major monoterpenes of Norway spruce to bark beetles using a fumigation assay, 2) the performance of male and female beetles in monoterpene-enriched diets, and 3) the role of symbiotic fungi in biotransformation of host tree monoterpenes.

Methods: Toxicity of monoterpenes was assessed by a) exposing beetles to various concentrations of individual monoterpene vapors and estimating LC_{50} (concentration of monoterpenes at which 50% of beetles were killed), and by b) using *in vitro* tunneling assay where the beetles were allowed to tunnel in "artificial galleries", medium amended with different concentrations of individual monoterpenes. After beetles were allowed to tunnel, the length of the tunnel made by beetles was measured, and their weight change was calculated. Further, to estimate the acceptance rate of the diet by beetles, c) boring behavior of beetles in monoterpene-amended media was observed. Additionally, to elucidate the role of fungus in the colonization of the host tree by beetles, we examined the beetles' boring behavior in fungus colonized media amended with monoterpenes. To determine the role of fungi in metabolizing host monoterpenes, different fungi were grown in monoterpene-amended media and the amount of remaining monoterpenes and their fungal biotransformation products were quantified using gas chromatography coupled to a flame ionization detector and to a mass spectrometer.

Results: As vapors, different monoterpenes showed variable toxicity to adult bark beetles: myrcene was the most toxic and (-)-bornyl acetate was the least toxic of all the monoterpenes. Tunneling assays showed that at a low dose, most of the monoterpene-amended diets had no effect on the performance of either sex. However, at high doses, several monoterpenes functioned as feeding deterrents to males but elicited no response in females. The tunneling length increased in all the treatments over time and all the beetles in various treatments gained significant weight with females gaining more weight than males. In the presence of fungus,

beetles' boring behavior in monoterpene-amended diets increased significantly compared to monoterpene diets without fungus. Overall, females showed a higher boring tendency than males in both fungus-free and fungus colonized diets. Bark beetle symbionts such as *Grosmannia penicillata* and *Grosmannia europhioides* significantly reduced the amount of (-)-bornyl acetate in the diet. (-)-Bornyl acetate was biotransformed to borneol and camphor. *Grosmannia penicillata* efficiently transformed (-)-β-pinene to oxygenated products such as terpinen-4-ol and α-terpineol.

Conclusion: This study reports that males and females respond differently to host defense chemicals and that each sex might implement a common strategy to overcome host defenses by partnering with fungi**.** The behavioral differences in both sexes can be a crucial factor in the management of this beetle species. Further research is needed to focus on the behavioral differences between males and females and to determine whether each sex has separate strategies to adapt to host defenses.

ZUSAMMENFASSUNG

Hintergrund: Der Buchdrucker *Ips typographus* ist einer der destruktivsten Waldschädlinge Europas geworden. Aufgrund des Klimawandels breitet er sich in Regionen außerhalb seines ursprünglichen Verbreitungsgebietes aus, und verursacht so große ökonomische und ökologische Verluste weltweit. Obwohl die Toxizität bestimmter Verteidigungsmetaboliten der Wirtspflanze, den Monoterpenen, gegenüber anderen Borkenkäferarten bekannt ist, wurde die Wirkung individueller Monoterpene auf *Ips typographus* für beide Geschlechter bislang kaum untersucht. Daher untersuchte ich 1) die Toxizität der wichtigsten Monoterpene aus der Gemeinen Fichte gegenüber dem Buchdrucker in einem Begasungstest, 2) die Performance männlicher und weiblicher Buchdrucker in mit Monoterpenen angereicherten Diäten, und 3) die Rolle symbiotischer Pilze bei der Biotransformation von Monoterpenen aus der Wirtsverteidigung.

Methoden: Die Toxizität der Monoterpene wurde ermittelt, a) indem die Käfer individuellen gasförmigen Monoterpenen unterschiedlicher Konzentrationen ausgesetzt und LC_{50} (Konzentration der Monoterpene, bei der 50% der Käfer starben) abgeschätzt wurde, b) mit Hilfe von in vitro Tunnelbautests, in denen die Käfer die Möglichkeit hatten, „künstliche Galerien“ in einem Medium anzulegen. Hierbei wurde das Medium mit unterschiedlich konzentrierten Monoterpenen angereichert und am Ende die Länge der Tunnel in den Galerien, welche die Käfer gebohrt hatten, sowie die Gewichtveränderung der Tiere, gemessen. Für die Beurteilung der Akzeptanz der Diät durch die Käfer, wurde c) das „Bohrverhalten“ (Anlegen der Tunnel) der Käfer in den mit Monoterpenen angereichten Medien beobachtet. Um die Rolle der Pilze bei der Kolonisation des Baumes durch die Käfer zu ermitteln, wurde das Bohrverhalten der Käfer in Medien geprüft, die sowohl mit Monoterpenen angereichert als auch mit Pilzen inokuliert wurden. Um zu untersuchen, inwieweit Pilze an der Metabolisierung der Monoterpene beteiligt sind, wurden unterschiedliche Pilze auf Medien angezogen, die mit Monoterpenen angereichert wurden. Schließlich wurde die Menge an verbleibenden Monoterpenen und deren Produkte, die durch die Biotransformation der Pilze entstehen, mittels Flammenionisationsdetektor-gekoppelter und Massenspektrometer-gekoppelte Gaschromatographie quantifiziert.

Ergebnisse: Die gasförmig applizierten Monoterpene zeigten eine variable Toxizität gegenüber adulter Buchdrucker, wobei Myrcen die höchste und (-)-Bornylacetat die geringste Toxizität aufwiesen. Bei Tunnelbautests mit geringer Dosis wurde bei den meisten Monoterpenen kein Effekt auf die Performance festgestellt, weder bei weiblichen noch bei männlichen Käfern. Allerdings zeigten einige Monoterpene in höherer Dosis eine abschreckende Wirkung auf männliche, nicht jedoch auf weibliche Käfer. Die Länge der Tunnel erhöhte sich in allen Behandlungen im zeitlichen Verlauf des Experimentes, und alle Käfer in den unterschiedlichen Behandlungen zeigten eine deutliche Gewichtszunahme, wobei weibliche Käfer eine stärkere Gewichtszunahme hatten als ihr männlichen Artgenossen. In Anwesenheit von Pilzen nahm das Bohrverhalten der Käfer in Monoterpen-angereicherten Diäten signifikant zu im Vergleich zu Monoterpen-angereicherten Diäten ohne Pilze. Generell zeigten die weiblichen Käfer eine tendenziell höhere Bohraktivität in den Diäten als die männlichen Käfer, sowohl mit als auch ohne Pilzwachstum. Manche der pilzlichen Symbioten des Buchdrucker, wie *Grosmannia penicillata* und *Grosmannia europhioides*, reduzierten signifikant die Menge von (-)-Bornyl acetat in den Diäten. (-)-Bornylacetat wurde dabei in Borneol und Camphor umgesetzt. *Grosmannia penicillata* metabolisierte darüber hinaus (-)-β-Pinen effizient in Oxidationsprodukte wie Terpinen-4-ol und α-Terpineol.

Schlussfolgerungen: Diese Studie hat gezeigt, dass männliche und weibliche Käfer unterschiedlich auf Verteidigungsmetaboliten ihrer Wirtspflanze reagieren und beide Geschlechter eine gemeinsame Strategie zur Überwindung der Wirtsverteidigung nutzen, indem sie eine Kooperation mit Pilzen eingehen. Die Verhaltensdivergenz zwischen den beiden Geschlechtern könnte einen entscheidenden Faktor in der Schädlingsbekämpfung dieser Käferart darstellen. Zukünftige Forschungen sollten die geschlechterspezifischen Verhaltensunterschiede und eventuelle differenzielle Anpassungsstrategien beider Geschlechter gegenüber der Wirtsverteidigung eingehender untersuchen.

1. INTRODUCTION

1.1. Background

Bark beetles have become a crucial disturbing factor in North American and European forests killing a huge number of trees and the damage is increasing at an alarming rate [1]. From 1971-1980, 2.1 million m^3 of pine and spruce timbers had been damaged by the bark beetles each year in Europe which increased to 14.5 million m^3 per year during 2002-2010 and it is expected to increase to 17.9 million m^3 per year by 2021-2030 [2]. Such outbreaks can alter forest ecosystems and biodiversity affecting the reduction of carbon and nitrogen storage while increasing soil temperature and water availability in the soil [3]. Moreover, bark beetle attacks can deteriorate the timber quality and appearance which causes huge economic losses to the timber producers. For instance, Southern pine beetle has caused an economic loss of around 375 million USD from 1977-2004 due to a reduction in timber prices [4].

1.2. Impact of bark beetles

Bark beetles are insect herbivores with economic and socio-political importance [1]. To date, more than 6000 species of Scolytids and nearly 1500 species of Platypodids beetles belonging to the weevil family have been described worldwide [5] but only a few species of them are actually able to kill healthy trees [1]. These insects help in forest rejuvenation and recycling of the ecosystem by invading and killing the old and wind thrown trees until they reach a threshold density. Above this threshold density, the more aggressive species can also attack the healthy trees [1], [6], [7]. Such outbreaks result in the destruction of millions of hectares of living forest resulting in vast economic losses and ecological imbalances [8]. Among the destructive species, *Dendroctonus ponderosae*, the mountain pine beetles in North and Central America and *Ips typographus,* the European spruce bark beetle are the most economically important pests in coniferous forest [1], [7].

1.3. Biology of the European Spruce bark beetle

The European spruce bark beetle, scientifically known as *Ips typographus* Linnaeus (Coleoptera: Scolytidae) is considered the major endemic forest pest across Euroasia [6], [7]. They mostly attack Norway spruce trees, *Picea abies,* as well as occasionally trees from the genera *Pinus*, *Abies,* and *Larix*. They maintain an association with a varied community of

bacterial and fungal symbionts [8] which are thought to help the beetle to exhaust tree defenses [9], detoxify tree defense chemicals [10], [11] and provide nutrients [12].

Bark beetles generally hibernate during winter and start to fly during April- June when the temperature increases above 15ºC. Males are the pioneer beetles which select new hosts. When a suitable host tree is located, the male releases aggregation pheromones composed of (-)-*cis*-verbenol and 2-methyl-3-buten-2-ol, produced by beetles and/or symbiotic microbes, which attract many conspecifics of both sexes. This results in a mass attack of the host tree to overcome the host's defenses. Once the tree's defense is overwhelmed, beetles switch to release an anti-aggregation pheromone, (-)-verbenone to divert the new coming beetles to neighboring trees to avoid intra-specific competition for limited resources [13]–[15]. Upon a successful attack, each male constructs a nuptial chamber under the bark and accommodates one to four females. After mating, females continue excavating vertical egg galleries [7]. The female lays eggs and inoculates fungi in the gallery. The hatched larvae make their own feeding tunnels perpendicular to the maternal gallery and obtain their nutrition by consuming fungus infected phloem tissues and develop through 3-5 instars. Larvae pupate in pupal chambers lined with spores of ophiostomatoid fungi and newly eclosed callow or teneral adults feed on the older sections of the gallery until they transform into sclerotized adults [16]. Once the brood beetles are fully developed, adults may emerge to initiate second generation attacks or hibernate under the bark or in the forest litter. Parent beetles can reemerge again to form sister broods under favorable conditions [7]. The full life cycle is illustrated in (Figure 1).

1.4. Conifer defense mechanism

Conifers evolved a sophisticated defense system comprising of physical, chemical and histological constitutive and induced protection mechanisms [17], [18]. The ultimate role of conifer defenses is to preserve the integrity of the tree by defending the nutrient-rich phloem, the vascular cambium, and the sapwood which is needed for transpiration [19]. Conifer defense system comprised of two phases- the constitutive defense and the inducible defense. The primary defense includes constitutive mechanical and chemical mechanisms. Mechanically, the tree repels invaders by strengthening of tissues with lignin and suberin polymers creating resistance to penetration, degradation or ingestion [18]. Chemical defenses

include toxic substances including specialized plant metabolites, proteins and enzymes and reservoirs of chemicals such as resins that can repel or physically entrap beetles [18], [20].

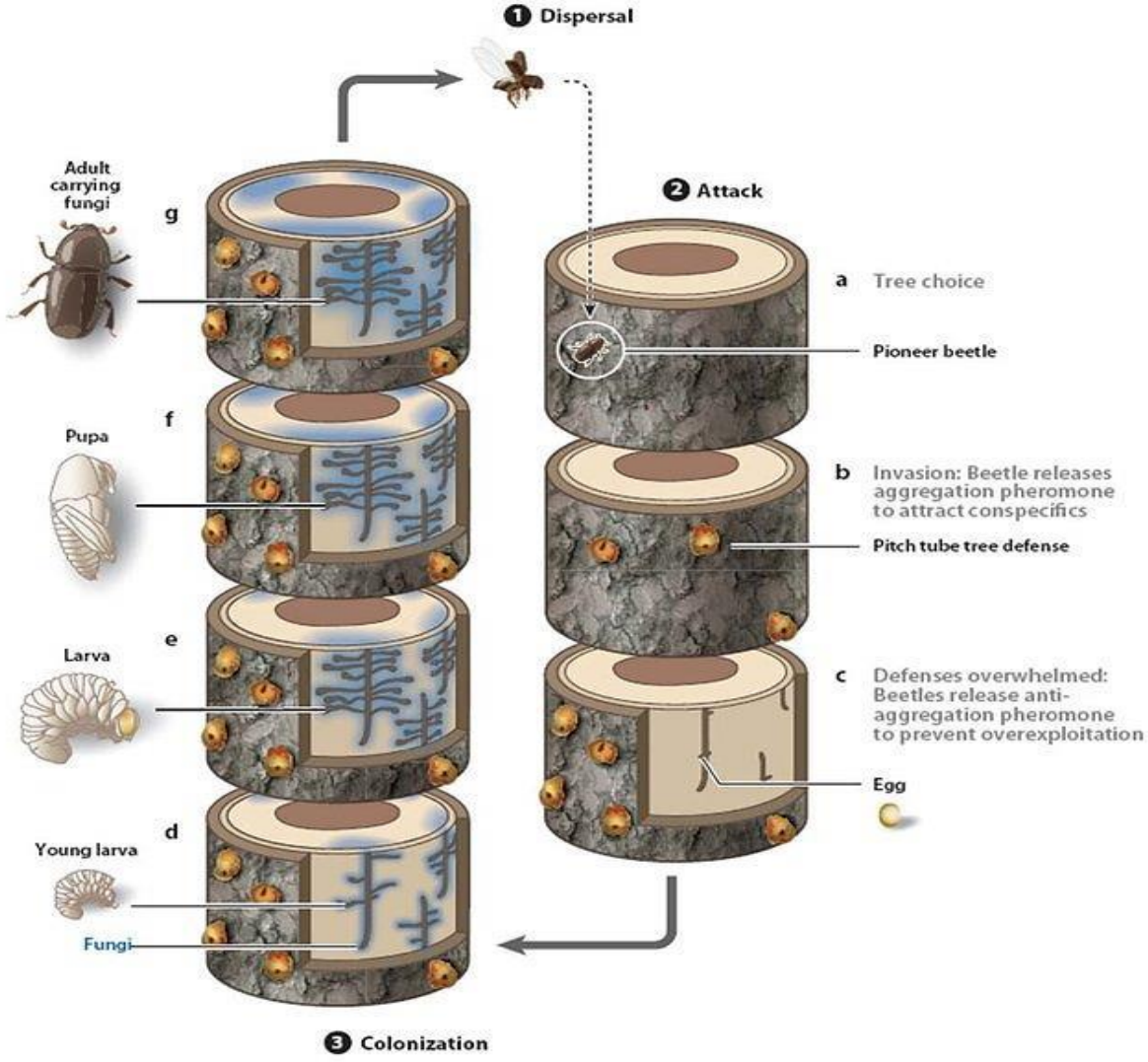


Figure 1: The life cycle of *Ips typographus* and its associated fungi. **(1)** Dispersal of adult beetles under favorable conditions. **(2)** Attacking period: **(a)** suitable tree is chosen by pioneer beetle, **(b)** Beetles enters into the tree and releases aggregation pheromones to attract conspecifics of both sexes to induce mass attack which exhaust the tree defense system, **(c)** after that beetles release as anti-aggregation pheromone to avoid over-exploitation, **(3)** Colonization period: **(d)** parent beetles construct vertical egg galleries, lay eggs and inoculate fungi, hatched larvae start to develop, **(e)** the developing larvae tunnel away from the oviposition gallery, feed on fungal spores and phloem tissues, **(f)** later they excavate pupal chambers and remain there during pupation, **(g)** after becoming adults they emerge carrying fungal spores on the exoskeleton. Adapted from [21].

Main constitutive mechanisms are located in the secondary phloem which contains sclerenchyma cells, calcium oxalate crystals and polyphenolic parenchyma cells. The sclerenchyma cells and the calcium oxalate crystals can act as mechanical barriers against the beetles. The polyphenolic parenchyma cells contain phenolic bodies and provides chemical

protection against the fungi [18]. Furthermore, the resin cells produce resins containing terpenes. The resins are stored under pressure in the extracellular lumen. When the bark is injured by insects, extracellular lumen releases the sticky resins which repel or trap the insects [22].

The second phase of defense systems is inducible structural and chemical mechanisms. Spruce trees mount an induced defense within 7 days after bark beetles tunnel into a tree. Until then an effective constitutive defense delays the progress of infestation. At the attack sites, production of reactive oxygen species and rapid cell death occurs due to hypersensitive reactions [23]. Wound periderm confines the wound region and cuts off the nutrient supply to the damaged tissues [18]. The inducible chemical mechanisms include proteins and non-protein based chemicals. Protein-based chemicals include lectins, enzymes such as chitinases or glucanases and enzyme inhibitors such as proteinases or amylase inhibitors. The enzymes (chitinases) can lignify tree cell walls. The enzyme inhibitors decrease performance of the invading organism by interfering with the digestion. The non-protein based chemicals include phenolics, terpenoid resins and alkaloids [18]. Attacks induce resinosis and additional traumatic resin duct formation, auto-necrosis and changes in polyphenolic parenchyma cells, and biosynthesis of different compounds through methylerythritol phosphate and shikimic acid pathways [18], [20], [24]. Phenolics can reduce the nutrient value of tissues by binding with amino acids and proteins. They can also reduce digestion of invaders by binding to digestive gut enzymes. The resin flow, with increased and altered terpenes composition, can inhibit the progress of the beetle into the tree by blocking the entry site and mask the release of pheromone from the entry site [18], [25], [26].

Four successive stages contribute to successful plant defense against the invaders. The first phase consists of repelling or inhibiting attacks with efficient constitutive protection. If this is not successful, the second stage is to kill or compartmentalize the attackers by the induced defense. The third phase of protection is to repair the damaged region to avoid secondary infections by opportunistic organisms. Eventually, acquired resistance can be generated locally and systemically in order to respond more efficiently during future threats [18], [27], [28]

1.5. Mode of action of terpenes

Terpenoid oleoresin is a complex blend of monoterpene (C_{10}), sesquiterpene (C_{15}) and diterpene (C_{20}) and other compounds in small quantities. Terpenes are stored in the resin ducts under pressure and once the invading bark beetle ruptures resin ducts in the bark, the resin ducts discharge the sticky resin. This can either immobilize the beetle or repel the beetle to move out of the bark. The resin flow can delay the beetle's progress. Once the resin is exposed to the air, the volatile monoterpenes evaporate and diterpenes become solidified and thus seal the wound [19]. A considerable amount of resin flow can inhibit the beetle's ability to trigger the pheromone-mediated arrival of conspecific beetles. This is possibly due to a combination of gummy resins that physically obstruct the release of volatile pheromones from the entrance site and high proportions of volatile host terpenes to pheromones that either mask or hinder attraction towards pheromones [25], [29]. Some terpenes are also important for bark beetle since they use these chemicals as primary host-finding cues [30]. It has been shown in several studies that terpenoids possess toxic effects on both beetles and fungi [31]–[33] and upon invasion by the bark beetles and their symbionts, the tree can increase the level of terpenoids with an altered composition of individual compounds and their concentrations [25], [29].

1.6. Biosynthesis of terpenes

Most of the terpenoids are synthesized by condensation of two C_5 units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The condensation occurs between one unit of DMAPP and one or more units of IPP which is catalyzed by specific prenyl transferases. IPP is derived from one of the two pathways, the mevalonate (MEV) pathway in the cytosol or metheyerythritol-4-phosphate (MEP) pathway in the plastid. The fusion of the two C_5 units by specific prenyl transferase enzymes (GPP-, FPP- and GGPP synthases) produces prenyl diphosphates (GPP, FPP, and GGPP) which are then modified to different mono-, sesqui- and diterpenes. The majority of terpenes are cyclical and many of them have a stereogenic center. The action of cytochrome P450 (CYP450) enzymes can further diversify the coniferous terpenes, especially diterpenes, with hydroxylation and further oxidation [20] (Figure 2).

Monoterpenes are a class of terpenes containing two isoprene units and with molecular formula $C_{10}H_{16}$. They can be monocyclic, bicyclic or acyclic. When the pyrophosphate group is eliminated from the GPP, it leads to formation of acyclic monoterpenes such as myrcene. Cyclization of GPP leads to the formation of 6-membered ring containing monocyclic monoterpenes such as limonene. Furthermore, two sequential cyclization reactions of GPP result in formation of bicyclic monoterpenes such as α-pinene, β-pinene (Figure 2 and 3).

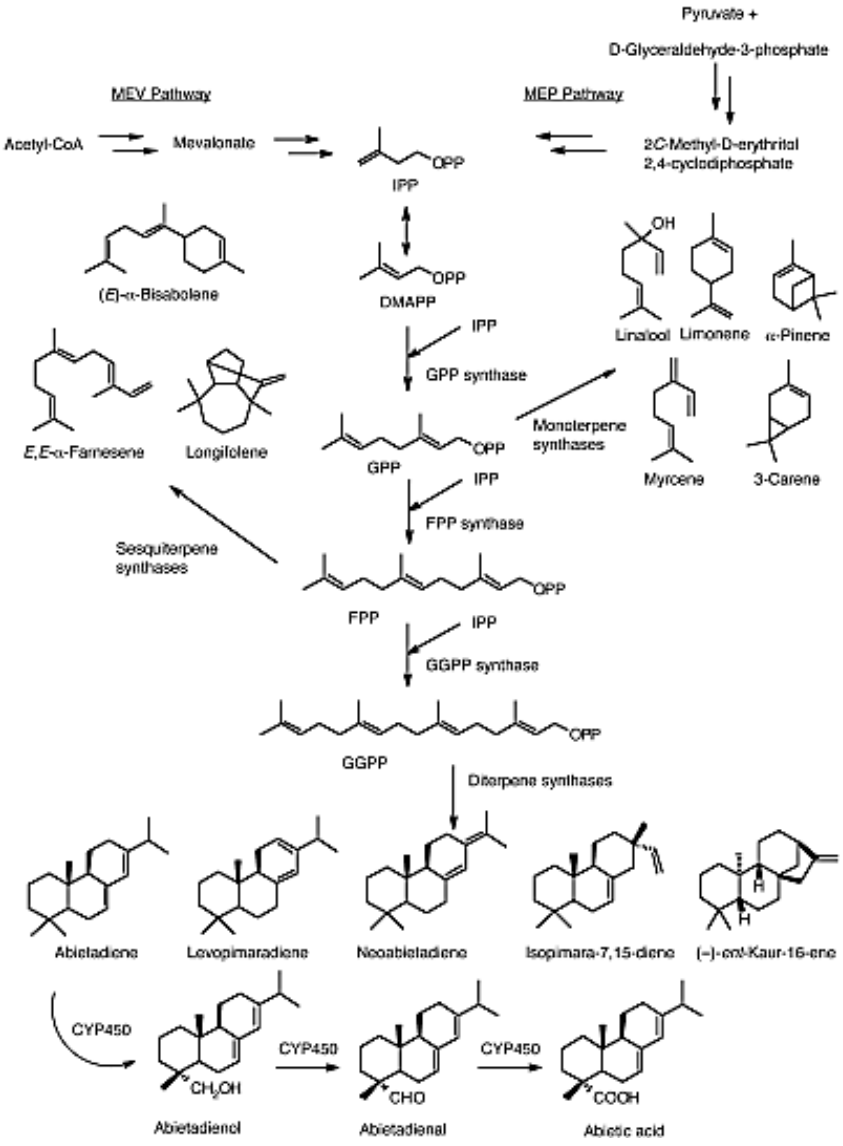


Figure 2: Biosynthesis of terpenoids in conifers. The fusion of one unit of DMAPP and one, two or three units of IPP yields in different mono-, sesqui- and diterpenes basic building blocks such as C(10), C(15), and C(20) which are catalyzed by specific prenyl transferase enzymes. These basic precursors are then further diversified into a large number of terpenoids with the help of specific terpene synthases and cytochrome P450 enzymes. Adapted from [20].

1.7. Monoterpenes in Norway spruce

Monoterpenes are volatile hydrocarbons and act as solvent in the tree's resin. They are essential for the colonization of the beetle bark as they are used as key chemical indicators and/or precursors for the biosynthesis of beetle pheromones [34], [35]. They are highly abundant in Norway spruce trees and constitute the largest volatile proportion of the resin mixture in both normal and induced conditions. It is also considered to be one of the primary factors towards host attraction [25], [29]. Moreover, both the symbiotic fungi and the beetles can convert the highly abundant monoterpenes into a less toxic oxidized form which are then utilized as pheromones by beetles for their own communication system [36]. *In vitro* bio-assays have shown that monoterpenes are anti-feedants, insecticidal and act as toxic fumigants, repellents, and also cause reproductive inhibition and neurotoxic effect on different bark beetles and other insects [24], [32], [37], [38].

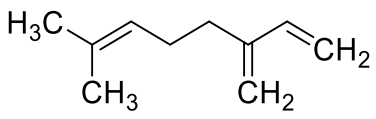
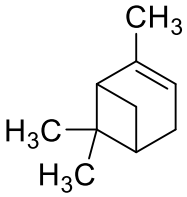
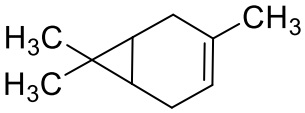
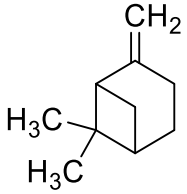
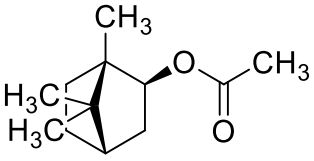
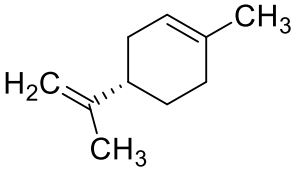
<table>
<tr><th>Acyclic</th><th colspan="2">Bicyclic</th></tr>
<tr><td> 
Myrcene</td><td> 
α-Pinene</td><td> 
3-Carene</td></tr>
<tr><td>Monocyclic</td><td rowspan="2"> 
β-Pinene</td><td rowspan="2"> 
Bornyl acetate</td></tr>
<tr><td> 
Limonene</td></tr>
</table>

Figure 3: Some of the highly abundant monoterpenes in Norway spruce.

1.8. Fungal associates of the spruce bark beetle

Symbiotic microorganisms play important roles in the life cycle of bark beetles particularly in overcoming tree defenses, usage of host plant substrates and provision of essential nutrients to

young adults. These symbionts include fungi, bacteria, and viruses [19]. Although *Ips typographus* does not have a specialized structure (mycangium) to harvest and transport microbial spores, it can transport microbes via non-grandular pit-like structures on the exoskeleton of elytra, punctures of the head and pronotum, and possibly in the gut [40]. Symbiotic fungi have a crucial role in successful host colonization and reproduction in *Ips typographus* [41]. The most frequently associated fungi of *Ips typographus* are *Endoconidiophora polonica*, *Grosmannia penicillata*, *Grosmannia europhioides* and *Ophiostoma bicolor* [42]. These symbiotic fungi can produce blue-grey or black stains on the infected wood due to their melanized hyphae and hence they are called blue-stain fungi. It has been shown that the *I. typographus* symbiont *E. polonica* can metabolize host toxins such as stilbenes and flavonoids in Norway spruce [9], [11]. Beetles benefit from both fungal metabolism of plant substrates as well as by consuming fungi directly [19] (figure 4). Fungal volatiles are known to support many of the associations between fungi and insects acting as a source of pheromones, kairomones, and allomones. Each fungus emits a specific blend of low molecular weight compounds with characteristic fragrances. It has been reported that *Ips typographus* can recognize specific volatiles emitted from fungi and these cues help them to maintain a specific fungal community [43].

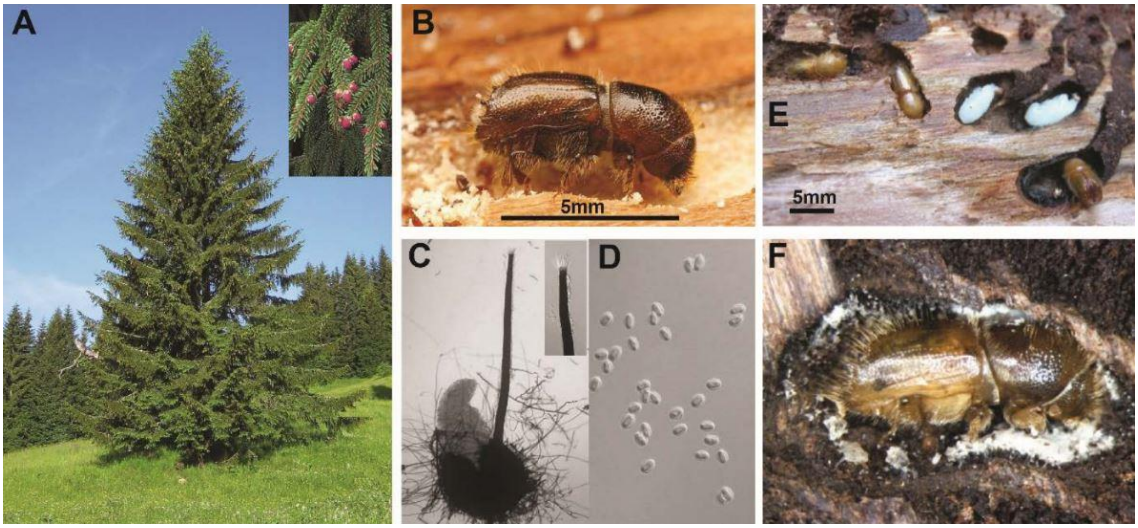


Figure 4: Norway spruce- *Ips typographus*- ophiostomatoid fungi tripartite interaction. **(A)** Norway spruce tree (*Picea abies*), **(B)** Under the bark, beetles feed on phloem which is rich in toxic plant defense chemicals such as monoterpenes, **(C, D)** Perithicia (sexual structure) of *E. polonica* produces sticky spores that facilitate the attachment of spores to the exoskeleton of the beetle, **(E)** Larvae feed on the fungus infected tissues for their nutrition and sexual maturation in the feeding galleries, **(F)** A callow adult lined with asexual spores of *G. penicillata*. Adapted from [36].

1.9. Aims of the study

Resinosis is one of the major strategies of the Norway spruce tree to fend off invaders. Monoterpenes constitute the largest volatile proportion of resin. However, little is known about the biological activity of monoterpenes on bark beetles. Therefore, the primary aim of this study was to determine the toxicity of different monoterpenes on *Ips typographus* by two types of *in vitro* bioassays- 1) fumigation assay and 2) no choice tunneling assay where beetles were allowed to tunnel on artificial diet enriched with monoterpenes. It is thought that ophiostomatoid fungi can metabolize the major monoterpenes in the Norway spruce tree to help the beetles to overcome the defense system. Therefore, the second aim of this study was to investigate the influence of symbiotic microbes in the tunneling behavior of bark beetles in monoterpene enriched artificial diet in the presence and absence of symbiotic fungi (boring probability assay) and to identify the metabolites produced from fungal biotransformation of monoterpenes using gas chromatography.

2. METHODS AND MATERIALS

2.1. List of chemicals

Chemical	Suppliers
3-Carene	Sigma- Aldrich
(-)-Limonene	TCI
Myrcene	Acros Organics
(+)-α-Pinene	Sigma- Aldrich
(-)-α-Pinene	Sigma- Aldrich
(+)-Limonene	Fluka
(-)-Bornyl acetate	Aldrich Chemistry
(-)-β-Pinene	Fluka
Bacteriological agar	Roth
Sabinene	Chem Cruz
α-Thujene	Carl Roth
Para- Cymene	Fluka
Terpinolene	Fluka
(-)-α-Phellandrene	Aldrich
1,8-Cineol	Sigma – Aldrich
α-Terpinene	Aldrich
γ-Terpinene	Aldrich
(-)-Camphene	Fluka
(+)-Camphene	Fluka
Potato dextrose broth	Sigma- Aldrich
D-(+)-Glucose anhydrous	Roth
Dimethylsulfoxide	Roth
Ethanol Absolute	VWR Chemicals
Cellulose	Sigma Life Sciences
Dichloromethane	Roth
Hexane	Merck
Nonyl acetate	Sigma- Aldrich

2.2. List of materials

Material	Suppliers
150mm glass Pasteur pipette	VWR international
200×300mm disposable bag	Roth
RNase free Tips (10, 20, 200, and 100 µl)	Star lab
Parafilm	Bemis
140mm grade 3 filter paper	Ahlstorm- Munsksjö
23mm grade 3 Whatman filterpaper	GE Healthcare Life Sciences
35×10mm Petri dish	Greiner bio one
Rubber band	ALCO
30ml Omnifix syringe	B. Braun
50ml Falcone tube	Sarstedt AG & Co.
15ml clear vial with a screw cap	Supelco
1.5ml amber glass vial	Macherey- Nagel Gmbh
1.5ml clear glass vial	Macherey- Nagel Gmbh
Pneumatic tube	Sang- a
Insect rearing tent (BugDorm-4S2260)	Mega View Science Co. Ltd.
20ml glass scintillation vial	Fisher scientific
13cm circular Petri dish	VWR international

2.3. List of instruments used

Instrument	Supplier
Electronic precision balance	Sartorius Lab Instrument Gmbh, Germany
Sterilizer (25 °C, 80 °C and 200 °C)	Heraeus Instrument
Vortex genie 2	Scientific Industries
Autoclave steam sterilizer	H+P Labortechnik Gmbh, Germany
Vibratory micro mill	Fritsch Gmbh, Germany
Stereomicroscope (Stemi 2000-CS)	Zeiss, Germany
Agilent 6890 series gas chromatograph	Agilent, Santa Clara, USA

Agilent 5973 quadrupole mass selective detector	Agilent, Santa Clara, USA
GC-MS-QP-2010 plus gas chromatograph	Shimadzu, Japan

2.4. Beetle rearing

For rearing bark beetles, a single Norway spruce (*Picea abies*) tree was cut from a nearly 60-year-old spruce plantation in southwestern Jena, Germany. The tree was cut into small logs of around 30 cm in diameter and 50 cm in height. Paraffin wax was applied on the cut ends of the logs to prevent desiccation. The waxed logs were kept in insect rearing tent (BugDorm-4S2260, MegaView Science Co., Ltd., Taichung, Taiwan) to prevent the emerging beetles from escaping and placed in the rearing chamber, which was maintained at 25 °C and 65% humidity with 18h-6h light-dark cycle. Twenty-five beetles from the previous generation were introduced into each log and allowed to breed. After 30 days, emerging offspring were collected manually with forceps and were separated into males and females prior to storage. After sex determination, approximately 50-60 beetles were stored in 50-ml Falcon tubes (SARSTEDT AG & Co. KG, Nümbrecht, Germany). Before putting the beetles in the tube, a small piece of filter paper was embedded in the tube with a few drops of Millipore water to maintain humidity inside the tube. The first 200-300 beetles were either stored or used for continuous rearing of next-generation beetles. All the collected beetles in the tubes were stored at 4 °C.

2.5. Separation of sexes

The separation of sexes of live beetles was done according to the method described by Schlyter and Cederholm [44]. Briefly, the beetles were separated based on the density of bristles on the pronotum of both sexes. Females have a higher density of bristles than males. Furthermore, the size of the frontal tubercle was also taken into consideration. Naturally male beetles have larger frontal tubercles than females. A stereomicroscope (Stemi 2000-CS, Zeiss, Jena, Germany) was used to observe the differences in both sexes. Some beetles had wearing and damage of the bristles as well as dirt and resin on the pronotum and hence sex differentiation was difficult. Therefore, those beetles were excluded from the study.

2.6. Grinding the bark into powder

A tree was cut from the forest and cut into small logs. After bringing them to the lab, the outer bark was removed and the inner bark (phloem part) was carefully peeled off with a hammer and chisel. The bark was cut into small pieces and was kept in plastic bags and stored at -80 °C until pulverization. The pulverization was done in vibratory micro mill PULVERISETTE 0 (FRITSCH GmbH, Germany). The whole instrumental setup was pre-cooled with liquid N_2. The bark pieces were pulverized at an amplitude of 2.0 for approximately 8-10 minutes with addition of liquid N_2 after every two minutes. The ground bark powder was then sieved through mesh no. 18 to separate the coarse particles from the ground powder. The fine powder was collected in 50 ml Falcon tubes and stored at -80 °C until used for the diet preparation.

2.7. Fumigant toxicity assay of monoterpenes on bark beetles

This bioassay was done to assess the toxicity levels of different monoterpenes on *Ips typographus*. For the fumigation toxicity assay, 20 ml scintillation vials were used. A total of 20 beetles (10 males and 10 females) were used for each concentration [32]. The concentrations used for the assay were 10, 50, 100, 200, 500, 750, and 1000 µl/l (µl of monoterpenes/ liter of the air volume of the vial). To achieve the respective concentrations, 0.2, 1, 2, 4, 10, 14 and 20 µl of monoterpenes were applied to the filter papers. In the control, no chemical was used and the filter paper was blank. One beetle was introduced in each vial before applying the monoterpenes. A 23 mm Whatmann no.3 circular filter paper was affixed in the inner top of the screw caps and impregnated with the calculated volume of monoterpenes. The vials were sealed with the caps immediately after the addition of monoterpenes to the filter paper. Then the vials were kept in the dark at room temperature for 24 hours. After 24 hours the beetles were removed from the vials and mortality was determined under a stereo-microscope. The beetles were gently touched on the antennae and legs with entomological tweezers to elicit the movement. Beetles not responding to the touch were considered as dead. The individual monoterpenes tested in this experiment were (+)-α-pinene, (-)-α-pinene, (-)-β-pinene, myrcene, 3-carene, (+)-limonene, (-)-limonene, and (-)-bornyl acetate. These compounds were selected based on their higher abundance relative to other monoterpenes in *Picea abies* [25]. Alongside the individual monoterpenes, three different blends of monoterpenes were also tested (Table 1). The blends were prepared based

on the level of monoterpenes in *Picea abies* (1) before the attack by *Ips typographus*, (2) after attack and (3) in trees that survived bark beetle attack as reported in Christian Schiebe et al. [25].

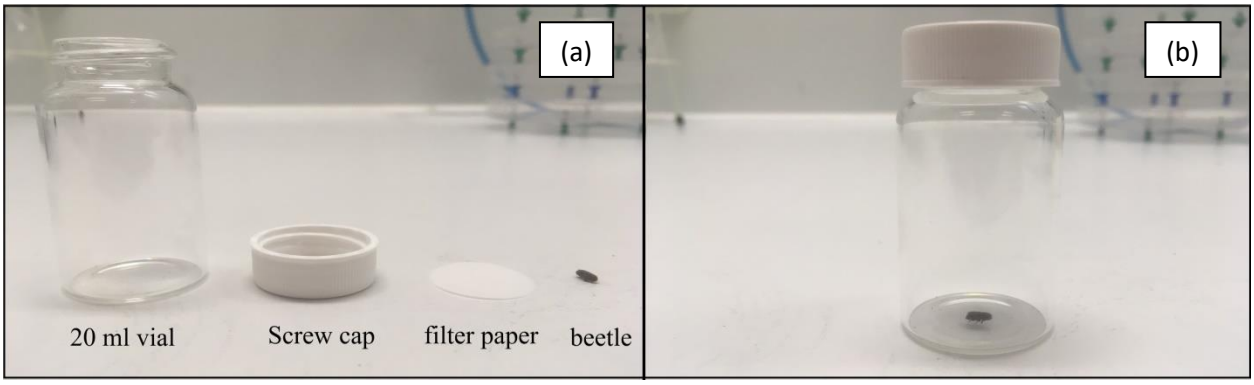


Figure 5: Experimental setup for fumigation toxicity assay. (a) Materials used in the experiment, (b) the complete setup of the experiment where a single beetle is placed at the bottom of the glass vials and the filter paper containing test chemical was secured on the top under the lid.

Table 1: Percentage of monoterpenes used for blends preparation.

Compounds	Purity (%)	CAS	Blend ratios		
			*Constitutive defense (%)	**Induced defense (%)	***Survived trees blend (%)
Tricyclene	>99	508-32-7	0.33	0.31	0.34
α-Thujene	80	2867-05-2	0.27	0.21	0.21
(-)-α-Pinene	>99	80-56-8	24.35	25	32.64
(+)-α-Pinene	>99	80-56-8	28.86	22.35	17.63
(-)-Camphene	>99	79-92-5	0.98	0.88	1.51
(+)-Camphene	>99	79-92-5	0.33	0.35	0.37
(-)-Sabinene	76	3387-41-5	0.38	0.76	0.58
(+)-Sabinene	76	3387-41-5	1.85	1.66	1.57
(-)-β-Pinene	>99	127-91-3	34.18	36.54	30.24
Myrcene	93.27	123-35-3	2.93	3.26	3.89
α-Phellendrene	>99	99-83-2	0.16	0.28	0.33
3-Carene	>99	13466-78-9	1.47	3.52	3.68
α-Terpinene	92.35	99-86-5	0.16	0.24	0.23
para-Cymene	>99	99-87-6	0.33	0.14	0.35
(-)-Limonene	>99	5989-27	0.82	0.83	2.75
(+)-Limonene	>99	5989-27	0.82	0.82	0.68
1,8-Cineol	>99	470-82-6	0.16	0.34	0.31
γ-Terpinene	96.88	99-86-5	0.33	0.44	0.47
Terpinolene	>99	586-62-9	0.65	1.26	1.39
Bornyl acetate	>99	76-49-3	0.65	0.82	0.83

***Constitutive:** level of monoterpenes present in spruce bark before beetle's attack, ****Induced:** level of monoterpenes after beetle's attack, *****Survived:** level of monoterpenes in trees that successfully defended the attack. The purity of the compounds was calculated by GC-MS analysis.

2.8. Bark beetle tunneling Assay

A performance experiment was carried out to assess the tunneling behavior of beetles in an artificial gallery and spruce bark diet enriched with different doses of monoterpenes and to ascertain the weight change of the beetles after feeding. The bioassay setup resembles the tunnels that beetles make in their natural system where the beetles can move either forward by feeding or tunneling or step back and stay at the starting point (Figure 6). This assay was performed in 11 cm long pneumatic tubes with an outer diameter of 6 mm, an inner diameter of 4 mm (Sang-A Pneumatic, Daegu, Korea). Each assay tube was filled with spruce bark diet prepared as follows: 7% (w/v) finely milled spruce inner bark powder was mixed with 1% fibrous cellulose (Sigma), 2% glucose (Roth), 4% Bactoagar (Roth) in water and autoclaved for 20 minutes at 121ºC. To ensure uniform distribution of the test chemicals in the diet, the mixtures were vortexed thoroughly and drawn up using a disposable sterile syringe (Omnifix 30 ml, B. Braun Melsungen, Germany). One end of the open assay tubes was then sealed with parafilm and the medium was allowed to cool down for 10- 15 minutes. Then the mixture of diet and test chemicals was loaded in the assay tube with the help of the disposable sterile syringe. Approximately 10 cm of the tube was filled with the diet leaving 1 cm empty to facilitate the insertion of the beetle into the tube. Before filling, the medium was mixed with 2% solvent mixture of DMSO and ethanol (mixed in 1:1) as control and with different test compounds separately. Stock solutions of the test compounds were prepared in the DMSO-ethanol solvent (1:1). The different concentrations of (+)-α-pinene, (-)-α-pinene, and (-)-β-pinene in the diet were as follows: 10, 5, 2.5, 1, 0.5, and 0.1 mg/g and for (-)-bornyl acetate 1, 0.1, 0.05, and 0.01 mg/g. For myrcene, 3-carene, and (-)-limonene, the final concentrations were 2, 1, 0.5, 0.1, 0.01 mg/g. The concentrations were selected based on the relative abundance of monoterpenes in the tree [25]. Before introducing the beetles to the tubes, their weights were recorded. Then the beetles were placed carefully in the assay tube (1 beetle per tube) with its head facing towards the diet. Then the opened end of the tube was sealed with tissue culture tape to facilitate the aeration from one end and also to prevent beetles from escaping. The length of the feeding tunnel in each tube was recorded after 6, 24 and 48 hours. According to previous studies, 6 hours is sufficient to see the difference in tunneling length and 48 hours for weight change in adult bark beetles[45], [46]. Each treatment was replicated 30 times (15 male and 15 female beetles). A maximum of 10 tubes were attached to a

whiteboard (Figure 6). The whole set up was then placed in the climate chamber for 48 hours. After 48 hours, the beetles were taken out from the tubes and their weights were recorded again.

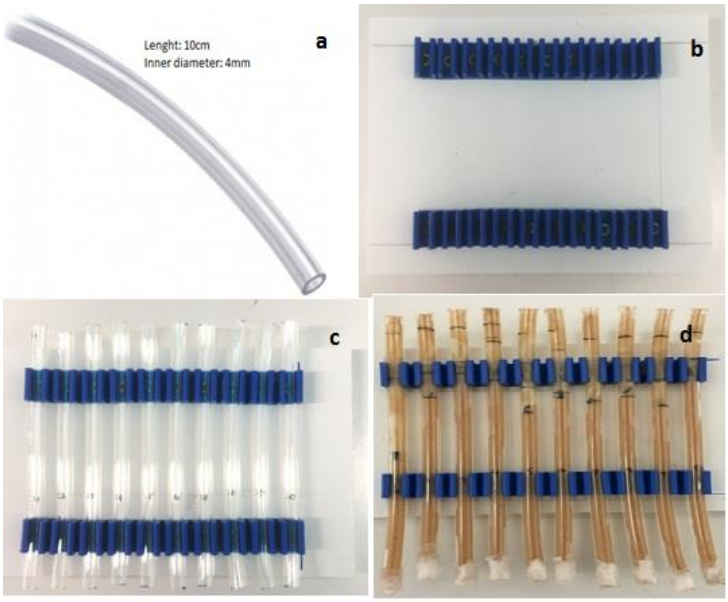


Figure 6: Bark beetle feeding assay apparatus: (a) pneumatic tube, (b) a wooden board to attach the tubes, (c) the tubes attached to the board and numbered, (d) beetles feeding on an artificial diet mixed with different monoterpenes.

2.9. Bark beetle boring behavior assay

This experiment was done to assess the boring behavior of the beetles in the diet mixed with different monoterpenes in the presence and absence of symbiotic fungus. It is known from a previous study that fungi can biotransform specific monoterpenes and convert them to oxidized monoterpenes and several other end products [43]. Therefore, we selected (+)-α-pinene, (-)-α-pinene, (-)-β-pinene and (-)-bornyl acetate for this experiment and excluded the other monoterpenes as those compounds are not metabolized into volatile byproducts by fungi. *Grosmannia penicillata* was used as this fungus has been shown to be efficient in bio transforming host tree monoterpenes in experiments compared to other fungi. For this experiment, 35×10 mm Petri dishes (Greiner Bio-one, Frickenhausen, Germany) were used and approximately, 3ml of spruce bark diet was poured in each Petri dish. The diet was prepared as before, 7% (w/v) finely milled spruce inner bark powder was mixed with 1% fibrous cellulose (Sigma), 2% glucose (Roth), 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 of DMSO and ethanol (mixed in 1:1) in controls and with different test compounds dissolved in DMSO: ethanol solvent (1:1) were added in treatments. The final concentrations of the test chemicals in the diet were similar to those used in tunneling bioassay. The media was allowed to cool down for 10-15 minutes and then the beetles were introduced in the experimental plates (1 beetle per plate). The plates were sealed with Parafilm and kept in the climate chamber for 48 hours. The beetles were monitored after 2, 4, 6, 24 and 48 hours and each time their boring rates were recorded. The responses of beetles were measured as a binary event. If beetles were inside the media, it was noted as "1" and if present outside, noted as "0". Each treatment was replicated 30 times consisting of 15 male and 15 female beetles. For treatment with fungus, 5 µl spore suspension of *G. penicillata* was added to each Petri dish.

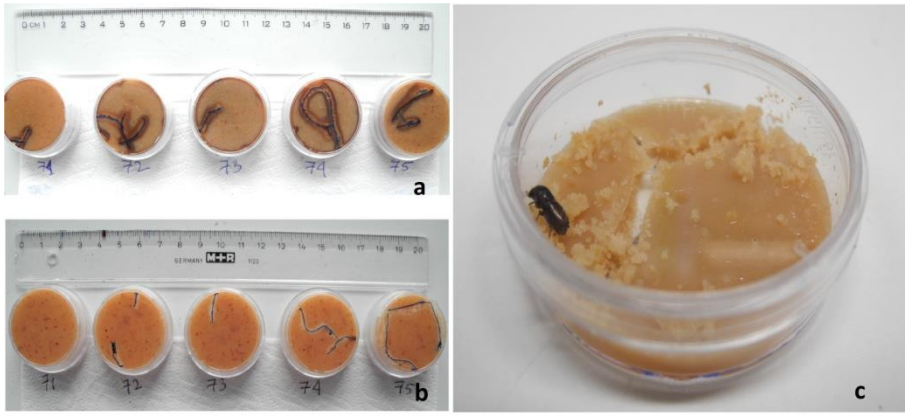


Figure 7: Boring probability assay. (a) Experimental setup with monoterpenes enriched medium inoculated with fungus, (b) Experimental setup without fungus, (c) Top view of the setup consisting approximately 3 ml of diet mixed with monoterpene in 35mm Petri dish and a single beetle is placed in each plate.

2.10. Extraction, collection, and analysis of fungal volatile compounds

2.10.1. Extraction and collection of fungal volatiles

As it is known from the previous studies that symbiotic fungi can metabolize monoterpenes, this experiment was performed to quantify the amount of monoterpenes metabolized by fungi and *de novo* produced metabolites after four days of fungal inoculation. (-)-α-Pinene, (+)-α-pinene, (-)-β-pinene and (-)-bornyl acetate were separately mixed with potato dextrose agar

media at a concentration of 0.5 mg/g of media. To achieve this concentration, 200 µl of test chemicals were added to 20 ml of PDA and vortexed thoroughly before pouring into the Petri dishes. Actively growing fungi were inoculated on Petri dishes with PDA+ test chemicals and incubated at 25 °C for four days. Petri dishes only with PDA+ test chemicals were used as the control for corresponding treatments. Each treatment was replicated five times. After 4 days of inoculation, 3 plugs of 6mm size were made from each Petri dish, weighed and transferred to 1.5 ml pre-sterilized vials. Then the plugs were homogenized with a plastic pestle and 1ml of extraction solvent (containing 10 µg nonyl acetate in 1ml of hexane) was added to the vials. The vials were vortexed for 30 seconds and approximately 800 µl supernatant was transferred to new glass vials. The fungal strains used in this experiment were *Endoconidiophora polonica*, *Grosmannia penicillata*, *Grosmannia europhioides*, *Ophiostoma bicolor*.

2.10.2. Quantitative analysis of fungal volatiles by gas chromatography-mass spectrometry and flame ionization detection

The solvent extracts were subjected to gas chromatography using an Agilent 6890 series gas chromatograph (Agilent, Santa Clara, CA, USA). 1 µl sample was injected using splitless mode. The mobile phase (carrier gas) was kept at a flow rate of 2 ml min^{-1}. The constituents were separated on a DB-5MS column (Agilent, 30 m × 0.25 mm × 0.25 µm) with a temperature gradient of 45 °C to 180 °C at 6 °C $min^{-1}$and then raised to 300 °C at 100 °C min^{-1}. For compound identification, the column outlet flow (He as carrier gas) was coupled to an Agilent 5973 quadrupole mass selective detector with interface temperature 270 °C, quadrupole temperature 150 °C, source temperature 230 °C and electron energy 70 eV. Each peak was identified by comparing its mass spectrum and retention time to those of authentic standards or spectra in reference libraries (NIST98 and Wiley 275). For compound quantification, the column outflow (H_2 as carrier gas) was coupled to a flame- ionization detector set at 300 °C. The amount of each compound was calculated from the peak area obtained in comparison with that of the internal standard and standardized to the weight of the agar plugs with normalized response factors for each compound.

2.11. Statistical analysis

All the statistical analyses were performed using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). For statistical analysis all the data were checked by the Shapiro- Wilk test for normality. For the fumigation toxicity test, the dose-response (non- linear regression) model was used. The log-transformed concentration values and normalized mortality data were used to determine the LC_{50}(Lethal concentration for killing 50% of beetles) values, standard error, 95% confidence interval, and Hill slope for each monoterpene. To analyze the differences in the LC_{50} of monoterpenes, an ordinary one-way ANOVA was done followed by Tukey's multiple comparisons on the log (LC_{50}) values. For the statistical difference between male and female beetles, unpaired t-test was done with Welch's correction.

For bark beetle tunneling assay, tunneling length and time course data were tested by Shapiro-Wilk test for normality and non- normal data were transformed with the log or log (1+Y) function. Transformed or non- transformed data were analyzed by using one or two- way ANOVA followed by Tukey's *post hoc* test for multiple comparisons (Geisser- Greenhouse's correction of sphericity for time-course data). For weight changes, data that did not follow Gaussian distribution were subjected to transformation to their absolute values or to log (1+Y). Then two- way ANOVA was used followed by Sidak's *post hoc* multiple comparisons test for comparisons between male and female groups. Antifeedant index (AFI) varying between -1 (stimulation) and +1 (repulsion) was calculated as follows (Kliepzig and Schlyter 1999): AI= (C-T)/(C+T), where C= mean tunneling length in the control group, T= mean tunneling length in treatment group. Then two- way ANOVA was used followed by Tukey's *post hoc* test for multiple comparisons. Effective threshold dose (ET) was calculated as the lowest dose for each compound at which the tunneling length becomes statistically lower than the control by one- way ANOVA followed by Dunnett's *post hoc* test for multiple comparisons.

For bark beetle boring probability assay, data were subjected to multiple logistic regression analysis to determine whether different doses of monoterpenes, sex, and the presence or absence of fungus (independent variables) can influence the boring probability of beetle (dependent variable) in monoterpene enriched medium. For binary events in the analysis, the

male has been coded as 1 and female as 0, the presence of fungus coded as 1 and absence of fungus as 0, boring inside the medium coded as 1 and not boring or staying outside the medium as 0. For each concentration of a single compound, we used a total of 30 beetles (15 male and 15 females). The specific logistic regression model fitted to the data was (including a model for two way interaction to check the effect of fungus on sex):

Logit [P(Y=1)] (Boring probability) = Ln[P(Y=1)/P(Y=0)] = b0 + b1+ b2 + b3 + b4 + b5 + b6

Here, b0 is constant. b1 (dose), b2 (sex), b3 (fungus), b4 (dose vs. sex), b5 (sex vs. fungus), b6 (dose vs. fungus) are logistic coefficients or estimates for the parameters, β1, β2, β3, β4, β5, β6 respectively. The results of boring probability assay were shown in terms of parameter estimates (β) and "odds ratio" which explains the relationship between two events. It can be either a negative or positive value. For instance, the boring probability is the main event and presence of fungus, different doses, and different sexes are secondary events. If the parameter estimates (β) is positive, it means the main event will increase in units by the increase in the odds ratio of 2nd event. If negative, then the main event will decrease. If the parameter estimates (β) is close to 1 or equal to 1, it means no change in the main event. The probability of success is calculated from the following equation:

Probability of success= (odds/1+ odds) × 100

The concentration of remaining monoterpenes and their fungal biotransformation products were subjected to ANOVA test followed by Sidak's *post hoc* test to test the differences among the different treatment combinations.

3. RESULTS

3.1. Fumigation toxicity and LC_{50} of tested monoterpenes

Non- linear regression analyses (Table 2) showed that different concentrations of monoterpenes had a positive relationship with the mortality of beetles and thus mortality rate increased with the increase in monoterpenes concentration. Here, LC_{50} varies with the identity of monoterpenes. Myrcene was the most toxic compound and (-)-bornyl acetate was the least toxic to *Ips typographus*. In general, the LC_{50} values for all the tested monoterpenes ranged from ~71µl/l to 2475µl/l, revealing a thirty-fold quantitative difference in the toxicity of monoterpenes between the least and most toxic monoterpenes. All the tested monoterpenes can be divided into three categories based on the degree of toxicity (LC_{50} values) as the most toxic, the intermediate toxic and the least toxic groups. Myrcene, (+)-limonene, 3-carene, the induced spruce monoterpene blend, (-)-limonene, the blend of trees that survived a bark beetle attack and the constitutive spruce blend were categorized as the most toxic group. (+)-α-Pinene, (-)-β-pinene, and (-)-α-pinene were assigned to the intermediate toxic group. (-)-Bornyl acetate was classified as the least toxic compound. In the most toxic group, myrcene was significantly more toxic compared to the other compounds ($p< 0.001$). Among the compounds in the intermediate group, (+)-α-pinene ($p< 0.001$) was significantly more toxic than (-)-β-pinene and (-)-α-pinene. The three blends (constitutive, induced and survived tree blends) showed varying LC_{50} values but they were not statistically different from each other. However, their LC_{50} values are lower than (-)-α-pinene, (+)-α-pinene and (-)-β-pinene which are the major components in the blends (Table 1). These results collectively showed that the addition of a small fraction of minor compounds such as myrcene, 3-carene and others lowered the LC_{50} values of the intermediate group and therefore blends are more effective than single compound.

3.1.1. Sex factor in the determination of LC_{50}

Sex was an important factor in determining LC_{50} values. Females were more susceptible to individual chemical vapors compared to males. There was no significant difference in mortality rate between males and females fumigated with myrcene, (+)-limonene and (-)-limonene, induced spruce blend, and constitutive spruce blend. (-)-α-Pinene showed the most significant difference ($p < 0.001$) in LC_{50} values between males (LC_{50}= 532.6) and females

(LC_{50}= 500). Similarly, (+)-α-pinene ($p < 0.01$), (-)-β-pinene ($p< 0.01$), 3-carene ($p < 0.05$) and (-)-bornyl acetate ($p < 0.05$) also showed significant difference in LC_{50} values between male and female groups with females being more susceptible than males to the chemical vapors.

Table 2: Toxicity of monoterpene volatiles against *Ips typographus*

Chemicals	LC_{50} [i] (combined sexes)	LC_{50} (individual sex)			95% CI[ii]	R^2	Hill Slope ± SE[iii]	df[iv]
Myrcene	71.46[a]	M F	71.96 60.79	ns	25.12 - 132.1	0.8933	1.138 ± 0.3139	5
(+)-Limonene	106.3[ab]	M F	106.5 105.3	ns	94.74 - 119.2	0.9955	2.110 ± 0.3139	5
3- Carene	110.8[ab]	M F	158.0 39.76	*	48.53 - 191.1	0.907	1.301 ± 0.3513	5
Induced blend	114.7[ab]	M F	108.6 123.8	ns	74.12 - 164.2	0.9555	1.931 ± 0.5003	5
(-)-Limonene	130.3[bc]	M F	142.4 118.2	ns	109.2 - 156.5	0.9889	2.531 ± 0.3982	5
Survived blend	176.4[bc]	M F	133.8 229.6	**	142.3 - 219.0	0.9869	1.832 ± 0.2382	5
Constitutive blend	184.5[bc]	M F	166.0 189.1	ns	149.1 - 231.2	0.9858	2.096 ± 0.3139	5
(+)-α-Pinene	223.4[c]	M F	282.8 181.2	**	176.1 - 287.7	0.9813	2.279 ± 0.3921	5
(-)-β-Pinene	516.1[d]	M F	546.1 500.0	**	463.7 - 555.4	0.9917	5.835 ± 1.180	5
(-)-α-Pinene	517.1[d]	M F	532.6 500.0	***	510.0 - 524.1	0.9997	6.102 ± 0.2450	5
(-)-Bornyl acetate	2475[e]	M F	2533 1176	*	2178 - 2884	0.9942	0.9123 ± 0.04606	6

[i]Lethal concentration (µL/L) required to kill 50% beetles; [ii]95% confidence interval of LC_{50} of combined sexes; [iii]Hill slope ± standard error; [iv]Degree of freedom; (*) = p<0.05, (**) = p<0.01, (***) = p<0.001, ns= not significant; M= Male, F= Female; LC_{50} values denoted with same lower case letters are not significantly different.

On the other hand, we observed an opposite trend for monoterpene blends especially for the "survived tree" blend where the LC_{50} for the males (133.8µl/l) differed significantly from females (229.6µl/l). These results collectively showed that toxicity of monoterpenes varies

between the sexes of the beetles, the concentrations, type of monoterpene and composition of the blends.

3.2. Bark beetle tunneling assay

The tunneling bioassay was initially carried out to estimate the toxicity of individual monoterpenes in artificial galleries filled with phloem based diet. Interestingly, none of the tested compounds caused mortality or any other effects on the beetle's vitality after 48 hours of feeding in the artificial diet. Rather, beetles in all the experimental groups gained weight regardless of the compound, sex, and doses. Generally, tunneling length increased slightly in all the tested chemicals at a lower dose (0.1 mg/g). At higher doses (1 mg/g to 10 mg/g; refer to individual compound; Figure 8), beetles made shorter tunnels. Overall, tunneling length was affected by all the considered factors such as type of compound, dose, weight gain, time and sex (Table 3).

Table 3: Statistical results of analysis of variance (ANOVA) on the influence of compounds, doses, weights, time points and sexes on the tunnel length made by adult beetles.

Factor	*F* value	*P*-value
Dose	19.34	*** (< 0.001)
Compound	10	*** (< 0.001)
Weight	6	*** (< 0.001)
Time	194.30	*** (< 0.001)
Sex	4.23	* (<0.05)

Data were transformed to log (1+Y) to approach normal distributions. The homoscedasticity of variances was tested by Bartlett's test. ANOVA was tested only on 0.1mg/g and 1mg/g as those were the only common doses among all the tested compounds.

3.2.1 Effect of dose and the type of compound on beetles' tunnel length

Concentration was an important factor influencing diet consumption by beetles. Except in (-)-bornyl acetate and (-)-limonene, beetles showed a concentration-dependent tunneling behavior for all the other compounds. Diet consumption slightly increased for certain chemicals at lower doses and in general resulted in decreased consumption at higher doses for all the compounds (Figure 8). Comparing the tunneling length of all the compounds at a specific low dose (0.1 mg/g), beetles made the longest tunnel in (-)-α-pinene compared to the control. At a

high dose (1 mg/g), only myrcene and (-)-bornyl acetate resulted in shorter tunnel length (Figure 9a and b) compared to their respective controls.

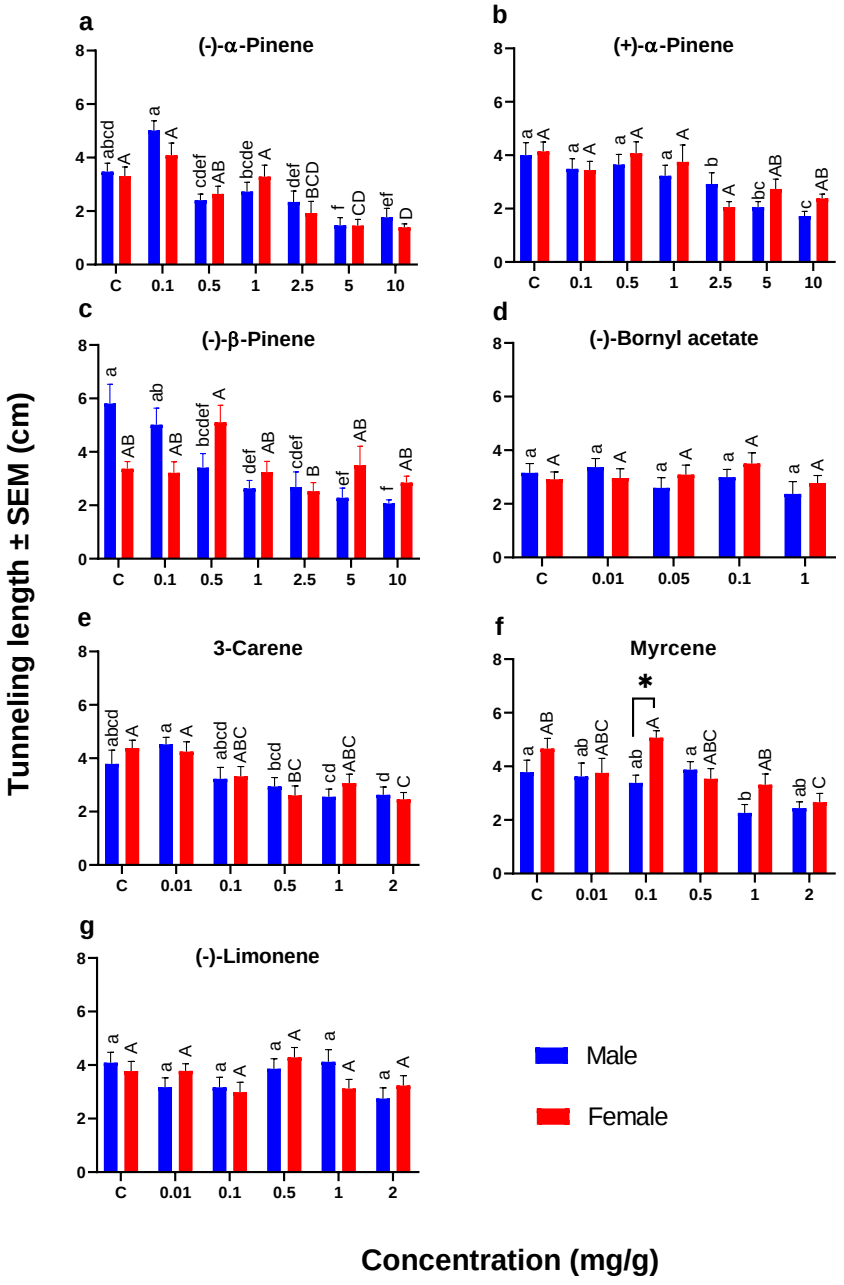


Figure 8: Tunneling length of *Ips typographus* in artificial galleries enriched with different doses of commonly found spruce tree monoterpenes. The X-axis shows different concentrations of monoterpenes and the Y-axis shows mean tunnel length in cm (n=15). C= control (only solvent mixed in artificial diet). The variation in tunnel length of adult beetles to different doses of each monoterpene was compared using two- way ANOVA followed by Tukey's *post hoc* test, data points with the same lower case letters (for males) and with same upper case letters (for females) are not significantly different. "*" denotes significant differences between males and females for a particular dose ($p < 0.05$).

Interestingly, different compounds showed activity at different concentrations. Among all the compounds, the lowest effective threshold dose (the lowest dose for each compound at which the tunneling length becomes statistically lower than the control) for 3-carene was at 0.5mg/g for both sexes (Table 4). Surprisingly, (-)-bornyl acetate and (-)-limonene did not affect adult bark beetle tunneling at all. No effective threshold can be found for these compounds and there was no significant difference in tunneling length among all the concentrations of these chemicals (Table 4, Figure 8d and g). At the lowest tested dose (0.1mg/g), the anti-feedant index was near zero (for males) or negative (for females) (Table 4 and Figure 10) indicating that the monoterpenes are neither feeding stimulants nor anti-feedants at the lowest tested dose. In spite of that, some of the compounds acted as weak feeding stimulus for females at the low dose (Figure 10). However, all the compounds at the higher dose (1 mg/g) acted as a slight antifeedant on males but most compounds elicited neutral response on females. The highest deterrent effect was observed for (-)-β-pinene at 1mg/g in males followed by myrcene. Over the time span of 48 hours, the beetle's diet consumption increased significantly over time.

Table 4: Effective Threshold (ET) and Antifeedant Index (AFI) values calculated for each compound.

Compound	ET mg/g (M)	ET mg/g (F)	AFI (M) at 0.1 mg/g	AFI (F) at 0.1 mg/g	AFI (M) at 1 mg/g	AFI (F) at 1 mg/g
(-)-α-Pinene	0.5	2.5	-0.18	-0.11	0.12	0.00
(+)-α-Pinene	2.5	2.5	0.07	0.09	0.17	0.05
(-)-β-Pinene	0.5	2.5	0.07	-0.01	0.38	-0.02
3-Carene	0.5	0.5	0.08	0.14	0.19	0.18
(-)-Bornyl acetate	no	no	0.03	-0.09	0.14	0.02
Myrcene	1	2	0.06	-0.04	0.25	0.17
(-)-Limonene	no	no	0.03	-0.06	0.14	0.09

Effective threshold dose (ET) was calculated as the lowest dose for each compound at which the tunneling length becomes statistically lower than the control (one- way ANOVA, Dunnett's *post hoc* test); M= male, F= female.

3.2.2. Weight change and sex factors

Weight change was an important factor affecting diet consumption by bark beetles. Overall, beetles gained weight in all the tested compounds. At the low dose (0.1 mg/g), beetles gained

more weight in myrcene enriched medium than in the control treatment ($p < 0.01$) and the lowest weight gain was observed in (-)-α-pinene enriched medium in comparison to the control group ($p < 0.01$) (Figure 9c). At the higher dose (1 mg/g), beetles gained more weight than the control group only in 3-carene amended medium ($p < 0.05$). Weight gain when feeding on other compounds was similar to the control group (Figure 9d). These clearly indicate that the tested monoterpenes, mixed in artificial spruce bark diet, were not anti-nutritional to beetles. Furthermore, sex was an important factor in beetles' weight gain. In most of the compounds, weight gain was significantly higher in females than in males (Figure 11). Although there was no significant difference in the tunneling length between males and females, females gained significantly more weight. Interestingly, in (-)-β-pinene and myrcene treatments, there was no significant difference between male and female weight gains (Figure 11a and f). Moreover, in (-)-bornyl acetate (at 1 mg/g; $p < 0.05$), (-)-α-pinene (at 10 mg/g; $p < 0.05$), and (-)-limonene (at 2 mg/g; $p < 0.05$) differences in the weight gain between males and females were significantly greater at higher doses compared to the lower doses of these chemicals (Figure 11 b, d, and g).

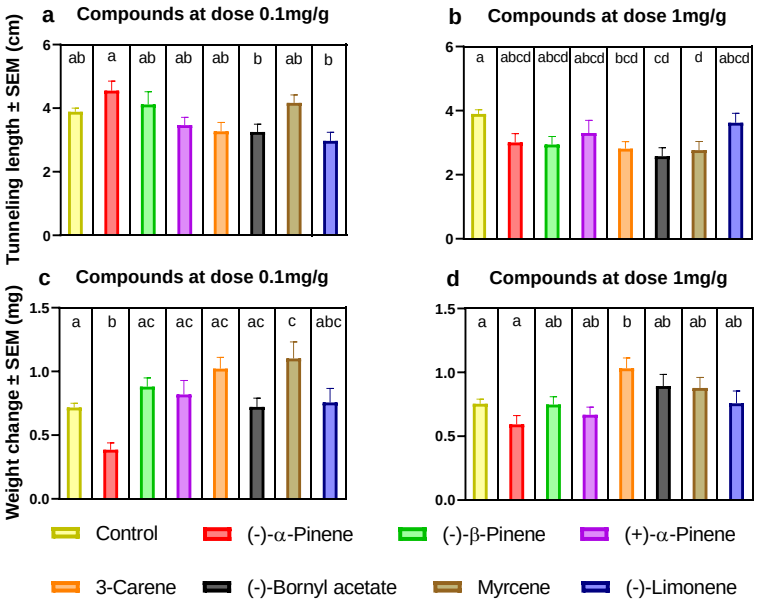


Figure 9: Tunnel length and weight gain variations in beetles among all the tested monoterpenes. (**a** and **b**) At low dose (0.1 mg/g) of most of the monoterpenes, beetles made tunnel length similar to the control but at high dose (1 mg/g) of 3-carene, (-)-bornyl acetate and myrcene beetles made significantly shorter tunnel than the control. (**c** and **d**) In general, beetles feeding on all the monoterpene-amended medium gained weight similar to the control. At the low dose of myrcene and at the high dose of 3-carene beetles gained the most weight. For each dose, data points denoted by the same letters are not significantly different (One- way ANOVA, Tukey's *post hoc* test)

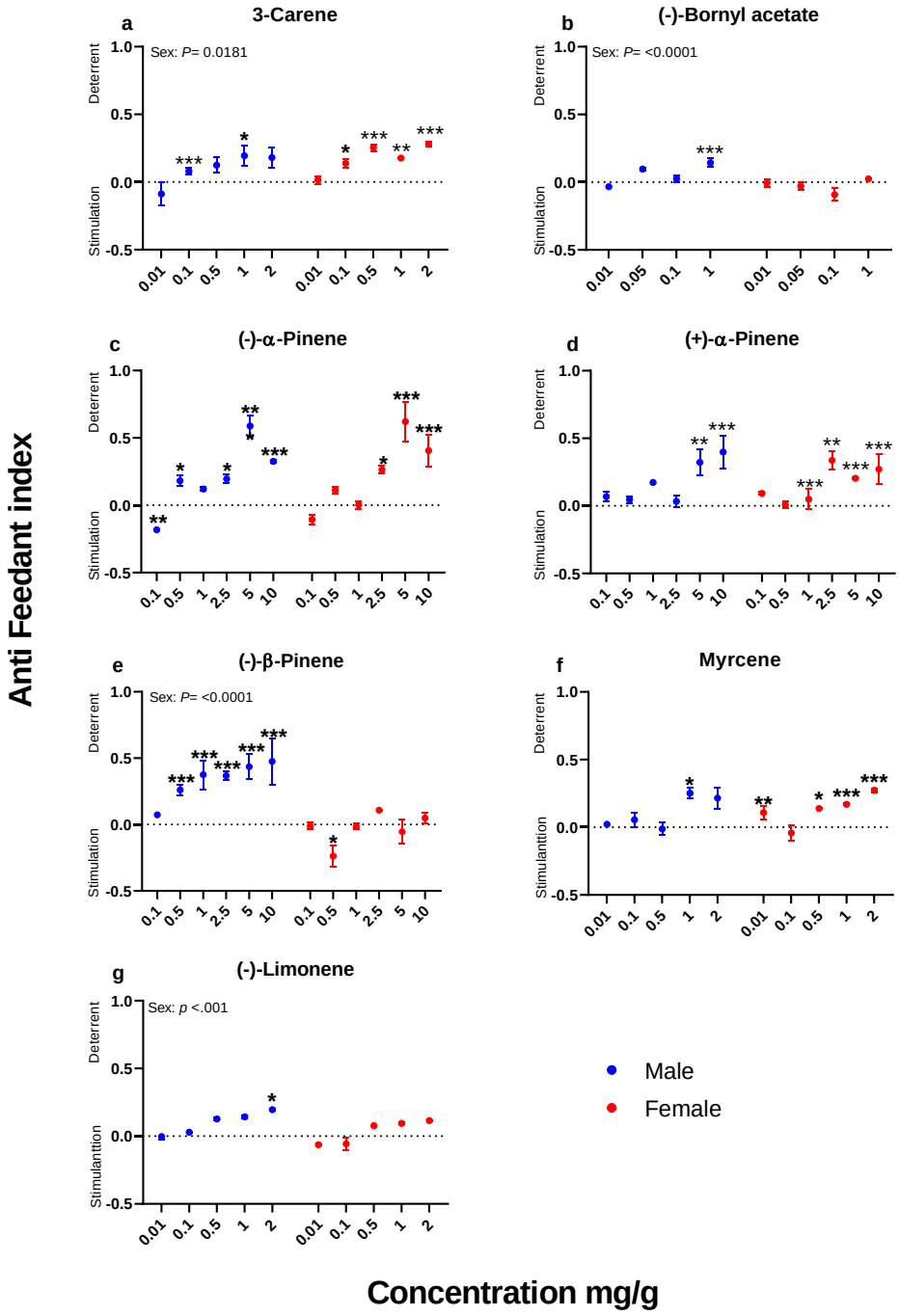


Figure 10: Anti-feedant index of the most common monoterpenes found in the bark of Norway spruce. Anti-feedant index (AFI) of -1 indicates strong feeding stimulant and +1 indicates a strong feeding deterrent which was calculated as means AFI= (C-T)/(C+T), where C= mean tunneling length of the control groups, T= mean tunneling length of treatment groups. Significance difference between control and each treatment group was denoted by asterisks- * (p<0.05), ** (p<0.01), *** (p<0.001) (Two- way ANOVA with Tukey's *post hoc* test).

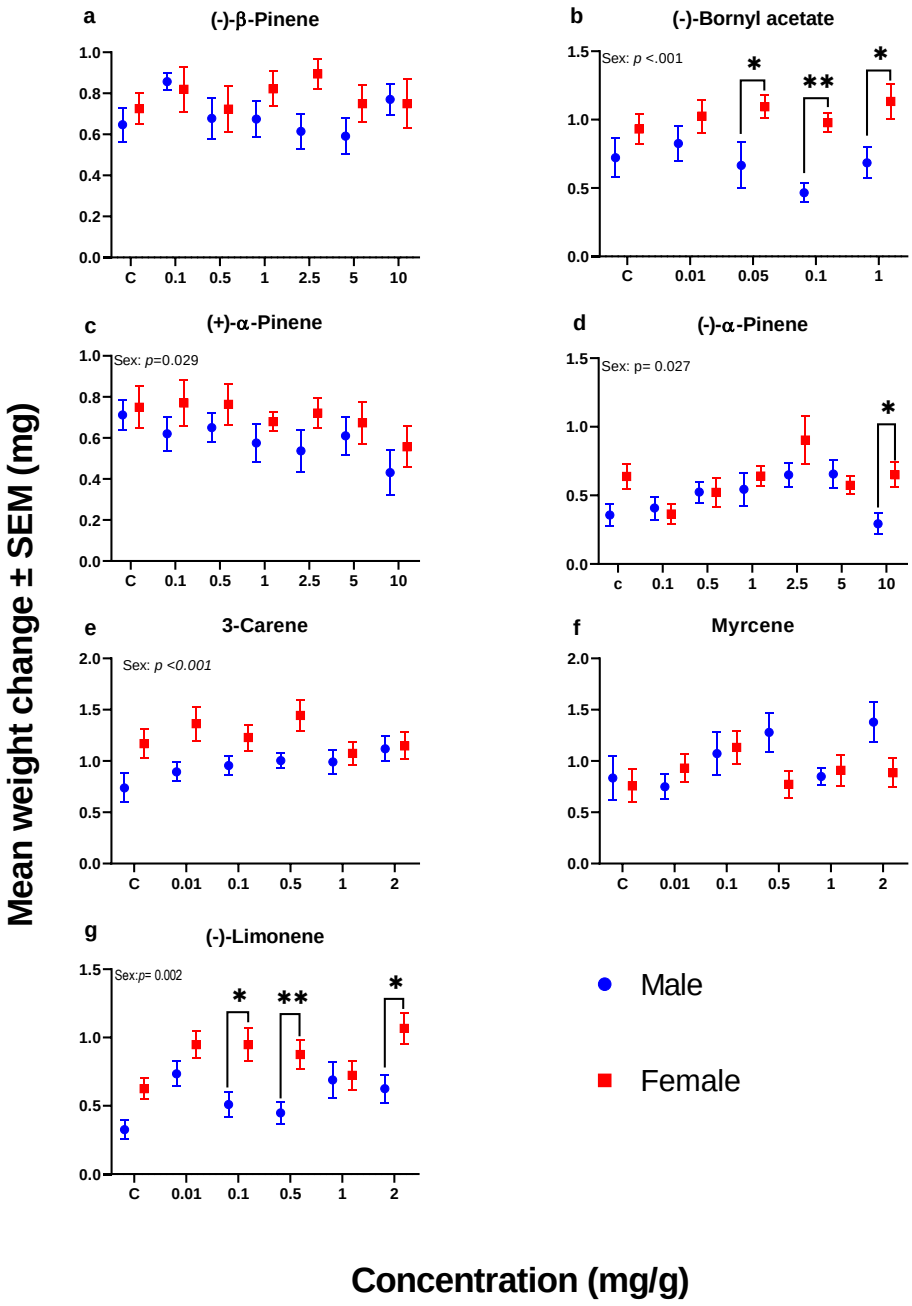


Figure 11: Effect of monoterpenes on beetle's weight change after 48 hours of feeding in the artificial diet. In general, all beetles gained considerable weight after 48 hours. For each compound, mean weight change after 48 hours for different concentrations and each sex was calculated (n= 15). C= Control (solvent+ diet); Data points denoted by * (p<0.05), ** (p<0.01) are statistically significant (two- way ANOVA, Sidak's *post hoc* test).

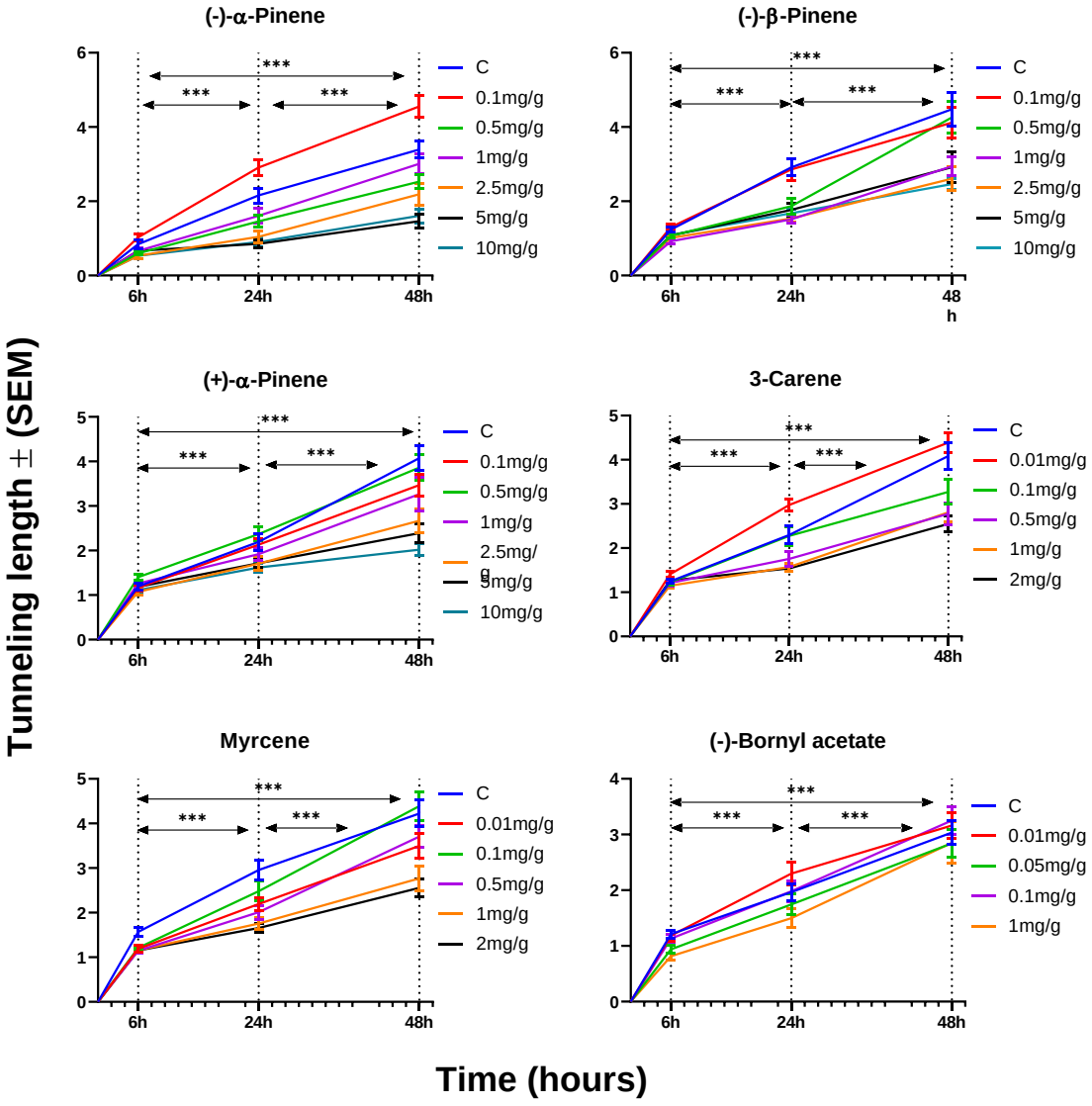


Figure 12: Time course analysis of bark beetles tunneling length in monoterpenes-amended artificial diet. In general, beetles tunneling length increased over time in all the monoterpenes-amended media. Significance differences between the time points were analyzed by two- way ANOVA followed by Tukey's *post hoc* test.

3.2.3. Tunneling length increased over time

Time was an important factor in the bark beetle tunneling assay. Beetles tunneling length in all the tested monoterpenes-enriched media was measured at 6h, 24h, and 48h after the introduction of beetles in the assay tube. In general, tunneling lengths in all the monoterpenes increased significantly over the course of time (Figure 12). Due to insufficient data, we excluded the result of (-)-limonene from the time-course analysis of tunneling length. Overall,

tunneling activity increased in all the beetles over time regardless of doses, sexes, and compounds.

3.3. Bark beetle boring behavior assay

From the previous experiments we found that tunneling length decreases at higher doses of monoterpene and hence we investigated if the presence of fungus in the monoterpene enriched medium can enhance the beetles' penetration or boring behavior.

3.3.1. Presence of fungus increased the beetles' boring behavior in monoterpene-amended diets

In general, the results of multiple logistic regression analysis suggest that presence of fungus in the diet resulted in a higher tendency of bark beetles to bore into the fungus colonized diet than the diet without fungus. For (-)-bornyl acetate, (-)-α-pinene and (+)-α-pinene, the full model considered all the three independent variables (fungus, dose and sex) together and was statistically significant (Hosmer- Lemeshow test; p = 0.47, 0.26 and 0.20 respectively; where *p*-value <0.05 indicates a faulty model). It is evident from the results that both the fungus and the sex factors affected the boring behavior of the beetles but not the dose. The model correctly classified approximately 84.33% of the cases for (-)-bornyl acetate, 80.71% for (-)-α-pinene and 85.35% for (+)-α-pinene. The "pseudo" R squared (0.42) estimation indicates that the model explained 42% of the variance in boring rate for (-)-bornyl acetate, and 22% (0.22) for (-)-α-pinene. The results predict that different doses of (-)-bornyl acetate in the medium were not the influencing factor in the beetle's boring behavior. Similarly, the different doses for (-)-α-pinene and (+)-α-pinene also did not regulate the boring behavior. The highest success probability of boring was in (-)-bornyl acetate amended medium and the lowest success probability was in (+)-α-pinene amended medium. The odds ratios signify that the presence of fungus could increase the beetle's boring behavior by 67.87 (98.55%), 42.01 (97.67%) and 3.51(77.83%) units in (-)-bornyl acetate, (-)-α-pinene and (+)-α-pinene enriched media respectively (Figure 13 and Table 5). For (-)-β-pinene, the full model considering all the three independent variables together was statistically significant (Hosmer- Lemeshow, *p*= 0.417). The results showed that only the fungus affects the boring behavior of the beetles but not the doses. The model correctly classified approximately 77.57% of the cases. The

"pseudo" R squared (0.30) estimates indicate that the model explained 30% of the variance in boring rate. The results predict that different doses of (-)-β-pinene in the medium didn't influence the beetle's boring behavior. Odds ratio shows that the presence of the fungus could increase the beetle's boring rate by 9.349 units and the success rate of probability of boring was 90.34% for (-)-β-pinene amended medium.

Table 5: Results from multiple logistic regression analysis predicting the probability of bark beetle boring into different monoterpenes-amended medium.

Compound	Predictors	Parameter estimates (β)	S.E[a]	*P* value[b]	Odds Ratio	95% C.I.[c]
(-)-β-Pinene	Dose	0.14	0.06	ns	1.16	1.03 - 1.32
	Sex	-0.1021	0.38	ns	0.9029	0.49 – 1.90
	Fungus	2.24	0.43	< 0.001 (***)	9.35	4.14 - 22.56
	Fungus vs. Sex	1.36	0.66	0.04 (*)	3.88	1.11 - 15.16
	Dose vs. Sex	-0.27	0.08	< 0.001 (***)	0.7603	0.64 - 0.89
(-)-Bornyl acetate	Dose	-0.65	0.44	ns	0.52	0.22 - 1.23
	Sex	-1.54	0.35	<.001 (***)	0.22	0.11 - 0.42
	Fungus	4.22	0.62	<.001 (***)	67.87	23.26 - 291.4
	Fungus vs. Sex	0.86	1.29	ns	2.37	0.10 - 27.72
(-)-α-Pinene	Dose	0.01	0.04	ns	1.01	0.91 - 1.09
	Sex	-0.58	0.28	0.038 (*)	0.56	0.32 - 0.97
	Fungus	3.74	0.60	<.001 (***)	42.01	15.24 - 174.1
	Fungus vs. Sex	0.30	0.22	ns	0.00	-0.13 – 0.47
(+)-α-Pinene	Dose	-0.02	0.049	ns	0.98	0.89 - 1.08
	Sex	-0.62	0.31	0.049 (*)	0.54	0.29 - 0.99
	Fungus	1.26	0.39	0.001 (**)	3.51	1.70 - 7.85
	Fungus vs. Sex	0.03	0.10	ns	1.03	0.85 - 1.25

[a] Standard Error of β
[b] *p* value of the individual parameter
[c] Confidence Interval of odds ratio = (Lower limit to Upper limit)
ns: not significant

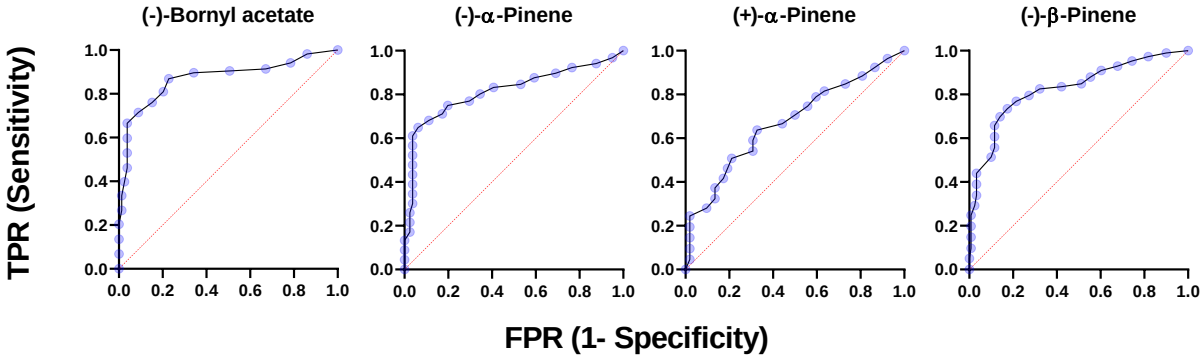


Figure 13: ROC (Receiver Operating Characteristic) curve of multiple logistic regression of monoterpenes in the boring probability assay. TPR= true positive rate; explaining all the successes under given parameters, FPR= false positive rate; explaining all the failure under given parameters such as the presence of fungus, doses, and sexes. The increase in blue dot lines over the red dot lines suggests the success of beetles boring probability when the fungus is present and the sex is female. The cutoff value was determined as 0.5 and all the successful events were considered as 1 and all the unsuccessful events as 0.

3.3.2. Females tunneled more readily into medium compared to males

In general, sex was an important factor in determining the boring behavior of bark beetles. All compounds showed that females had higher acceptance of the medium than males even in medium without fungus. The odds ratio of sex indicates that the boring probability increased by 0.22 units (18.03%) (p <0.001, β= -1.54) for (-)-bornyl acetate, 0.56 units (35.91%) for (-)-α-pinene (p <0.05, β= -0.58) and 0.54 units (35.06%) for (+)-α-pinene (p <0.05, β= -0.62) when the beetle was female in the medium without fungus (table 5). In the presence of fungus, females accepted the medium more readily than male in (-)-bornyl acetate (β= -1.70, probability of success = 15.25%, and p< 0.05) and (+)-α-pinene (β= -2.33, probability of success = 8.84%, and p< 0.05) amended media but for (-)-α-pinene both males and females had similar probability of boring (β= 0.03, p= 0.08). In (-)-β-pinene enriched media without fungus, although boring probability was higher for females than males, it was not statistically significant. The presence of fungus increased the male beetle's boring likelihood by 3.88 units or 79.51% (p <0.05) compared to the diet without fungus. Furthermore, in (-)-β-pinene amended media without fungus; females' boring activity was higher than males even at higher doses. Taken together, the results clearly showed that the tunneling behavior increased when the sex of the beetle was female and when fungus was present in the diet.

3.4. Quantification of monoterpenes from the monoterpene-amended medium colonized by bark beetle associated fungi

From the tunneling or feeding assay results, we found that the major monoterpenes in Norway spruce were not toxic to the beetles. However, it was evident from the results of boring behavior assay that the presence of symbiotic fungi can increase the boring activity of beetles in the medium containing monoterpenes. Hence, we aimed to see if the symbiotic fungi can reduce the amount of monoterpenes in the diet. Four major monoterpenes in Norway spruce were added separately to different Petri dishes containing PDA medium on which symbiotic fungi were inoculated. After 4 days of inoculation, the concentration of the remaining monoterpenes on fungus colonized medium was quantified by GC-FID. In total four different fungi were tested. From the GC-FID analysis, we did not observe a significant reduction in the amount of all the monoterpenes in the fungus colonized medium except for (-)-bornyl acetate. In different treatment combinations of (-)-bornyl acetate with different fungi, only *G. penicillata* (66.96%, $p < 0.05$; t= 3.25, dF= 29) and *G. europhioides* (86.09%, $p < 0.05$; t= 3.65, dF= 29) reduced the concentration of (-)-bornyl acetate significantly in comparison to the control (Figure 14 a).

3.5. Identification of fungal biotransformation products of monoterpenes by GC-MS

As it was known from previous studies that symbiotic fungi can metabolize the major monoterpenes, we aimed to identify the volatile fungal biotransformation products of monoterpenes from the solvent extracts of fungus inoculated medium enriched with monoterpenes. The identified biotransformed products of (-)-bornyl acetate were borneol and camphor. All the four fungi grown on PDA supplemented with (-)-bornyl acetate produced borneol and the significantly highest concentration of borneol ($p < 0.001$) was produced by *G. europhioides* (Figure 15) The other fungi such as *O. bicolor*, *E. polonica* and *G. penicillata* produced lower amounts of borneol than *G. europhioides* but the amount of borneol in *O. bicolor* and *E. polonica* were significantly lower than the others. Except for *E. polonica*, all the other fungi produced a similar amount of camphor.

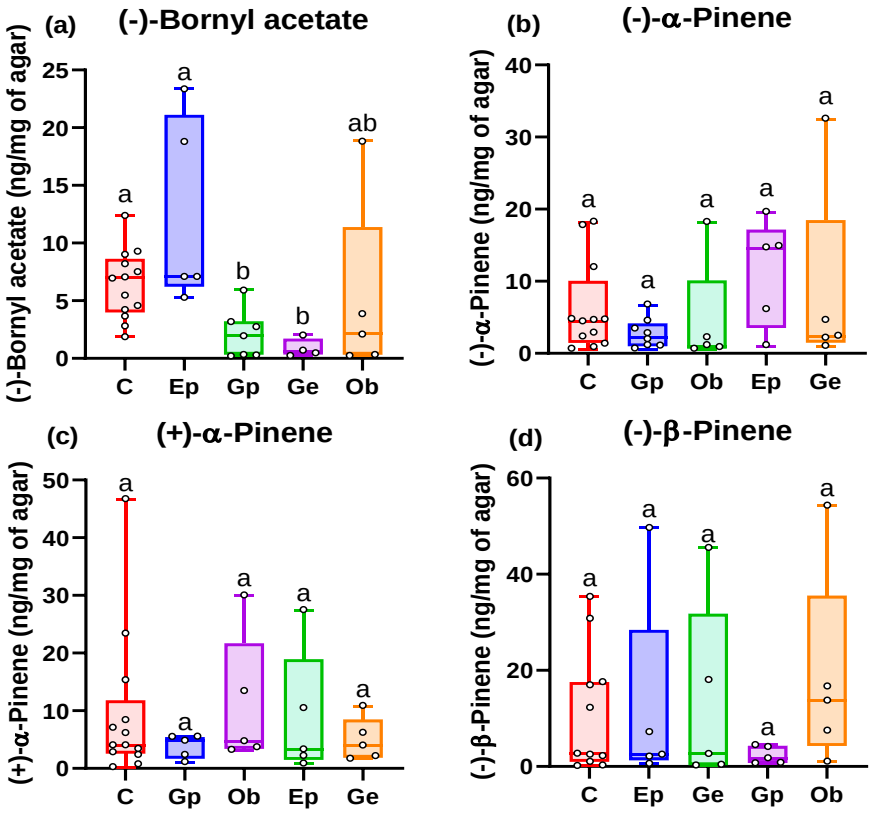


Figure 14: Concentration of remaining monoterpenes left in the PDA medium after four days of fungal growth. C= control containing only the specific monoterpene. For each compound, groups of data points denoted by the same letters are not significantly different.

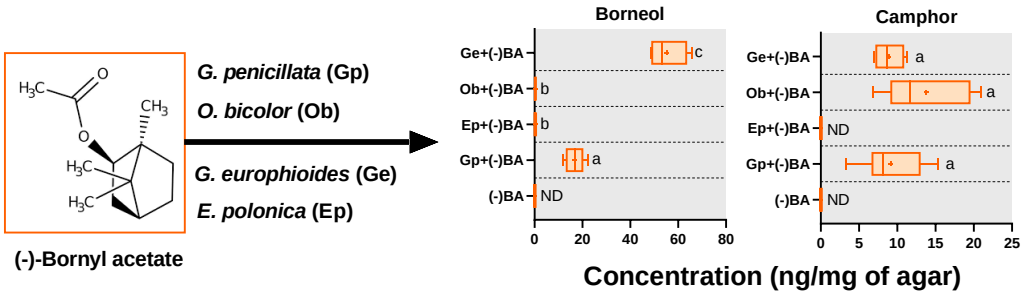


Figure 15: Oxygenated monoterpenes were produced from the biotransformation of (-)-bornyl acetate by fungi. Borneol and camphor were the only identified biotransformation products. ND: not detected. Data points with similar letters are not significantly different.

The major fungal biotransformation products of (-)-α-pinene, were terpinen-4-ol and α-terpineol. Only *G. penicillata* grown on (-)-α-pinene amended PDA medium produced these oxygenated biotransformation products and these compound were not detected from other fungi grown on the same media. 2-phenylethanol and 2-phenylethyl acetate were also detected as fungal volatiles. The concentration of 2-phenylethanol was significantly higher in *G.*

penicillata culture than in culture of the other fungi. 2-phenylethyl acetate was significantly higher in *E. polonica* (Figure 16). *cis*-Verbenol, the precursor of verbenone was identified only from (-)-α-pinene amended PDA medium without fungus and on *E. polonica* inoculated (-)-α-pinene amended PDA medium. Verbenone was found on all the treatments and in significantly higher amounts in *G. europhioides* inoculated (-)-α-pinene amended PDA medium compared to the control and other fungi.

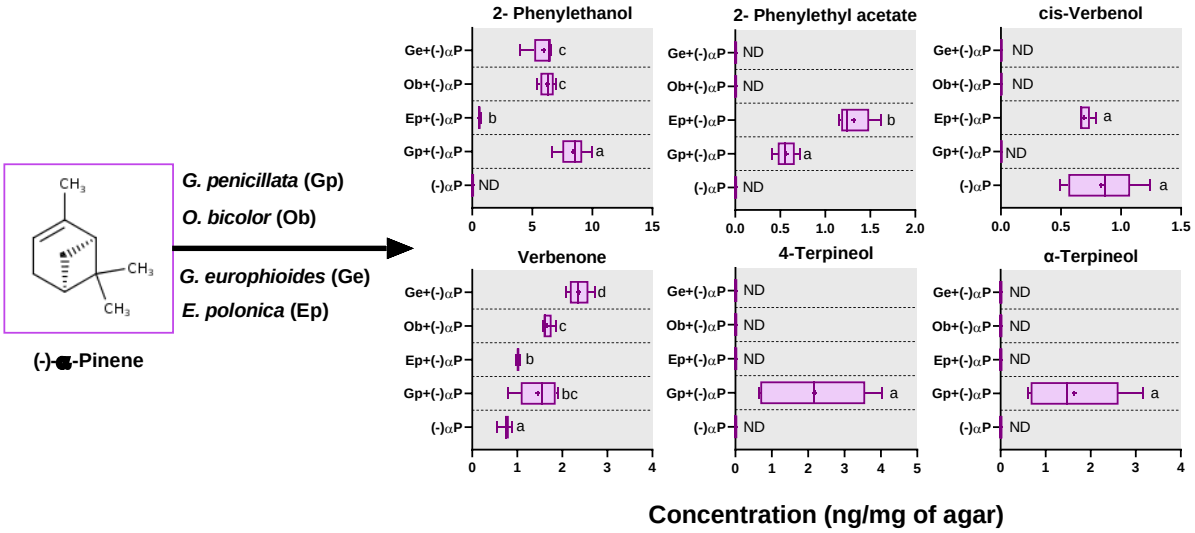


Figure 16: Volatiles produced from the biotransformation of (-)-α-pinene by different fungi. Terpinen-4-ol and α-terpineol were the major biotransformation products of (-)-α-pinene. *G. penicillata* was the most effective fungus in the biotransformation of (-)-α-pinene. ND: not detected. Data points with similar letters are not significantly different.

Similar to (-)-α-pinene, the major biotransformed products from (+)-α-pinene and (-)-β-pinene, were 4-terpineol and α-terpineol. *Grosmannia penicillata* was significantly more effective compared to the other fungi in biotransformation of (-)-α-pinene and (-)-β-pinene (Figure 17 and 18). Similar to (-)-α-pinene, *cis*-verbenol the precursor of verbenone was identified only from (+)-α-pinene amended PDA medium without fungus and on *E. polonica* inoculated (-)-α-pinene amended PDA medium. Similar to (-)-α-pinene, verbenone was produced in higher amounts in *G. europhioides* medium compared to the control and other fungi. The concentration of 2-phenylethanol was significantly higher in *G. penicillata* than in the other fungi. 2-phenylethyl acetate was significantly higher in *E. polonica* (Figure 17). Furthermore, in (-)-β-pinene supplemented medium, *G. penicillata* produced a significantly higher amount of 2-phenylethanol than the other fungi. Moreover, *E. polonica* yielded a significantly higher concentration of 2-pheylethyl acetate in (-)-β-pinene amended medium.

Finally, *O. bicolor* produced a significantly higher amount of camphor and *G. europhioides* produced significantly more borneol in (-)-β-pinene amended medium (Figure 18).

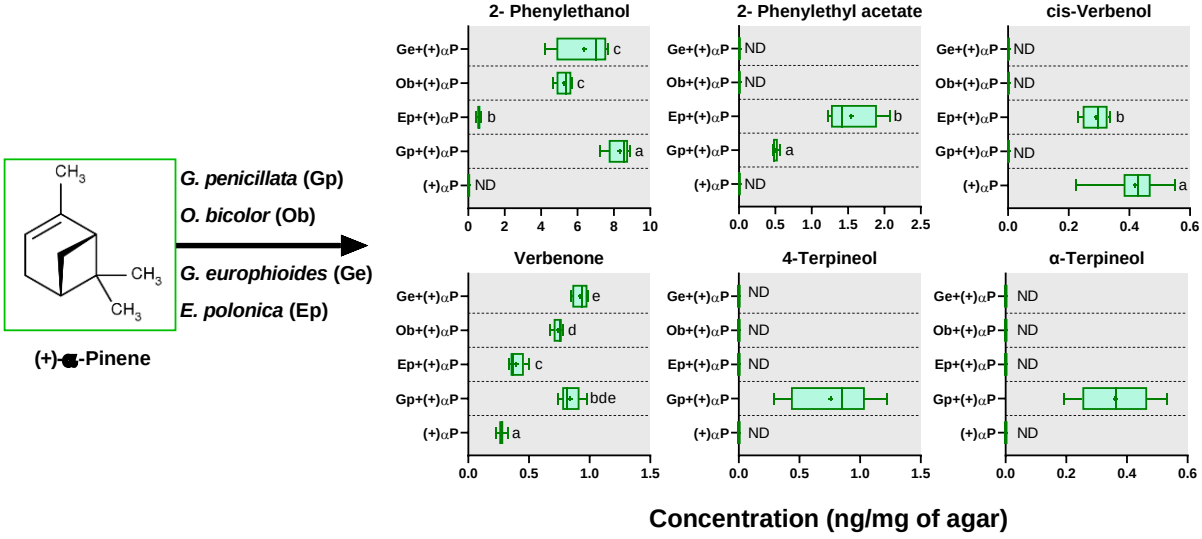


Figure 17: Fungal volatiles and oxygenated monoterpenes were produced from biotransformation of (-)-α-pinene by different fungi. Terpinen-4-ol and α-terpineol, verbenone were the biotransformation products. *G. penicillata* was the most effective in biotransforming (-)-α-pinene. ND: not detected. Data points denoted with similar letters are not significantly different.

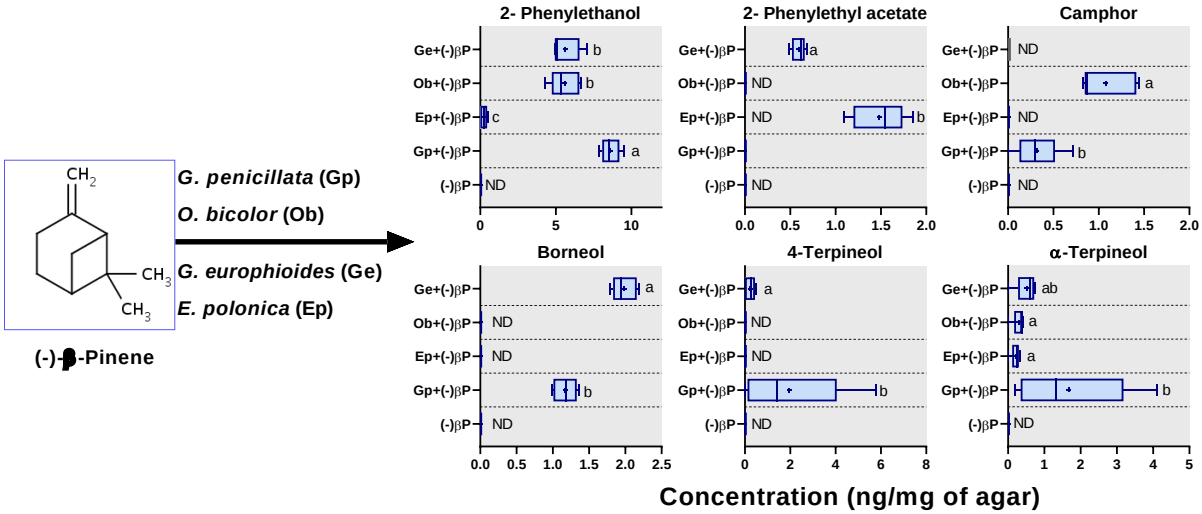


Figure 18: Fungal volatiles and oxygenated monoterpenes were produced from the biotransformation of (-)-β-pinene by different fungi. Terpinen-4-ol and α-terpineol were the major and camphor, and borneol were the minor biotransformation products. *G. penicillata* was the most competent fungus in the biotransformation of (-)-β-pinene. ND: not detected. Data points represented with similar letters are not significantly different.

4. DISCUSSION

4.1. Vapor phase toxicity of monoterpenes to bark beetles

Monoterpenes can be exposed to bark beetles as volatiles, through physical contact and by feeding as well. In this study, we tested the toxicity of common monoterpenes as volatile/fumigant and also through feeding. We considered only the mortality as a form of toxicity and excluded other toxicological behaviors in beetles such as paralysis, seizures, agitation, hyperactivity, etc. In our study, we used only the adult beetles and hence our whole study was focused on the effect of monoterpenes on adult beetles. Here, we reported the results of fumigant toxicity as LC_{50} values in µl/l unit which can give an idea of a relative ranking of monoterpenes based on their toxicity levels. The average length of a bark beetle galley in natural habitat is 120 mm [47] and the width or diameter of a tunnel is 3mm. Therefore an empty single paternal gallery contains ca. 0.8 ml headspace volume. Based on the volume of a tunnel in a natural gallery, it is possible to correlate the toxic concentrations in this study with the concentrations of the monoterpenes present naturally in resin ducts. In general, the constitutive doses may not be toxic but induced doses appear to be lethal to the bark beetles. For example, the concentration of (-)-α-pinene in spruce tree during constitutive phase is approximately 1mg/g and during induced phase is approximately 10 mg/g [25]. Considering 0.8 ml volume of a tunnel, the constitutive defense concentration of (-)-α-pinene in the spruce tree (i.e. 1 mg/g or 1000 µl/l) may not be toxic to the beetles in volatile form as LC_{99} of (-)-α-pinene is 1100 µl/l (approx.). However, the induced concentration of (-)-α-pinene (i.e. 10 mg/g or 10,000 µl/l) can be very toxic to the beetles. Therefore the present study experimentally confirms that prevailing hypothesis that the induced defense with altered ratio and concentrations of defense chemicals are very effective in inhibiting the colonization or even killing bark beetles.

Based on the chemical structure, the most toxic compound we tested was myrcene which was the only acyclic compound with most unsaturation. The bicyclic compounds such as both enantiomers of α-pinene and β-pinene were less toxic than myrcene. The least toxic compound, (-)-bornyl acetate was the only oxygenated compound in our study. The structure activity relationship studies showed that chemical structures and molecular parameters of terpenes are correlated to their insecticidal activity to lice, cockroaches and Triatominae bugs.

It was also showed that acyclic monoterpens or monoterpenes having fewer rings in their structure elicited higher activity and monocyclic monoterpenes were more toxic than bicyclic monoterpenes [48].

Several studies reported the mode of monoterpene toxicity on insects. At molecular level monoterpenes can affect several components of insects' nervous system. A high concentration of monoterpene can decrease the amount of cyclic adenosine monophosphate which is a secondary messenger in intracellular signal transduction and also block tyramine and octapamine receptor [49]. Monoterpenes can also inhibit the enzyme acetylcholinesterase which catalyzes the breakdown of several neurotransmitters [50]. Some of the monoterpenoids have been shown to bind to GABA receptors due to their structural similarity and chemical properties [31].

4.2. The blends of monoterpenes are more toxic than the individual compounds

The number of *Ips typographus* beetles colonizing severely weakened trees can drastically ascend to epidemic levels, usually after windstorm or long drought cycles, and then destroy large areas of Norway spruce forests [51]. To find a suitable host, the pioneer beetles use semiochemicals from both host and non- host trees as they have a similar number of olfactory receptor neurons for detecting both host and nonhost chemical odors [52], [53]. Beetles are able to recognize the host suitability for breeding and this suitability depends on host defense chemicals and phloem nutrients etc. Primary guidance towards suitable host can be an attraction from host monoterpenes or other stress signals from individual trees [54]. However, the decision to accept or reject the host depends on host chemical properties [25]. It has been found that host trees increase their monoterpene contents in the bark as a defense strategy and induce the production of defense chemicals to tackle the beetles' attack [29]. A recent study showed that the trees that survived after the bark beetle attack had a strong induced defense against *Ips typographus*. It is noteworthy that these trees were attacked by the pioneer beetles but were not joined by conspecifics in mass number. Some of the beetles were either trapped in resin flow or abandoned their entry holes perhaps due to the severity of defense chemicals. Notably, those survived trees had an increase in (-)-α-pinene, myrcene and 3-carene [25] which was found to be highly toxic to the adult beetles. Some other studies also reported the pivotal role of induced defense in repelling the beetles' mass attack [29], [55], [56]. In Norway

spruce, the most abundant monoterpenes during the constitutive defense phase prior to beetle's attack are (-)-α-pinene (approximately 24%), (+)-α-pinene (approx. 29%), (-)-β-pinene (approx. 34%). In our study, these compounds showed a low range of toxicity among all the tested compounds. Nevertheless, the toxicity trend of these three compounds against *Ips typographus* seems consistent with mountain pine beetles [32]. Besides, (+) and (-) enantiomers of limonene and 3-carene possess mid to high range of toxicity to mountain pine beetles which is also consistent with our study. Conversely, myrcene which is found relatively in lower amount (approx. 3%) in Norway spruce tree showed high toxicity with LC_{50} of 71.46 µl/l. Myrcene had been shown to be increased in amount as a consequence of induced defense response in the tree. A similar study on monoterpene toxicity also reported high toxicity of myrcene with LC_{50} of 69 µg/g when applied topically on *Ips typographus* [57]. Moreover, myrcene and limonene have been reported to be highly toxic to *Dentroctonus rufipennis* and *Dendroctonus simplex* [58].

Among the three different blends we used in our study, the induced defense blend showed medium to high range of toxicity and the other two blends were in low to mid-range. The monoterpene blends were clearly more toxic than the dominant monoterpenes such as (-)-α-pinene, (+)-α-pinene, (-)-β-pinene indicating that blends of monoterpenes are more effective than individual compounds and also the composition and the ratio of individual compounds determine the toxicity of the respective blends used in this study. However, monoterpenes ratio in the blends depends on the specific genes expression in trees which ultimately leads to the increase or decrease of individual monoterpenes in the total monoterpene profile. Norway spruce contains a multi-member monoterpene synthase gene family and the expression of these genes regulates the diversity and variation in monoterpene profiles in the host tree. The expression of monoterpene synthase genes is not same in all the individual trees [59]. The monoterpene profiles in an individual tree can be changed in response to beetles attack due to inducible changes in monoterpene synthase genes expression [60] and many of these genes can produce multiple monoterpenes within the same tree thus contributing to the diversity of monoterpene profile. Previous studies have functionally characterized four monoterpene synthase genes that encode for 3-carene synthase, myrcene synthase, (-)-α-pinene synthase, (-)-β-pinene synthase enzymes [59], [60]. These enzymes contribute to the production of monoterpenes that are highly increased during induced responses. The induced defense is

considered to be more important than constitutive defense for trees in response to the bark beetles- fungal symbionts attack [28]. Conifers also release a large amount of airborne volatiles during induced phase which may interfere with the host selection by bark beetles [38], [56]. Similarly, we found that the induced mixture was more toxic than the other mixtures. Collectively, trees employ induced defense strategy by increasing the production of toxic resins to resist the bark beetles attack and to repel them.

4.3. Males were more tolerant to individual monoterpene vapor than females

Another important aspect of our study was that males, pioneer beetle in host selection, were more tolerant to the monoterpene vapors than females. Though there was no significant difference between male and female survival rates at high doses of monoterpenes, the male showed a higher survival rate at low doses of highly abundant monoterpenes such as (-)-α-pinene, (+)-α-pinene and (-)-β-pinene. Male beetles can produce *cis*-verbenol from (-)-α-pinene which they employ as primary aggregation pheromone. Besides, they can also produce *trans*-verbenol from (+)-α-pinene which is used in regulating the attack density [61], [62]. Moreover, they can also produce myrtenol from α-pinene and *trans*-myrtanol from β-pinene [62], [63]. These compounds are reported to be synthesized by the beetles itself by using the monoterpene precursors. Furthermore, an earlier study reported that beetles possess myrcene resistant gut bacteria [64]. Therefore, the higher survival rate of male beetles in the presence of highly abundant monoterpene vapors can be due to the fact that they could convert those monoterpenes to their oxygenated forms. Since the males are the pioneers in identifying suitable healthy or stressed host tree which generally contains high amounts of defense chemicals in the early stages of attack, it is plausible to say that males inheritably have high tolerance to defense chemicals compared to females. Females of *Dendroctonus ponderosae*, the pioneer sex in host selection, were reported to survive better than males when they were exposed to monoterpene vapor [32]. Male cockroaches were reported to be four times more susceptible than the females to monoterpene vapors [65]. However, an opposite scenario has been observed for blends where females were more tolerant than males. Survived tree blend showed females were significantly more tolerant than males. Nevertheless, beetles ability to degrade the monoterpenes is correlated to the expression of cytochrome P450 enzymes. Keeling *et al.* reported that the mountain pine beetle possesses cytochrome P450 enzymes in

their antennae that hydroxylates or epoxidizes α-pinene, β-pinene, limonene, 3-carene [66]–[68]. *Ips pini* contains cytochrome CYP9T2 that hydroxylates myrcene to ipsdienol [69] and in male *Ips paraconfusus*, cytochrome CYTP9T1 was reported to be up-regulated up to five folds after feeding on monoterpene enriched phloem diet [70]. However, the mechanism of tolerance to monoterpenes by a specific sex such as male beetles in *I. typographus* is not known.

4.4. Monoterpenes mixed with artificial diet are not toxic to beetles

On the contrary to fumigant toxicity assay, we have observed a different scenario in the tunneling bioassay. In tunneling assay, beetles were kept in the artificial diet enriched with different monoterpenes for up to 48 hours and yet we did not observe mortality in beetles even at higher doses. Previous studies have shown that tunneling assay in monoterpenes, oxygenated monoterpenes, and in phenolics enriched media did not cause any mortality in *Ips typographus* [45], [71], [72]. This could be due to the fact that these chemicals show toxicity on insects through several routes such as through inhalation, through feeding or ingestion, and dermal contact. Toxicity through inhalation normally occurs faster than ingestion since it can affects the nervous systems directly [48]. Through feeding, insects can metabolize and excrete the toxicants more efficiently than through inhalation. Studies showed that monoterpenes toxicity were higher during fumigation assays compared to contact and feeding assays in darkling beetles *Alphitobius diaperinus* and in Colorado potato beetles *Leptinotarsa decemlineata* [73], [74]. Overall, beetles tunneling activity was higher in lower doses which was decreased with the increase in dose concentrations. Several other studies showed that conifer phloem, their outer bark extracts and even those of angiosperms contain secondary metabolites that exhibit feeding stimulant behavior in bark beetles such as *Ips paraconfusus, Dendroctonus frontalis, dendroctonus ponderosa, Scolytus multistriatus and Scolytus rugulosus* [75]–[78]. In our experiment, we allowed the beetles to tunnel up to 48 hours and measured their tunneling length after 6h, 24h and 48h and notably their tunnel length significantly increased after each observation. Furthermore, beetles feeding on all the monoterpenes added media gained weight similar to or higher than the control groups. Therefore, unlike phenolics which are anti-nutritional to the bark beetles, monoterpenes had no effect on the nutrition of bark beetles [72].

4.5. Females gained more weight compared to males and each sex behaved differently to monoterpene enriched diet

We observed a significant difference in feeding behavior between male and female beetles. For males, almost all the tested monoterpenes acted as either neutral or weak stimulant at lower doses but acted as weak anti- feedant at higher doses. On the other hand, higher doses of most of the monoterpenes had neutral effect on females' tunneling behavior. Our results appeared to be consistent with the previous studies that diet with monoterpenes are feeding stimulant for females but feeding deterrent for males [45]. Another important finding from our study was that females gained more weight than males generally, though there were no significant differences in the tunneling length between males and females. Feeding is not equal to the tunneling length and diet is removed as beetles often tunnel but do not necessarily mean that they feed the same amount [45], [71]. Therefore high weight gain in females could be explained by increased consumption of the diet by females during tunneling. On the other hand, due to the sensitivity to monoterpenes, males might have consumed less diet albeit made tunnel length similar to females. Since males are pioneers in host selection, they are more sensitive to repellents and antifeedants. The females were reported to be 10 fold less sensitive to (-)-α-pinene than males [53], [79].

Host selection in bark beetles depends on several aspects of tree physiology of which plant secondary metabolites are very important. The choice of host tree by pioneer beetles depends on the concentration of the defense compounds and beetle species. Besides, after landing on the tree, the feeding activity increases or decreases based on the concentration gradient of the host chemicals [54]. Naturally male and female beetles of *Ips typographus* have different roles in the host tree and their sensitivity to the host chemicals can also differ from each other. The differences in sex-specific sensitivity to host monoterpenes could lead to variation in boring, tunneling and feeding behavior of each sex. Males initiates the nuptial chamber by boring into the bark, the females then continue excavating long oviposition chamber to lay eggs. During oviposition, females that make longer egg galleries are usually exposed to the phloem monoterpenes to a greater extent than males. Thus sex-specific roles could explain the behavioral difference in bark beetles.

4.6. Symbiotic fungi are a key factor in host colonization by bark beetles

It is evident that the boring probability of females in general was higher than males in both media with fungi and without fungi. As discussed previously that females are more adapted to the host monoterpenes and males are more sensitive, therefore females boring probability is expected to be higher compared to males in the media without fungi. Even in fungus colonized media, females showed slightly higher boring tendency than males. In general, bark beetles preferred the diet colonized by fungus irrespective of different concentration of amended monoterpenes. Their boring behavior increased even at high doses of monoterpene enriched medium when fungus was present. This shows that in the presence of fungus, the type of monoterpenes and their concentrations have no influence on the boring behavior of beetles. It can be due to the fact that the fungi can biotransform the monoterpenes producing oxidized monoterpenes that are less or not toxic to the beetles [80], [81]. Besides, previous studies showed that bark beetles possess olfactory sensory neurons (OSNs) such as verbenone OSN, isopinocamphone OSN, and 4-thujanol OSN through which they can detect the oxidized monoterpenes [36]. This shows that the biotransformation products are ecologically important for bark beetles and might help them to evaluate the quality of the food sources, infestation status of tree (high or low density attack), defense status of the tree [82], [83]. The biotransformed volatile cues may also indicate fully colonized trees and signal the incoming new beetles to find an alternative host to avoid intraspecific competition or crowding. For example, verbenone, an anti-aggregation pheromone which is produced in part by fungi, exert repellent effects on incoming bark beetles [84], [85]. Furthermore, fungi produce certain alcoholic compounds that attract the beetles [36].

4.7. Symbiotic fungi metabolize tree defense chemicals

Fungi did not reduce the amount of (-)-α-pinene, (+)-α-pinene and (-)-β-pinene after 4 days post- inoculation. However, the amount of (-)-bornyl acetate was reduced significantly in fungus colonized medium. No reduction in the concentration of (-)-α-pinene, (+)-α-pinene and (-)-β-pinene in fungus colonized media was unexpected as several reports suggested that fungi degrades/ reduce the amount of MTs in the bark [36], [86], [87]. Probable reasons could be that the extraction method was less efficient, the concentration of monoterpenes added in the medium might not be enough or four days of fungal growth was too short for

biotransformation of monoterpenes. Another reason might be that the added monoterpenes were not equally distributed all over and perhaps localized in a particular region of the media. However, assessment of monoterpene degradation by fungi and their biotransformation compounds indicates how bark beetles can overcome monoterpene toxicity in the host tree using fungi and the role of fungal biotransformation production in the chemical communication with conspecifics.

5. OUTLOOK AND FUTURE EXPERIMENTS

This thesis provides considerable evidence that male and female beetles behave differently towards host defense chemicals. Future experiments should focus on investigating the role of specific sex in the life cycle of *Ips typographus*. Therefore, the tunneling performance of larvae and callow adult beetles on monoterpenes-amended diets has to be investigated. Olfactometer assays have to be done to elucidate the behavior of beetles towards monoterpene biotransformation products. Since sex specific response was found in toxicity assays, it would be worth to investigate the sex specific olfactory preferences towards fungal volatiles, host monoterpenes and its fungal biotransformation products. It will also be interesting to assess the boring probability of beetles in other symbiotic fungi grown media since those fungi are also efficient in metabolizing monoterpenes and phenolics. Phenolics are also abundant in Norway spruce tree along with terpenes. A little is known about the toxicity of phenolics and oxygenated monoterpene volatiles. More research has to be done with phenolics amended media to investigate the performance of male and female *Ips typographus*. Besides, investigation should be done to elucidate the reason of higher weight gain in females which can indicate the possible differences in gut microbiome composition between the sexes. Thus, sex specific role of beetles can be a crucial factor in pest management strategy.

The quantification of remaining monoterpene concentration in fungi inoculated media was less efficient and therefore we could not observe a significant reduction in most of the monoterpenes tested. The monoterpenes mixed with the diet were perhaps suspended in a particular region of the media and were not equally distributed. Better optimizations of experimental procedures are needed.

6. CONCLUSION

In this thesis, I showed a clear difference in the performance between male and female adult beetle which was merely focused in previous studies. Male beetles are more sensitive to the monoterpenes added in the diet than the females in terms of feeding, weight gain and boring tendency. Though the olfactory neurons in the beetles' antenna are similarly wired in both male and female beetles, it is not yet well known how the chemical information is processed in the higher brain centers. As sex specific responses have been found, sex specific preventive measures can be developed and tested to control the population of this beetle species. An earlier study shows that addition of non- host volatiles increases the beetles' sex ratio towards females in pheromone bait [53].

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ACKNOWLEDGMENTS

At first, I would like to express my sincere gratitude to **Dr. Dineshkumar Kandasamy** for his continuous support, mentoring and assistance during my master thesis and other research projects, and for always taking the time to help and give useful feedback. It was a wonderful experience for me to learn under his supervision. I thank him for sharing his experiences, scientific discussions and fun stuff.

I would like to thank **Prof. Dr. Jonathan Gershenzon, Dr. Almuth Hammerbacher** and **Dr. Franziska Eberl** for constant advice and support. Furthermore, I would like to thank **Prof. Dr. Jonathan Gershenzon** for giving me the opportunity to carry out my thesis work and to participate in other exciting projects.

I am also very thankful to all the members of the MPI- Chemical Ecology in Jena, especially **Emily Puckett** for her regular feedback and help, **Dr. Chhana Ullah** and **Dr. Grit Kunert** for their advice and feedback with statistical analysis and experiments.

Thanks to **Prof. Dr. Jonathan Gershenzon** and **Prof. Dr. Ralf Oelmüller** for reviewing this thesis.

Extremely grateful to my family members – Mother, Father and Brothers for always being there for me regardless of the time difference providing constant emotional support. I really couldn't have gotten this far without their tireless encouragement.

Finally, thanks to the entire Department of Biochemistry at the Max Planck Institute for Chemical Ecology for creating a supportive and encouraging working environment.

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Date: 17.12.19 Rashaduz Zaman