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Biotransformation of host tree (Norway spruce) phenolics by the bark beetle (*Ips typographus*) symbiotic fungi

Master Thesis

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Abbreviations

µl - microliter

µm - micrometer

µl/l - microliter/liter

µg/g - microgram/gram

µg/ml - microgram /milliliter

ANOVA - Analysis of variance

CA - Cinnamic acid

cm - centimeters

Ctrl - Control

DMSO - Dimethyl sulfoxide

Ep - *Endoconidiophora polonica*

g - Gram

GC-FID - Gas chromatography-flame ionization detection

GC- MS - Gas chromatography-mass spectrometer

Gp - *Grosmannia penicillata*

LC-MS - Liquid chromatography-mass spectrometer

Le - *Leptographium europhioides*

Ml - milli liter

mg - milli gram

mm - mili meter

MM - Minimal media

ng/g - nano gram/gram

ng/mg - nano gram/milli gram

ng/ml - nano gram/milli liter

NA - Nonyl acetate

Oa - *Ophiostoma ainoae*

Ob - *Ophiostoma bicolor*

Op - *Ophiostoma piceae*

PDA - Potato dextrose agar

Pre ctrl - Pre control

Pst ctrl - Post control

SBA - Spruce bark agar (7%)

TDU-GC-MS - Thermal desorption unit-gas chromatography-mass spectrometer

TMBE - Methyl tert-butyl ether

Abstract

Insects are often associated with multiple microbial symbionts that exhibit either mutualistic, commensalistic, or antagonistic properties toward their host. An important and widely studied insect-symbiont relationship is that between blue-stain (ophiostomatoid) fungi and bark beetles. Aggressive bark beetle species such as *Ips typographus* have become a matter of concern across Europe because their large-scale outbreaks have led to the rapid destruction of coniferous forests. They owe this ability primarily to the various associated, virulent ophiostomatoid fungi, which are essential for the colonization of their host tree, *Picea abies*. These fungi degrade various tree secondary metabolites, to produce volatile compounds that can attract bark beetles to overwhelmed or weakened trees. In this thesis, we focused mainly on specific volatile organic compounds (VOCs) that are produced by *I. typographus*-associated filamentous fungi from phenolic acids and the behavioral response of the beetles to these fungal VOCs. We initially compared the virulence of six fungi associated with *I. typographus*, and among them *Grosmannia penicillata* and *Ophiostoma bicolor* were the most virulent fungi. Both the fungi metabolized cinnamic acid completely and produced VOCs such as styrene, benzaldehyde, and benzyl alcohol. The beetles were strongly attracted to styrene-producing *G. penicillata* when the fungus was grown on cinnamic acid amended media. In contrast, benzaldehyde and benzyl alcohol producing *O. bicolor* was avoided by adult beetles. Furthermore, adult males were strongly attracted to pure styrene, but not to pure benzaldehyde and benzyl alcohol. Overall, our results suggest that bark beetles are attracted to virulent fungi via fungal volatile metabolites of host tree phenolics, and that fungal VOCs produced could indicate the suitability of the tree physiology for bark beetle colonization.

Methods: Virulence of fungi was tested by measuring the lesion length and area caused due to fungal inoculation in spruce tree logs. The concentration of phytohormones, and phenolic acids present in the fungal lesions were quantified using LC-MS. *In vivo* and *In vitro* analysis was performed to identify fungal biotransformation products of cinnamic acid using GC-FID/MS and TDU-GC-MS. The toxicity of phenolic acids against *G. penicillata* and *O. bicolor* was tested by measuring their growth rate in media supplemented with different phenolic acids. To test the toxicity of cinnamic acid to adult bark beetles, a tunnel test was conducted where the beetles were allowed to feed on a diet amended with cinnamic acid for 48 hours. The behavioural response of adult beetles to fungal VOCs produced by biotransformation of cinnamic acid, was tested using olfaction-based trap bioassay.

Results: Virulence of ophiostomatoid fungi is highly variable. *Grosmannia penicillata* and *O. bicolor* were the most virulent fungi whereas, *O. piceae* was the least virulent fungus. Virulent fungi induced significantly higher concentrations of phytohormones such as jasmonic acid and jasmonic acid isoleucine. Interestingly, the concentration of cinnamic acid was lower in the lesions caused by *G. penicillata* and *O. bicolor*. The decrease in cinnamic acid in fungal infected bark correlated with the production of volatile phenolics such as styrene, benzyl alcohol, and benzaldehyde. *In vitro* analysis revealed that biotransformation of cinnamic acid by *G. penicillata* produced styrene, and *O. bicolor* produced benzaldehyde and benzyl alcohol. The growth of both fungi was least in cinnamic acid when compared to ferulic and *p*-coumaric acid. Interestingly, low

concentrations of cinnamic acid promoted the growth of *G. penicillata*. The presence of cinnamic acid in the fungal medium increased the attraction of adult males and females towards *G. penicillata*. On the contrary, beetles preferred *O. bicolor* grown on a cinnamic acid-free medium over it grown on cinnamic acid enriched medium. Adult male *Ips typographus* showed strong attraction towards the fungal VOC, styrene at a specific dose. The beetles did not show attraction or aversion to benzaldehyde and benzyl alcohol or to *O. bicolor* grown in media with cinnamic acid.

Conclusion: Volatile organic compounds produced by symbiotic fungi elicit a behavioral response in *I. typographus*. Styrene, benzaldehyde, and benzyl alcohol are produced by fungal biotransformation of cinnamic acid which is in turn derived from an essential amino acid phenylalanine. The fungal blend also contains endogenously produced volatile alcohols and esters. It is hypothesized that the presence of fungal VOCs could indicate appropriate food sources rich in essential amino acids that are vital for insect development. Collectively our results suggest that volatiles such as styrene could indicate the nutritional value of the food source, rich in essential amino acids and antioxidants.

Zusammenfassung

Insekten sind oft mit verschiedenen symbiotischen Mikroben assoziiert, welche entweder mutualistische, kommensalistische oder antagonistische Eigenschaften gegenüberihres Wirtes zeigen. Eine wichtige und weithin untersuchte Insekten-Mikroben-Symbioseist die zwischen Bläuepilzen (in der Gruppe der Ophiostomatales) und Borkenkäfern. Vor allem einzelne aggressive Borkenkäfer wie die Art *Ips typographus* sind in ganz Europa zu einem Problem geworden, da sie in sehr kurzer Zeit hohe ökonomische Schäden in Nadelwäldern verursachen. Diese Fähigkeit verdanken sie vor allem den verschiedenen assoziierten, virulenten Pilzen (va. Ophiostomatales) welche für die Besiedlung ihres Wirtsbaumes (*Picea abies*) essentiell sind. Diese Pilze sind in der Lage, die verschiedenen und für Borkenkäfer oftmals schädlichen, sekundären Stoffwechselprodukte der Bäume abzubauen, um diese zu flüchtige Verbindungen (Volatile) umzuwandeln. In dieser Arbeit konzentrierten wir uns vor allem auf spezifische flüchtige organische Verbindungen (VOCs), welche durch symbiotische mit *I. typographus* assoziierte filamentöse Pilze aus Phenolsäure gebildet werden, aber auch wie die Käfer selbst auf diese Volatile reagieren. Hier verglichen wir zunächst die Virulenz von sechs mit *I. typographus* assoziierten Pilzen, und konnten zeigen, dass vor allem *Grosmannia penicillata* und *Ophiostoma bicolor* die höchste Virulenz auswiesen. Beide verstoffwechselten Zimtsäure vollständig und produzierten daraus VOCs wie Styrol, Benzaldehyd und Benzylalkohol. Die Käfer wiederum wurden stark vom Styrol produzierenden *G. penicillata* angezogen, wenn dieserauf einem mit Zimtsäure angereicherten Medium gezüchet wurde. Im Gegensatz dazu wurde der Benzaldehyd und Benzylalkohol produzierende *O. bicolor* von Käfern gemieden. Männliche Käfer wurdenstark von reinem Styrol angezogen,aber nicht von anderen Zimtsäure-Metaboliten. Insgesamt deuten unsere Ergebnisse darauf hin, dass *I. typographus* von seinen Pilzen über flüchtige Pilzmetabolite angezogen wird, welche aus Wirtsbaumphenolen produziert werden. Zusätzlich können solche Pilzvolatile viele Informationen über den Zustand und damit die Attraktivität eines Baumes für Borkenkäfer geben.

Methoden: Die Virulenz der Pilze wurde durch die Messung der durch die Beimpfung in Fichtenstämmen verursachten Läsion (Länge und Fläche) getestet. Die Konzentration von Phytohormonen und Phenolsäuren in den Pilzläsionen wurde mittels LC-MS quantifiziert. In-vivo- und In-vitro-Analysen wurden durchgeführt, um die Biotransformationsprodukte der Zimtsäure durch Pilze mittels GC-FID/MS und TDU-GC-MS zu identifizieren. Die Toxizität vonr Phenolsäuren gegen *G. penicillata* und *O. bicolor* wurde durch Messung der Wachstumsrate der Pilze in mit verschiedenen Phenolsäuren angereicherten Medien getestet. Um die Toxizität von Zimtsäure für erwachsene Borkenkäfer zu testen, wurde ein Tunneltest durchgeführt, bei dem die Käfer 48 Stunden lang ein mit Zimtsäure versetztes Futter fressen konnten. Die Verhaltensreaktion erwachsener Käfer auf VOCs, welche durch die Biotransformation von Zimtsäure durch *G. penicillata* und *O. bicolor* entstehen, wurde mit einem olfaktorischen Versuchsaufbau getestet.

Ergebnisse: Die Virulenz der verschiedenen getesteten Pilze ist sehr unterschiedlich. *G. penicillata* und *O. bicolor* zeigten die höchste Virulenz, während *O. piceae* die niedrigste aufzeigte. Vor allem Pilze mit hoher Virulenz induzierten eine signifikante Erhöhung der Konzentrationen von Phytohormonen wie Jasmonsäure und Jasmonsäureisoleucin.

Interessanterweise war die Konzentration von Zimtsäure in den von*G. penicillata* und *O. bicolor* verursachten Läsionen niedrig. Der Rückgang der Zimtsäure in pilzbefallener Rinde korrelierte mit der Produktion von flüchtigen Phenolen wie Styrol, Benzylalkohol und Benzaldehyd. Die In-vitro-Analyse ergab, dass bei der Biotransformation von Zimtsäure durch *G. penicillata* Styrol, und durch *O. bicolor* Benzaldehyd und Benzylalkohol gebildet wurden. Das Wachstum beider Pilze war mit Zimtsäure am geringsten, verglichen mit Ferulasäure und p-Cumarsäure. Interessanterweise förderten niedrige Konzentrationen von Zimtsäure aber das Wachstum von *G. penicillata*. Das Vorhandensein von Zimtsäure im Pilzmedium erhöhte die Attraktivität für die adulten *I. typographus* bei *G. penicillata*. Im Gegensatz dazu bevorzugten die Käfer *O. bicolor* Zimtsäure freiem Medium gegenüber Zimtsäure angereichertem Medium. Männliche *I. typographus* wurden stark vom Biotransformationsprodukt Styrol angezogen. Die Käfer zeigten keine Präferenz gegenüberBenzaldehyd und Benzylalkohol sowie gegenüber *O. bicolor*, auf Zimtsäure reichen Medium.

Schlussfolgerung: Flüchtige organische Verbindungen, die von symbiotischen Pilzen produziert werden, lösen bei *I. typographus* eine Verhaltensreaktion aus. Styrol, Benzaldehyd und Benzylalkohol werden durch Biotransformation von Zimtsäure durch Pilze erzeugt, welche aus der essentiellen Aminosäure Phenylalanin gewonnen wird. Die Mischung aus Pilzen enthält auch endogen produzierte flüchtige Alkohole und Ester. Es wird vermutet, dass das Vorhandensein flüchtiger Alkohole auf entsprechende Nahrungsquellen hinweisen könnte, die reich an essentiellen Aminosäuren sind, welche für die Entwicklung von Insekten lebenswichtig sind. Insgesamt deuten unsere Ergebnisse darauf hin, dass flüchtige Stoffe wie Styrol den Nährwert der Nahrungsquelle anzeigen könnten, welche reich an essentiellen Aminosäuren und Antioxidantien ist.

1. Introduction

1.1. Insect-microbe interactions

Interactions between insects and microbes have been extensively studied. Insects are contingent on microbes to perform basic functions like accessing vital resources and exploiting different habitats. Some microbes help insects gain access to new genomic variation, allowing them to survive in new adaptive zones (Six, 2013). Most microbial associates have the ability to produce a wide range of metabolic products that could cater to the metabolic needs of their insect hosts. (Hansen & Moran, 2014). The extent of insect microbial interactions depends on whether the association is obligatory or facultative, these interactions can range from being ectosymbiotic (outside the body) to endosymbiotic (inside the body). Endosymbionts can either be intra- or extra-cellular. The degree of these interactions also reflect evolutionarily a more or a less intimate relationship, leading to a highly stable or unstable association (Zimmermann et al., 2016). The transmission of microbial symbionts can happen in two major ways either horizontally or vertically. Horizontal transmission takes place by the uptake of new symbionts from the environment by each host generation, whereas for vertical transmission symbionts are most often transferred from parents to the offspring mostly through female germ lines (Bright & Bulgheresi, 2010). Both endosymbionts and ectosymbionts can be transmitted horizontally or vertically. Insects evolve multiple adaptations both morphologically and at cellular levels to gain a selective advantage to sustain their symbionts (Wielkopolan & Obrepalska-Steplowska, 2016). Ectosymbionts are mostly free-living organisms and are ubiquitous to many insect species. They benefit their host species by providing them with vital nutrients, protection against pathogens, tolerance to abiotic stressors, detoxification of plant chemicals, and production of insect semiochemicals. Hence, the effects of the associated microbes on their insect host are highly dependent on the circumstances and vary with change in environment (Klepzig & Six, 2004), (Gunther et al., 2015).

Recent studies have shown that adaptations in insects to accommodate symbionts, particularly in systems having strong influences on Earth's ecosystems, are affected by anthropogenic changes (Six, 2013). One such system that has major effects on the forest ecosystems and is of ecological relevance is bark beetles and their ectosymbiotic ophiostomatoid fungi.

1.2. Impact of bark beetles

The conifer bark beetles attack and kill mature spruce and pine trees especiallyin hot and dry seasons. Bark beetles are phloem-feeding insects. They help to maintain the forest ecosystem by attacking old and wind-thrown trees, in turn rejuvenating the forests by recycling nutrients (Raffa et al., 2008). In recent decades due to climate change and global increase in temperatures, the population of the aggressive bark beetles has increased significantly reaching its threshold resulting in the destruction of healthy trees. Due to such rapid changes and their expansions to susceptible host tree species, bark beetles have become ecologically and economically crucial (Bentz et al., 2010; Raffa et al., 2008). The aggressive species that are capable of attacking and killing healthy trees include *Dendroctonus ponderosae*, the mountain pine beetles in North and Central America and *Ips typographus*, the European spruce bark beetle (Bakke, 1988; Raffa et al.,

2008). These bark beetles have become a prominent disturbance factor in North American and European forests resulting in the death of a large number of trees with an alarming rate of increase (Raffa et al., 2008). Bark beetles outbreaks resulted in the destruction of 2.1 million m^3 of pine and spruce plantations in Europe from 1971-1980, which increased to 14.5 million m^3 per year during 2002-2010 and this number is expected to increase to 17.9 million m^3 per year by 2021-2030 (Seidl et al., 2014).

Such frequent outbreaks result in the alteration of forest ecosystems and biodiversity, effectively reducing carbon and nitrogen storage while increasing soil temperature and water availability (Hlásny, 2019). Timber producers also incur economical losses due to the deteriorated quality of timber from trees attacked by bark beetles. The European spruce bark beetle, *Ips typographus L. (Coleoptera: Scolytidae)* is a serious pest on Norway spruce, *Picea abies* and occasionally on trees from the genera *Pinus*, *Abies*, and *Larix* (Bakke, 1988) (Wermelinger, 2004). The success of this species is often related to its association with multiple ophiostomatoid fungi (Kirisits, 2004). As a result, studies focus mainly on the symbiotic relationship between the European spruce bark beetle, *I. typographus* and ophiostomatoid fungi and on finding effective solutions to combat growing populations of these ecologically concerning pests.

1.3. Multitrophic interactions

The European spruce bark beetle is associated with a variable community of bacterial and fungal symbionts, which are assumed to aid the beetle in exhausting tree defenses, detoxifying tree defense chemicals and providing nutrients (Kandasamy et al., 2016), (Zhao et al., 2019), (Wadke et al., 2016). The first note of the association of blue-stain fungi with trees killed by bark beetles was made back more than 100 years ago by Von Schrenk (Six & Wingfield, 2011). Only later was there speculations that the blue-stain fungi play an important role in the death of the trees attacked by bark beetles (**Figure 1.1**).

Some microorganisms are dependent on various vectors like insects or animals to reach their new hosts. These associations are thought to be possible because of chemical signaling and screening involving volatile and non-volatile chemicals (Biedermann & Kaltenpoth, 2014). For example, bark and ambrosia beetles carry beneficial fungi to convert indigestible substrates to high-quality nutrients (Dowd, 1992). Fungi-growing ambrosia beetles are able to recognize their symbiotic partners through a unique bouquet of chemical signals that help beetles to differentiate between symbionts and non-symbionts (Hulcr et al., 2011).Chemical signals and cues are known to be oldest means of communication to exchange information within and between species (Leonhardt et al., 2016). Features like low molecular masses (< 300 Da) and high vapor pressures facilitate these lipophilic volatile based signals to diffuse and evaporate, making them ideal for both short and long-range intra- and inter-specific interactions (Kanchiswamy et al., 2015) (Schulz-Bohm et al., 2017).

The volatile compounds produced by microbes are referred to as microbial volatile organic compounds (mVOCs). They include compounds belonging to different chemical classes such as alcohols, aldehydes, acids, esters, ketones, lactones, nitrogen, and sulphur containing compounds (Lemfack et al., 2018). The rate of their production varies among microorganisms and also depends on factors such as their growth conditions, nutrient availability, growth stage,

oxygen availability, temperature, moisture level, and pH (Zhou et al., 2018). These compounds are often biosynthetic by-products, waste products, or detoxification products produced by microbes. Interestingly, recent studies have demonstrated that many mVOCs have specific biological activities and are not just waste products (Ossowicki et al., 2017). Although, the role of microbes especially the blue-stain fungi in the life cycle of *I. typographus*, has been studied, the, specific functions or benefits of microbes to this beetle are not well understood (Six & Wingfield, 2011).

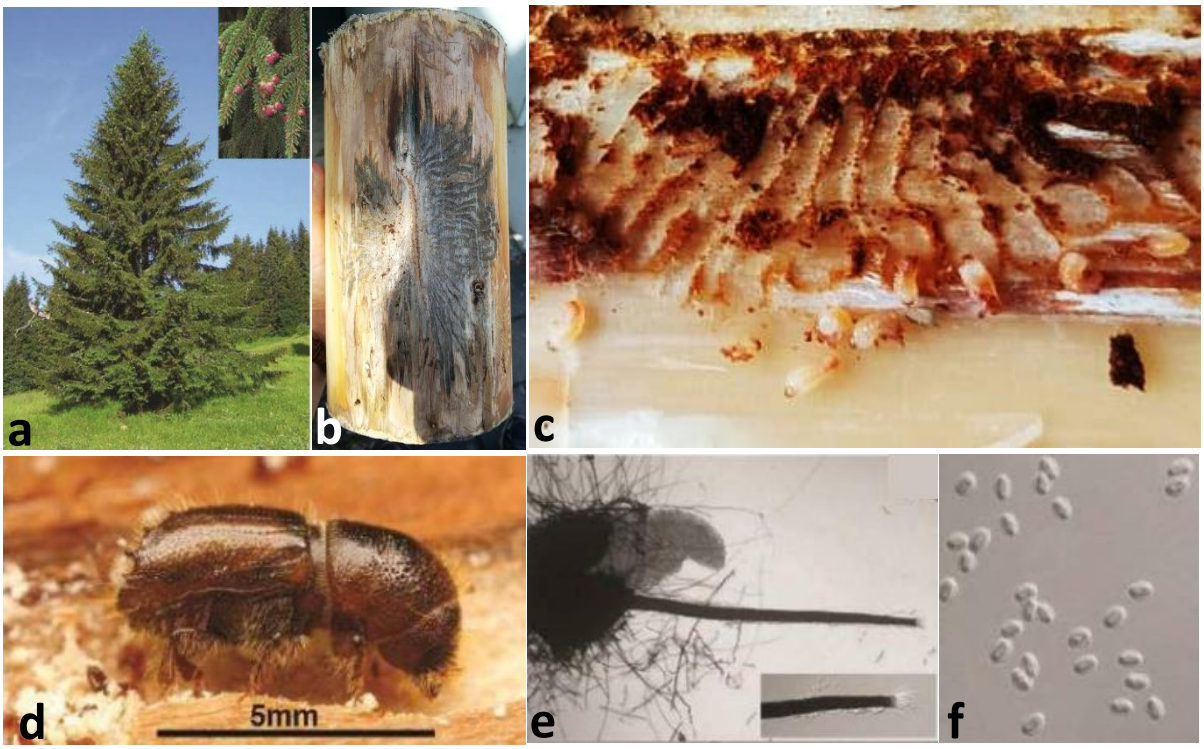

Figure 1.1: Norway spruce- *Ips typographus*- ophiostomatoid fungi interaction. **a)** Norway spruce tree, *P. abies*, **b)** Blue-stain fungi in a beetle gallery, **c)** Larvae feed on the fungus infected tissues for their nutrition, **d)** Under the bark, beetles feed on phloem rich in plant secondary metabolites **e), f)** Perithecia (sexual structure) of *E. polonica* produces sticky spores that facilitate the attachment of spores to the exoskeleton of the beetle. Adapted from (Kandasamy, 2019).

1.4. Life cycle of bark beetles

Bark beetles usually hibernate through winter and start to disperse and fly when the temperature is above 15°C. Male pioneer beetles are responsible for the selecting suitable host tree for colonization. When a suitable host tree is located, the males construct nuptial chambers and release aggregation pheromones comprising (-)-cis-verbenol and 2-methyl-3-buten-2-ol to attract conspecifics of both sexes. This results in the aggregation of thousands of beetles resulting in a mass attack on the host tree, helping them to overcome the host's defenses. Once the tree's defenses are overwhelmed and enough beetles are recruited, beetles start to release anti-aggregation pheromones like (-)-verbenone to prevent the new beetles from attacking the same trees in order to avoid intra-specific competition and instead divert them to neighboring trees (Vite et al., 1972).

Once the attack is successful, each male in the constructed nuptial chamber can accommodate one to four females. After mating, females make vertical oviposition tunnels (Bakke, 1988). Females lay eggs along the sides of the tunnel and cover them with frass and inoculate fungi. Larvae hatch and make feeding tunnels that run perpendicularly away from the maternal tunnel. The larvae obtain their nutrition by consuming the phloem tissues infected by fungus and develop through 3-5 instars. Larvae pupate in pupal chambers, which are lined with spores of ophiostomatoid fungi. The newly enclosed callow or teneral adults feed on the fungus-infected bark tissues until they transform into sclerotized adults (Six, 2012). Once the adult beetles are fully developed, they emerge and initiate the second generation of attacks or instead hibernate under the bark or in forest litter, if the temperature is low before starting new attacks in the spring. Under favorable conditions, adult beetles that emerge can re-enter the same tree to form sister broods (Bakke, 1988). The full life cycle is illustrated in (**Figure 1.2**).

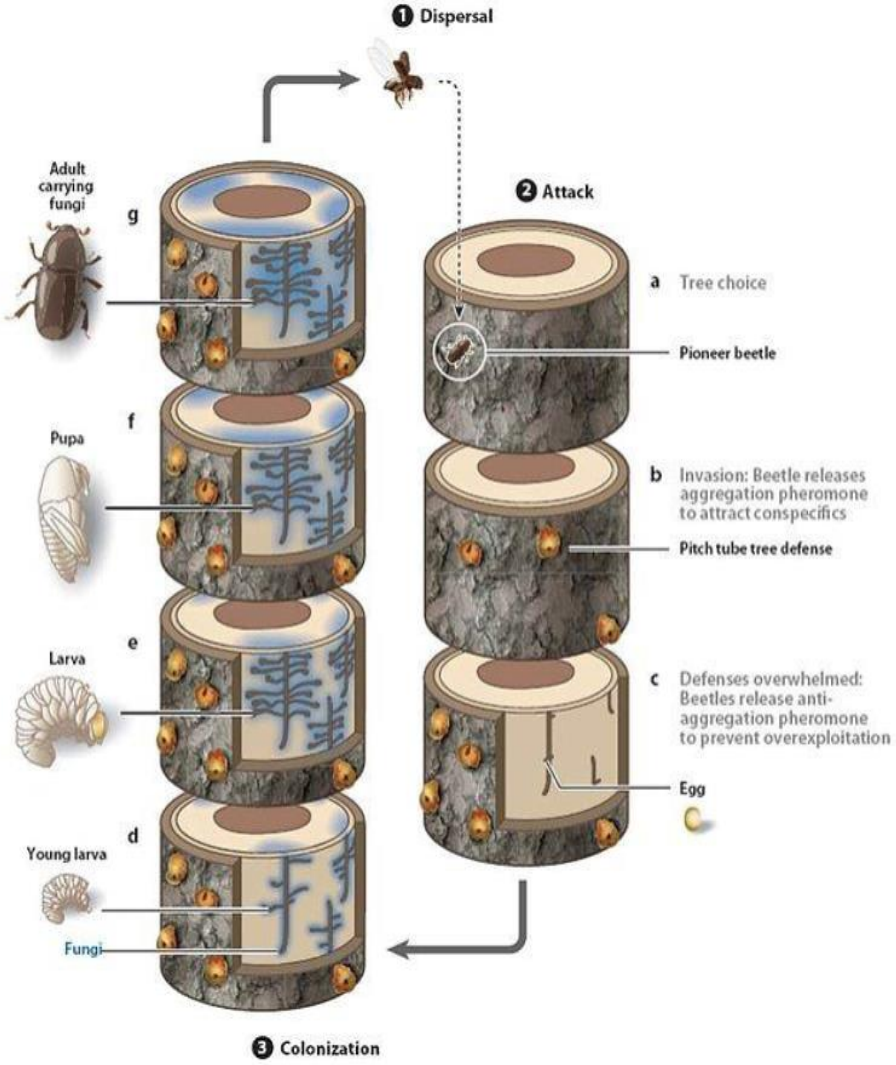

Figure 1.2: The life cycle of *Ips typographus* and its associated fungi. **(1)** Dispersal of adult beetles under favorable conditions. **(2)** Attacking period: **a)** suitable tree is chosen by pioneer beetle, **b)** Beetles enter into the tree and release aggregation pheromones to attract conspecifics of both

sexes to induce mass attack which exhausts the tree defense system, **c)** after that beetle's 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 (Six & Wingfield, 2011).

1.5. Ectosymbiotic fungi of bark beetles

The success of aggressive species such as *Ips typographus* is often credited to their symbiosis with multiple ophiostomatoid fungi. Unlike other bark beetles, *I. typographus* lacks the presence of a glandular mycangium, a specialized cavity for harvesting spores. Instead, the sticky fungal spores are embedded in pit-like non-glandular mycangia, lining the exoskeleton (Furniss et al., 1990). Reproductive structures of these fungi have special adaptations for attachment to the beetle's exoskeleton, including long stalks bearing thick masses of sticky spores (**Figure 1.1**). The ectosymbiotic community of *I. typographus* is dynamic and their composition changes with time and space (Kandasamy et al., 2016). Based on their abundance across the distribution range, four species of fungi *Grosmannia penicillata*, *Ophiostoma bicolor*, *Endoconidiophora polonica* and – *Leptographium europhioides* are considered to be their frequent associates (Kirisits, 2004; Linnakoski et al., 2012).

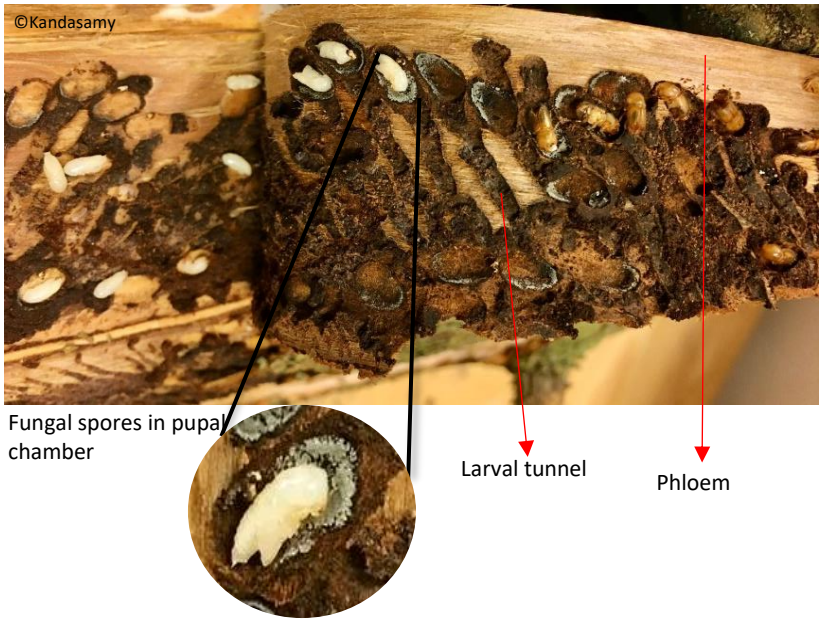

Figure 1.3: Pupae surrounded by fungal spores in the phloem under the bark. Bark beetles spend most of their life cycle under the bark consuming phloem infected with fungi.

These symbiotic fungi produce blue-grey or black stains on the infected wood due to their melanized hyphae and hence they are called blue-stain fungi. After being inoculated in the bark, they modify the phloem by detoxifying host defense compounds. They are thought to release soluble carbohydrates, amino acids, and other essential nutrients from cells for the benefit of

larvae, as the larvae obtain their nutrition by feeding on fungi (**Figure 1.3**) (Bleiker & Six, 2007). However, these fungi can also modify their environment by releasing a suite of volatile signals that mediate interactions with bark beetles and probably influence their behavior (Zhao et al., 2019). Hence, the ophiostomatoid fungi are thought to play an important role in aiding beetle colonization and reproduction by enabling them to deal with high levels of host tree toxins(Kandasamy et al., 2019).

1.6. Conifer defense mechanism

Conifers have evolved a sophisticated defense system to protect themselves against biotic stress. It usually comprises physical, chemical, and induced mechanisms for the protection (Bohlmann et al., 2000). Conifers produce toxic secondary metabolites as immune responses against fungal and bacterial pathogens. The ultimate goal of the trees defenses is to preserve the integrity of the tree and defend the nutrient-rich phloem, vascular cambium, and the sapwood important for transpiration (Vega, 2015). Conifer defense has two phases - the constitutive and the inducible defenses. The primary constitutive defense consists of mechanical and chemical defense. Mechanically, the tree strengthens the tissues with lignin and suberin polymers to prevent penetration, degradation, and ingestion by invaders. Chemical defenses include toxic substances like specialized plant metabolites, proteins and enzymes. It also includes structures like chemical reservoirs of resins that can repel or physically entrap beetles (Franceschi et al., 2005; Keeling & Bohlmann, 2006).

Most of the living bark in mature trees is made up of the secondary phloem, which is the main site for inducible defenses. There are three types of cells in the secondary phloem: lignified sclerenchyma cells, calcium oxalate crystals containing cells, and polyphenolic parenchyma (PP) cells (Paal, 2015). The sclerenchyma cells and the calcium oxalate crystals act as mechanical barriers against the beetles. The polyphenolic parenchyma cells contain phenolic bodies and provide chemical protection against fungi (Franceschi et al., 2005). Despite their powerful multifaceted defenses, conifers periodically face extensive outbreaks of tree-killing bark beetles. Beetles and their symbionts break down tree resistance in different ways. Beetles play a central role in tree-killing, and they actively select a suitable host tree and bore straight into the cambial area. Additionally, the beetles' tunneling activity results in considerable mechanical damage. Beetle symbionts such as blue-stain fungi contribute to the death of host trees by colonizing healthy bark beyond the beetle tunnels. Several species of the fungi are known to kill healthy trees on their own if they are inoculated in sufficient numbers (Franceschi et al., 2005) (Paal, 2015). Recent molecular and biochemical studies have shown that specialized tree metabolites such as terpenes and phenolics can be metabolized by bark beetle-associated fungi and bacteria (Hammerbacher et al., 2013) (Wang et al., 2013). The contribution of beetle symbionts to defense exhaustion, suggests that both bark beetles and symbionts contribute to defense depletion.

Spruce trees launch induced defenses against beetles and fungi within 7 days after bark beetles' tunneling into a tree. The constitutive defense delays the progress of infestation until then. At the site of the attack, reactive oxygen species are produced, and rapid cell death occurs due to hypersensitive reactions (Bleiker & Uzunovic, 2004). The wounded periderm confines the wound region and cut off the nutrient supply to the damaged tissues leading to the formation of lesions.

The inducible chemical mechanisms include proteins and non-protein based chemicals. Some of the important non-protein based chemicals include phenolics, terpenoid resins and alkaloids. The attack results in the biosynthesis of different compounds through methylerythritol phosphate and shikimic acid pathway (Franceschi et al., 2005) (Raffa & Berryman, 1983).

1.7. Phenolic acids in Norway spruce tree

Attack from herbivores initiates a suite of physical and chemical defenses in plants. (Freeman, 2008) (Kaur & Kariyat, 2020). The defense mechanism is initiated through a cascade of signaling molecules, starting from the activation of phytohormone pathways, leading to the synthesis of physical defense and the production of defensive chemical compounds. The bark of most gymnosperms functions as the first line of physical defense, delaying or stopping the establishment of pathogens. They have specialized polyphenolic parenchyma cells for the synthesis and accumulation of phenolic compounds, which have antifeedant and antifungal activities (Beckman, 2000) (Singh et al., 2021).

Secondary metabolites consist of a class of small molecule organic compounds that principally include polyphenols, terpenes, and nitrogenous organic compounds. They play an important role in plant resistance to various plant-feeding insects (Jiang et al., 2021). The idea of plant-insect interactions being positively or negatively affected by polyphenols was first proposed by Fraenkel in 1959 (Fraenkel, 1959). Several thousand polyphenolic compounds that are found in plants are synthesized mostly via biosynthetic pathways such as shikimic acid, and phenylpropanoid or polyketide pathway. Polyphenolic compounds have a basic structure that consists of a benzene ring with a hydroxyl group attached and without any nitrogen-based functional group (Singh et al., 2021). The amino acid, L-phenylalanine is primary product of the shikimic acid pathway and forms the basis for downstream synthesis of other polyphenols (Singh et al., 2021). Phenylalanine metabolism is particularly relevant in trees, especially conifers as they divert large amounts of carbon into the biosynthesis of Phe-derived compounds, particularly lignin which is an important constituent of wood.

Major groups of polyphenols, including flavonoids, phenolic acids, stilbenes, and lignans play a critical role in plant chemical defense (Singh et al., 2021). Numerous studies have investigated the defensive as well as stimulatory roles of such metabolites on insect herbivores. For instance, grain aphid (*Sitobion avenae F.*) infestation in winter triticale (*Triticosecale Wittm*) seedlings induces bioactive compounds such as phenolic acids that provide resistance against them. Ferulic acid in rice imparts resistance against brown plant hopper (*Nilaparvata lugens Stål*) (Singh et al., 2021). An increase in phenolic acids and flavonoids, especially quercetin has been observed in white cabbage (*Brassica oleraceae L*) upon infestation by cabbage butterflies (*Pieris brassicae L.*) and flea beetles. Some of the most common phenolic acid defense compounds of Norway spruce are shown in **Figure 1.4**. Phenolics get oxidized due to tissue damage caused by herbivores. The oxidation of phenolics can also occur the insect gut due to the presence of high pH conditions. Phenolics in an oxidative state can bind to proteins resulting in the precipitation of proteins in the insect gut thereby, affecting the absorption of nutrients (Felton, 2005).

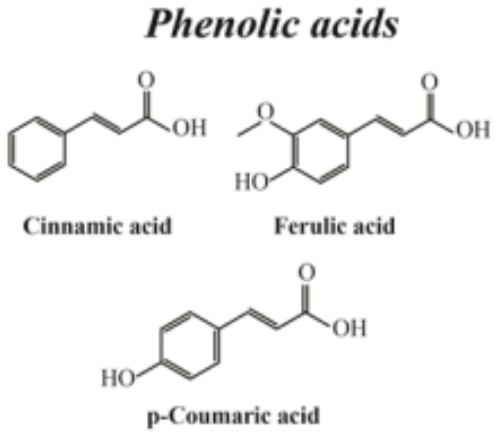

Figure 1.4: Representative structures of some common phenolic acids of Norway spruce that we focused on in this thesis.

1.8. Biosynthesis of phenolic acids

Phenolics are involved resistance against conifer natural enemies such as bark beetles and their associated fungi (Lieutier, 2004) (Paal, 2015). The shikimic acid pathway is a major pathway in conifers linking carbohydrate metabolism to the biosynthesis of aromatic amino acids, which in turn give rise to phenolics (Ralph et al., 2006). In conifers, phenolics are synthesized through the phenylpropanoid pathway (Hu et al., 2018). The aromatic amino acid L-phenylalanine is converted to the first-formed phenolic acid, cinnamic acid by the action of phenylalanine ammonia-lyase (PAL) in the phenylpropanoid pathway. After the recognition of the attack, possibly through surface receptors (Steinbrenner et al., 2020) plants activate phytohormones such as jasmonic acid (JA), salicylic acid, and ethylene which act as signaling molecules that spread through the plant away from the wound site. The phytohormones induce an increase in the expression of phenylalanine-ammonia-lyase, PAL; the chief enzyme necessary for regulation and operation of the shikimate phenylpropanoid pathway, which in turn diverts amino acids from primary metabolism towards the secondary metabolite production (Koornneef & Pieterse, 2008). Further, cinnamic acid 4-hydroxylase catalyzes the conversion of cinnamic acid to form *p*-coumaric acid. Cinnamic acid also leads to the formation of salicylic acid. *p*-Coumaric acid further leads to the formation of caffeic acid. Ferulic acid is formed by methylation of caffeic acid. Further methylation of ferulic acid gives rise to sinapic acid (**Figure 1.5**). These phenolic acids also play significant roles in anti-herbivore defense (Singh et al., 2021).

Biosynthesis of flavonoids, stilbenes, and lignans are through the phenylpropanoid pathway in conifers. *p*-Coumaryl acid, ferulic acid and sinapic acid are the precursors for lignin synthesis, which is an important compound for mechanical defense and the structural integrity of the tree. Compounds such as proanthocyanidins and flavanols, are produced in the phenylpropanoid pathway by *P. abies* as a response to fungal infection (Pascual et al., 2016).

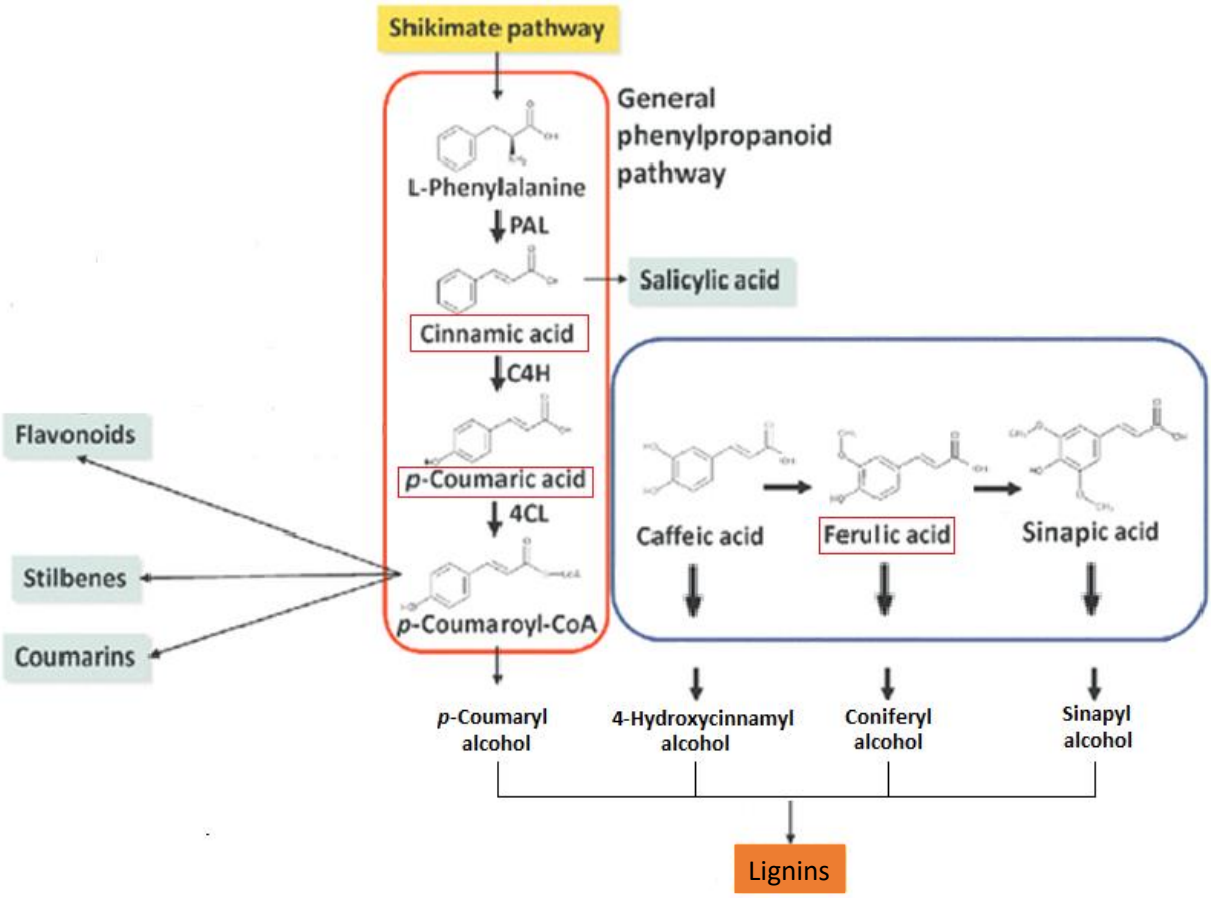

Figure 1.5: Simplified schematic representation of phenylpropanoid pathway leading to the synthesis of different polyphenols i.e., phenolic acids, flavonoids, stilbenes and lignin in conifers. Adapted from (Pascual et al., 2016) (Singh et al., 2021).

1.9. Fungal volatiles

We know that conifers have evolved a strong and complicated defense system to protect themselves from bark beetles and their associated fungi. Some ectosymbionts can detoxify host defense compounds like phenolic acids to produce some volatile organic compounds (VOCs). Detoxification of such host defense compounds can directly affect the development of bark beetles. These host chemicals could otherwise be toxic to adult beetles, larvae, or eggs (Raffa & Berryman, 1983) (Raffa et al., 2005).

Trees respond to fungal infection by increasing the biosynthesis of secondary metabolites at the infection site. Blue-stain fungi like *E. polonica* are known to overwhelm the tree's defenses by inducing the production of polyphenols and depleting tree defenses by metabolizing terpenes and phenolics faster than the tree can produce them (Zhao et al., 2019) (Hammerbacher et al., 2011) (Hammerbacher et al., 2013). In some insect-microbe associations, microbes transform metabolites of the host plant to volatile signals used by the insect host to locate feeding or breeding sites (Biedermann & Kaltenpoth, 2014) (Kandasamy et al., 2021). The fungal symbionts of bark beetles emit volatile compounds by oxidation of host tree monoterpenes, that could be used by the beetles as cues to find breeding sites with beneficial symbionts (Kandasamy et al.,

2021). As bark beetle-associated fungi can break down secondary metabolites such as terpenes to produce fungal VOCs, it can be speculated that same would be true for other secondary metabolites such as phenolic acids. Each fungus emits a specific blend of low molecular weight compounds with characteristic fragrances. It has been reported that *I. typographus* can recognize specific volatiles emitted from fungi and these cues help them to maintain a specific fungal community (Kandasamy et al., 2019) (Kandasamy et al., 2021). The fungal volatiles emitted could also aid in host location, broadcast host vulnerability, orient insects towards nutrient rich sources and assist in communication via recognition signals to distinguish between native and non-native microbes. (Kandasamy et al., 2019). However, the functional role of other fungal volatile are unknown, although the behavioral activity of some ambrosia beetles to their native fungal symbionts' volatiles have been studied (Hulcr et al., 2011).

1.10. Aims

The role of ophiostomatoid fungi associated with *I. typographus* to overcome the tree defense and leading the tree to the point of no return has being extensively studied. Recent studies have shown that a highly virulent bark beetle-associated fungus, *E. polonica* metabolized polyphenols and also use these compounds as a carbon source (Hammerbacher et al., 2013). However it is not known if different fungal associates of *I. typographus* have similar strategies for detoxification.

The aim of this thesis was to 1) identify and quantify phenolic acids in the bark, 2) identify volatile organic compounds produced by biotransformation of host tree phenolic acids by ophiostomatoid fungi *in vivo* and *in vitro,* 3) test the toxicity of phenolic acids to fungi and beetles and to test if phenolic acid spromote or inhibit the growth of fungi, and 4) study the behavioral response of adult *I. typographus* to different fungi and to volatile fungal metabolites of cinnamic acid.

2. Methods and Materials

2.1. List of chemicals

Chemical name	Formula	Suppliers	CAS no.
Acetone	(C_3H_6O)	VWR Chemicals	67-64-1
Ammonium carbonate	$(NH_4)_2CO_3$	Sigma Aldrich	506-87-6
L-Asparagine monohydrate	$(C_4H_{10}N_2O_4)$	Duchefa Biochemie	5794-13-8
Benzaldehyde	(C_6H_5CHO)	Fluka	100-52-7
Benzyl alcohol	(C_7H_8O)	Sigma Aldrich	100-51-6
Bacteriological agar		Roth	9002-18-0
p-Coumaric acid	$(HOC_6H_4CH=CHCO_2H)$	Sigma- Aldrich	501-98-4
trans-Cinnamic acid	$(C_9H_8O_2)$	Sigma- Aldrich	140-10-3
Dimethylsulfoxide (DMSO)	(C_2H_6OS)	Sigma- Aldrich	67-68-5
Dichloromethane	(CH_2Cl_2)	Roth	75-09-2
Ferulic acid	$(C_{10}H_{10}O_4)$	Sigma- Aldrich	537-98-4
D-(+)-Glucose anhydrous	$(C_6H_{12}O_6)$	Roth	50-9907
Hexane	(C_6H_{14})	Merck	110-54-3
Methanol absolute	(CH_3OH)	Merck	67-56-1
Mineral oil		Sigma- Aldrich	8042-47-5
Nonyl acetate	$(C_{11}H_{22}O_2)$	Sigma- Aldrich	143-13-5
Potassium dihydrogen phosphate	(KH_2PO_4)	Roth	7778-77-0
Potato dextrose agar		Sigma- Aldrich	
Qualitative retention time standard		Restek	
Styrene	(C_8H_8)	Sigma- Aldrich	100-42-5
Tert-butyl-methyl ether (TMBE)	$(C_5H_{12}O)$	Acros Organics	1634-04-4
Trifluoro-methyl cinnamic acid	$(C_{10}H_7F_3O_2)$	Alfa Aesar	2062-25-1
Yeast nitrogen base (no $(NH_4)_2SO_4$, without amino acids)		Sigma- Aldrich	

2.2. List of materials

Materials	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
10mm grade 1 Whatman filterpaper	GE Healthcare Life Sciences
35×10 mm Petri dish	Greiner Bio One
150×25 mm glass Petri dish	Paul Marienfeld GmbH & Co. KG
30ml Omnifix syringe	B. Braun
50ml Falcone tube	Sarstedt AG & Co.
15ml clear vial with a screw cap	Supelco
5ml Screw cap plastic tubes	Sartedt AG & Co. KG
5ml amber glass vial	Macherey- Nagel Gmbh
5ml clear glass vial	Macherey- Nagel Gmbh
1.5ml amber glass vial	Macherey- Nagel Gmbh
1.5ml clear glass vial	Macherey- Nagel Gmbh
200µl glass capillaries	Blaudrand Intra Mark
Pneumatic tube	Sang – A Pneumatic
Transparency film (A4, 210x297 mm)	Sigel
Insect rearing tent (BugDorm-4S2260)	Mega View Science Co. Ltd.
Insect rearing box ((WxHxD) 32.7x 21.5 x22.7 cm)	Emsa
13cm circular Petri dish	VWR International
Poly dimethyl siloxane tubes (PDMS)	Prepared inhouse

2.3. List of instruments

Instruments	Supplier
Electronic precision balance	Sartorius Lab Instrument Gmbh, Germany
Incubator (25°C)	Heraeus Instruments
Sterilizer (200 °C)	Heraeus Instruments
Vortex genie 2	Scientific Industries
Autoclave steam sterilizer H+P	Abortechnik Gmbh, Germany
Vibratory micro mill (PULVERISETTE)	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
TD-20 thermal desorption unit	Shimadzu, Japan
GC Cryo-Trap	Tenax®
Agilent Qtrap 6500 mass spectrometer + Liquid chromatograph	Agilent, Santa Clara, USA
Paint shaker	Skandex
Tabletop centrifuge 5810	Eppendorf

2.4. Beetle rearing

Approximately 40-year-old Norway spruce, *P. abies* trees from a spruce plantation in southwestern Jena, Germany, were used for rearing the beetles. The trees were cut into small logs of around 30 cm in diameter and 50 cm in height. The cut ends were coated with paraffin wax to prevent the logs from drying out or desiccation of the cut ends. The waxed logs were placed in either insect rearing tents (BugDorm-4S2260, MegaView Science Co., Ltd., Taichung, Taiwan) or in Emsa clipon boxes (10.6 liters, (WxHxD) 32.7x 21.5 x22.7 cm) kept in the rearing chamber, which was maintained at 25 °C and 65% humidity with 18h-6h light-dark cycle. Twenty beetles, 10 males and 10 females from the previous generation were introduced for breeding in each log. Emerging offspring were collected after 30-35 days of infestation manually with forceps in case of tents or from collection cups in case of boxes. The collected adults were then separated into males and females prior to storage. A stereomicroscope (Stemi 2000-CS, Zeiss, Germany) was used to determine the sex of the beetle (Schlyter & Cederholm, 2009). Approximately 40-50 beetles were stored at 4 °C in 50 ml Falcon tubes (SARSTEDT AG & Co. KG, Nümbrecht, Germany) after sex determination. A piece of lightly wet tissue

paper (Rotizell Tissue Tücher) was placed into falcon tubes before the beetles were put into them to maintain humidity inside the tube. Around 200-300 beetles that emerged first from the rearing were used for continuing the rearing of next-generation beetles and the remaining beetles were stored at 4 °C until required for experiments.

2.5. Sex Determination

The sex determination of live beetles was done according to the description provided by Schlyter and Cederholm (Schlyter & Cederholm, 1981). Briefly, the beetles were separated based on the density of bristles present on the pronotum. Females have a higher density of bristles compared to males. Furthermore, the size of the frontal tubercle was also taken into consideration; male beetles tend to have larger frontal tubercles than females. A stereomicroscope (Stemi 2000-CS, Zeiss, Jena, Germany) was used to differentiate both the sexes. Beetles that had damaged bristles and presence of dirt and resin on the pronotum were excluded from the study, due to the difficulty in sex determination.

2.6. Grinding the bark into powder

Some logs from the tree cut down for rearing were used for making bark powder. Firstly, the outer bark was shaved using a draw knife and the inner bark (phloem part) was carefully peeled off using a hammer and chisel. The bark was then cut into small pieces and placed in liquid nitrogen (N_2) before placing them in boxes and storing them at -80 °C until pulverization. The pulverization was done using a vibratory micro mill PULVERISETTE (FRITSCH GmbH, Germany) and the instrumental setup was pre-cooled using liquid N_2. The bark pieces were placed in the mill along with liquid N_2 and pulverized at an amplitude of 1.8 per minute and 3 seconds interval, for approximately 8-10 minutes with the addition of liquid N_2 after every two minutes. The ground bark powder was sieved through mesh no. 18 to facilitate the removal of coarse particles from the ground powder. The sieved fine powder was then collected in pre-cooled 50 ml Falcon tubes and stored at -80 °C until used.

2.7. Metabolism of host tree phenolics by ophiostomatoid fungi

Five logs from a freshly felled tree (harvested from same place as the trees used for rearing) of approximately 30x50 cm were taken and used for inoculations with different ophiostomatoid fungi grown on PDA at 25°C for 5 days. Two rings were drawn along the circumference of the log each being 15cm away from the cut ends of the logs. Eight equidistant marks were made along the circumference of the rings, and at each mark the bark plugs were removed using a cork borer of diameter 1 cm (no.4 boring tool), and agar plugs with different fungal species were placed and sealed with the same bark plugs. The plugs were removed with the cork borer in an alternating manner between the two rings to prevent overlapping of the fungal lesions and potentially hindering their growth. Seven different types of bark beetle symbiotic fungi *Grosmannia penicillata*, *Grosmannia europhioides*, *Endoconidiophora polonica*, *Ophiostoma bicolor*, *Ophiostoma piceae*, *Ophiostoma ainoae* growing on PDA were used to inoculate the bark. A sample was initially collected as a pre-control (“pre-ctrl”) from each log before infecting with fungi by cutting with a number 4 boring tool and placing in liquid nitrogen. In addition, control medium (“ctrl”) was made by sealing a PDA plug with no fungi into the tree. The 5 logs were then incubated for 14 days in an incubation chamber maintained at 25° C and 60% humidity similar to the conditions provided for rearing beetles. After the incubation period, the phloem of each of the logs was peeled carefully around the fungal bores, lesions made by wounding of the bark due to infections were marked on transparent film (A4, 210 x 297 mm, Sigel) and phloem

samples from each of the infection regions were collected and placed in liquid nitrogen until stored at -80°C. Additionally, a post-control sample was collected which was taken from the uninfected part of the logs after the 14 day incubation period to see the systemic effect on the tree defense due to fungal infection. The samples collected were stored at -80°C until they were ground to a fine powder and returned in -80°C until used for analysis.

2.7.1. Preparation of extraction solvents and details of gas chromatograph methods

Extraction performed	Analysis method	Extraction solvent/ surface	Internal standard	Material extracted or used as volatile source
Phenolic acids; Phytohormones	LC-MS	Methanol (100%)	100 ng/ml Trifluoro-methyl-cinnamic acid (phenolic acid); 40 ng/ml D_6-JA,D_4-SA and D_6-ABA, 8ng/ml D_6-JA-Ile	Bark powder
Phenolic acid	GC-MS, GC-FID	Tert-butyl-methyl ether (MTBE)	50 µg/ml Nonyl acetate	Bark powder
Head space volatiles	TDU-GC-MS	Polydimethylsiloxane (PDMS) tubes	-	Fungi grown in media with phenolic acid (used as volatile source)
Fungal volatile extracts	GC-MS, GC-FID	Hexane	10 µg/ml Nonyl acetate	Fungi grown in media with phenolic acid (used as volatile source)
Phenolic acid extracts	LC-MS	Methanol	100 ng/ml Trifluoro-methyl-cinnamic acid	Fungal plugs grown with phenolic acid in media

Table 2.1: Extractions performed for LC-MS, GC-MS, GC-FID, TDU-GC-MS. Using different extraction solvents and internal standards.

Machine	Sample	Company	Column used	Injection volume	Split	Oven details					
						Initial		Ramp 1		Ramp 2	
						Temp (°C)	Run time (mins)	Temp (°C)	Run time (mins)	Temp (°C)	Run time (mins)
GC-MS	Bark and fungal sample	Agilent	OPTIMA 5-0.25µm, 30m*0.25m ID, Fused silica capillary column - Macherey-Nagel	2 µl	Split -less	45	3	210	30.5	300	34.4
GC-FID	Bark and fungal sample	Agilent	OPTIMA 5-0.25µm, 30m*0.25m ID, Fused silica capillary column - Macherey-Nagel	2 µl	Split -less	45	3	210	30.5	300	34.4
TDU-GC-MS	Volatile from fungal plates	Shimadzu	OPTIMA 5-0.25µm, 30m*0.25m ID, Fused silica capillary column - Macherey-Nagel	-	1-100	45	3	210	30.5	300	34.4

Table 2.2: Details of different gas chromatography methods used for extracting volatiles from either bark samples inoculated with fungi (Bark) or fungal samples from *in vitro* analysis.

2.7.2. Phytohormone and phenolic acid analysis from spruce bark

Labeled glass vials were pre-weighed and approximately 100-150 mg of frozen powdered bark was added to the vials placed on dry ice and weighed again. One ml of extraction buffer (**Table 2.1**) was added to the vials and placed in the paint shaker for 30 seconds initially and then in the plate shaker for 30 minutes. Then, the vials were centrifuged for 10 minutes at 3320 rcf, and supernatant was collected and then analyzed using LC-MS/MS. Samples were analyzed on the API 6500 LC-MS**,** 1 µl Injection volume. The mobile phase used was 0.05 % formic acid and acetonitrile. The column used was from Agilent, a Zorbax Eclipse XDB C18 (4.6x50mm, 1.8 µm).

2.7.3. Volatile analysis for the spruce bark inoculated with fungi

Approximately 100 mg of frozen bark powder was placed in pre-weighed 4 ml glass vials, and the vials were cooled prior by placing them on dry ice. One ml of the MTBE + internal standard prepared according to (**Table 2.1**) was added using a dispenser to the vials containing bark powder. The vials were vortexed for 5-10 s. The vials were left at room temperature for around 30 minutes and then weighed again. For the weight of ether, an average of at least five vials without any biological material was taken. For extractions, the vials were placed in a rotating shaker for 20-22 hours. Then 0.4 ml of 0.1M $(NH_4)_2CO_3$ (pH 8.0) was added to each vial and vortexed for 10-20 seconds, to purify the extract from other small organic acids (Martin et al., 2002). The samples were then centrifuged at rcf 3320 for 10 minutes using a tabletop centrifuge (Eppendorf centrifuge 5810). The samples were frozen at -80°C for 2-3 hours to allow the crystallization of some impurities. Later the samples were placed on dry ice and the supernatant was extracted in clean 2ml brown glass vails using a micropipette and 200 µl glass capillaries. Samples were stored at -20°C until injected into GC-MS and GC-FID.

The solvent extracts were subjected to gas chromatography using an Agilent 6890 series gas chromatograph (Agilent, Santa Clara, CA, USA). 2 µl sample was injected using split-less mode. The mobile phase (carrier gas) was kept at a flow rate of 2 ml min^{-1}. The constituents were separated on an OPTIMA 5 column (5% phenyl, 95% dimethylpolysiloxane, a standard non-polar capillary column for GC analyses) (**Table 2.1**) with a temperature gradient. For compound identification, the column outlet flow (Helium 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 pure standards along with the help of a qualitative retention time standard (**Figure S1**, Supplementary) and spectra in reference libraries (NIST98 and Wiley 275). For compound quantification, the column outflow (H2 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 in comparison with that of the internal standard and standardized to the weight of the bark powder with normalized response factors for each compound. To ensure the accuracy of the compounds identified, a qualitative retention time standard (composed of a mixture of alkanes) and pure reference standards were used and the retention index was calculated (**Figure 2.1**).

$$RI = 100 \left[\frac{\log Rt(i) - \log Rt(z)}{\log Rt(z) - \log Rt(z+1)} \right] + 100(z)$$

Figure 2.1: Retention index is calculated for the compound of interest where **RT(i)** is the retention time of the compound **RT(z)** is the retention time of the alkane (from the retention time standard used) eluting before the compound, **RT(z+1)** is the retention time of the alkane (from qualitative retention time standard, **Figure S1**, Supplementary) eluting out after the compound and **Z** is the number of carbon atoms of the alkane eluting before the compound. (Cammann & Co-workers, 2010).

2.8. Analysis of fungal volatile compounds produced by biotransformation of cinnamic acid in synthetic medium

As it is known from previous studies that, symbiotic fungi can metabolize phenolic acids to produce volatile compounds, this experiment was performed to quantify the amount of phenolic acids metabolized by fungi to produce by-products. Cinnamic acid dissolved in dimethyl sulfoxide (DMSO) was mixed in 1.5% of minimal media (**Table 2.3**) at a concentration of 50 µg/ml and 25 µg/ml of media. Actively growing fungi, *G. penicillata* and *O. bicolor* were inoculated on these media and incubated at 25 °C for 21 days for 50 µg/ml cinnamic acid and 14 days for 25 µg/ml in the dark. Petri dishes with fungi growing in minimal media only with DMSO and no cinnamic acid were used as one of the controls. The other control was minimal media plates with cinnamic acid but with no fungal growth (**Table 2.4**). Each treatment was replicated five times. At the end of the experiment, Petri dishes were first used to collect headspace volatiles and then extracted for volatiles (**Figure 2.2**).

Stock solutions for minimal media (filter-sterilized)			
Compound	**Stock concentration**	**Final concentration**	**Volume needed (400 ml agar)**
Glucose	20%	2.0%	40
L-Asparagine monohydrate	2%	0.3%	60
Yeast nitrogen base (without ammonium sulfate, without amino acids)	6%	0.6%	40
To prepare 400 ml Minimal Media(MM) agar			
Pre-autoclave: make 260 ml 2.3% water agar (will result in 400 ml 1.5% agar MM after adding final stock solutions)			
Compound	**Mass (g)**	**Volume of water (ml)**	
Potassium dihydrogen phosphate (KH_2PO_4)	0.4	260	
Bactoagar	6.0		
Post-autoclave: bring filter-sterilized solutions to room temperature and add to slightly cooled (60°C) agar			
Solution	**volume (ml)**	**Final volume (ml)**	
Water agar + KH_2PO_4	260	400	
Glucose 20%	40		
L-Asparagine 2%	60		
YNB 6%	40		

Table 2.3: Recipe and instructions for making minimal media (MM) agar that was used to grow fungi.

Sample name	Minimal Media condition	Fungi inoculated	Concentration
Gp+MM+CA50	Cinnamic acid	*G. penicillata*	50µg/ml
Gp+MM50	DMSO	*G. penicillata*	50µg/ml
Gp+MM+CA25	Cinnamic acid	*G. penicillata*	25µg/ml
Gp+MM+25	DMSO	*G. penicillata*	25µg/ml
Ob+MM+CA50	Cinnamic acid	*O. bicolor*	50µg/ml
Ob+MM+50	DMSO	*O. bicolor*	50µg/ml
Ob +MM+CA25	Cinnamic acid	*O. bicolor*	25µg/ml
Ob+MM+DMSO25	DMSO	*O. bicolor*	25µg/ml
MM+CA50	Cinnamic acid	-	50µg/ml
MM50	DMSO	-	50µg/ml
MM+CA25	Cinnamic acid	-	25µg/ml
MM 25	DMSO	-	25µg/ml

Table 2.4: Samples names and their compositions, MM- minimal media. DMSO- Dimethyl sulfoxide.

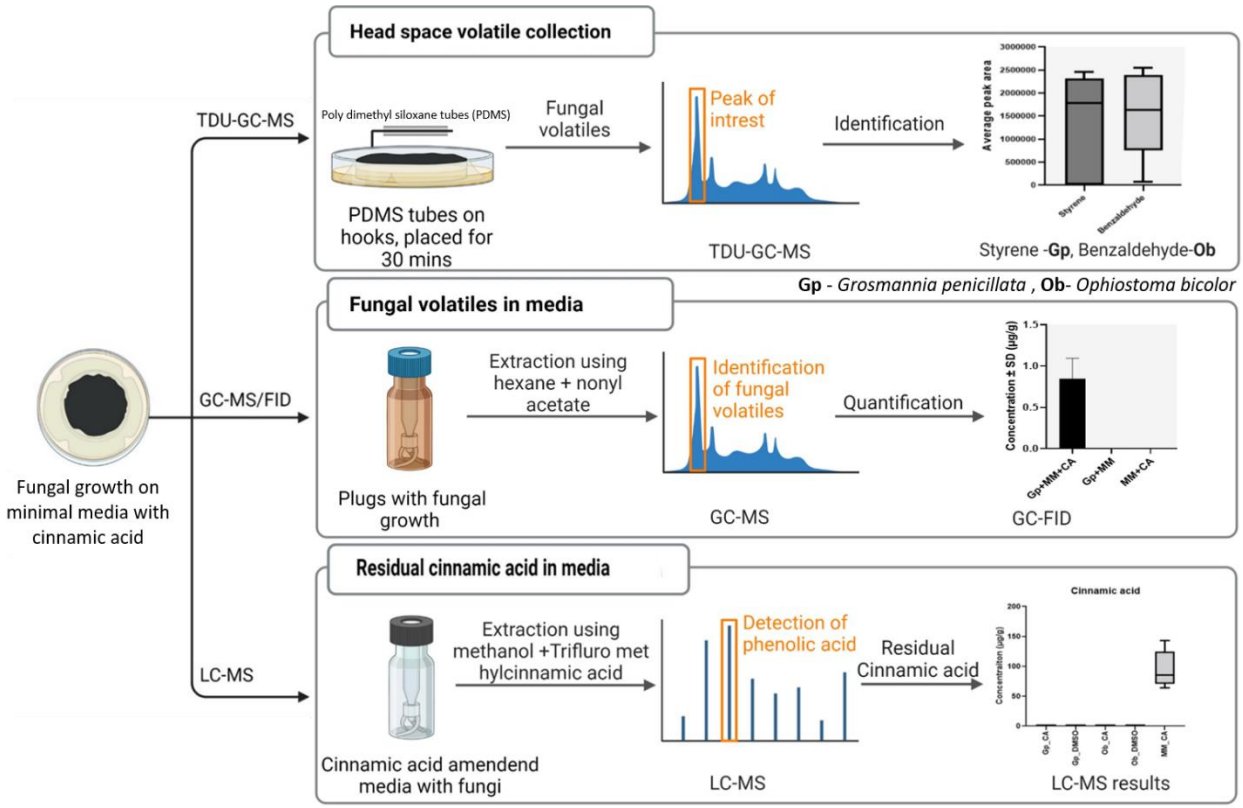

Figure 2.2: Workflow of various chemical analyses presented in this thesis.

2.8.1. Collection of headspace volatiles using PDMS tubes

The headspace volatile collection was performed using polydimethylsiloxane (PDMS) (**Table 2.1**) sorbent silicone tubes. Petri plates with fungal colonies of *G. penicillata* and *O. bicolor* containing 1.5% minimal media (**Table 2.3**) amended with 50 µg/ml and 25 µg/ml cinnamic acid and DMSO (**Table 2.4**) were used for headspace volatile collection. Manually crafted metal hooks with two to three 5mm PDMS tubes inserted in them were used for volatile collection. Before adding the

PDMS tubes, the metal hooks, 1.5 ml brown glass vials and the forceps to be used were all placed in a 200°C oven for 20 minutes to get rid of any residual volatile compounds. The heated hooks, vials, and forceps were cooled under the laminar airflow bench. The PDMS tubes were inserted on the metal hooks and the hooks were placed upright in the agar such that the PDMS tubes did not touch the agar or Petri plate lid (**Figure 2.3**). The hooks with PDMS tubes were left inside Petri plates for 30 minutes to allow efficient adsorption of volatiles. The plates were resealed with paraffin. After the hooks were carefully removed from the plates and the adsorbed PDMS tubes were transferred to labeled brown glass vials and closed tightly. The vials were stored at -20°C until analysis.

Volatiles collected on PDMS tubes were analyzed using a GC-2010 plus gas chromatograph coupled to a MS-QP2010 quadrupole mass spectrometer equipped with a TD-20 thermal desorption unit (Shimadzu, Japan) and a GC Cryo-Trap (Tenax®). A single PDMS tube was placed in a 89 mm glass thermal desorption tube and desorbed at a flow rate of 60 mL min^{-1} for 8 min at 200 °C under a stream of N_2 gas. The desorbed substances were focused in a cryogenic trap at −60 °C. The Tenax® adsorbent was heated to 210 °C and the analytes were injected using split mode (1:100) onto an OPTIMA 5-0.25µm GC column (**Table 2.1**) using helium as carrier gas.

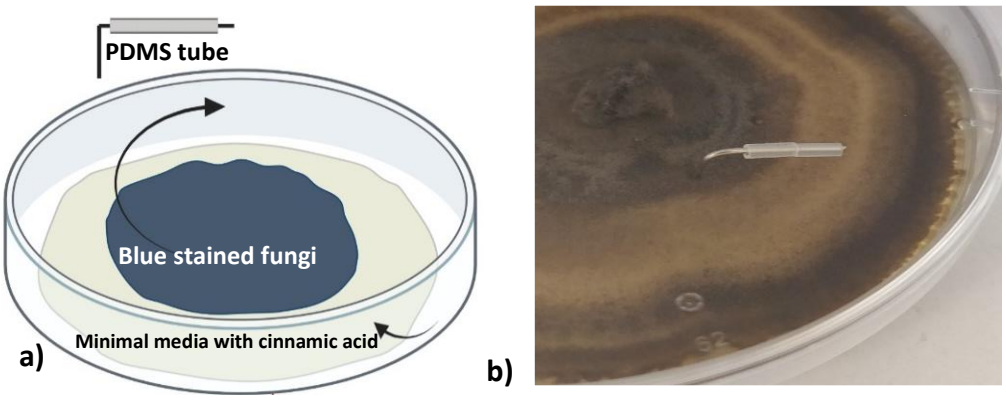

Figure 2.3: **a)** Illustration of headspace volatile collection inside a Petri dish containing live blue-stain fungi **b)** PDMS tubes mounted on metal hooks were placed inside the plate upright and left inside the closed plate for 30 minutes to allow optimal adsorption on the surface of the PDMS tubes. The PMDS tubes were collected and analyzed using a TDU-GC-MS.

2.8.2. Identification and quantification of fungal biotransformation metabolites of cinnamic acid

The same Petri dishes used for headspace volatile collection were also used for the extraction of volatiles from the media containing fungal breakdown products of cinnamic acid. For the extraction, three to four plugs of 6 mm size were taken from each Petri dish, weighed, and transferred to 1.5 ml pre-sterilized glass vials. Then the plugs were homogenized with a plastic pestle and extracted using 1ml of hexane containing 10 µg/ml nonyl acetate for GC-FID/MS analysis (**Table 2.1**). The vials were then vortexed for 30 seconds and then centrifuged for 5 minutes at 15060 rpm and the supernatant was transferred to new glass vials. The extracts were analyzed using GC-MS (Agilent, Santa Clara, USA) along with pure standards and retention time

standards (**Table 2.2**). Quantification of the samples was performed using GC-FID (Agilent, Santa Clara, USA). Styrene and benzyl alcohol were quantified using GC-FID whereas benzaldehyde was quantified using GC-MS instead (**Table 2.2**). GC-MS was used for quantification of benzaldehyde due to the presence of non-ionizable impurities/stabilizing agents in hexane that co-elute with benzaldehyde making it difficult to quantify benzaldehyde using GC-FID (**Figure 2.4**). An external calibration curve for benzaldehyde was used to quantity benzaldehyde in the extracts.

2.8.3. Extraction and analysis of remaining cinnamic acid using LC-MS

To confirm that the phenolic volatiles produced by the fungi were due to the breakdown cinnamic acid, the same Petri plates were used for extraction. Three to four agar plugs of 6mm size with fungal growth were weighed and placed in pre-sterilized glass vials. Then the plugs were homogenized with a plastic pestle and then extracted using 1 ml of methanol containing 10ng/ml of trifluoro methyl cinnamic acid as internal standard. The samples were analyzed using an API 6500 LC-MS, 1 µl Injection volume. Mobile phase used was 0.05 % formic acid and acetonitrile. Column used was an Agilent Zorbax Eclipse XDB C18 (4.6 x 50mm, 1.8 µm).

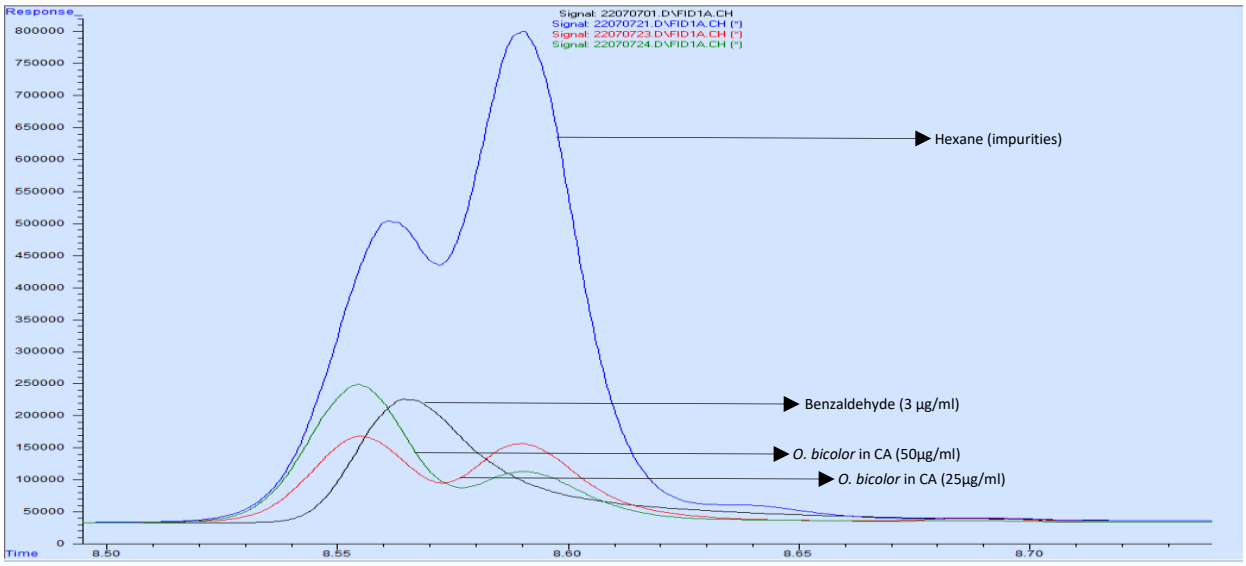

Figure 2.4: GC-FID chromatograms showing the presence of non-ionizable impurities/stabilizers in the extraction solvent hexane used. The peaks in hexane overlap the benzaldehyde peak that might be present in the samples. Hence, GC-FID was not used to quantify benzaldehyde, instead, quantification of benzaldehyde was done using GC-MS data, using an external calibration curve. CA- cinnamic acid.

2.9. Test of phenolic acid toxicity on fungi and beetles

The toxicity of phenolic acids was tested for fungi and adult beetles. We tested the effect of phenolic acids on the growth of fungi, weight change, tunneling behaviour, and the mortality of adult beetles.

2.9.1. Toxicity of phenolic acids to fungi

The effect of phenolic acids on bark beetle-associated ophiostomatoid fungi was tested by checking the growth rate of fungi grown on 50 µg/ml, 5 µg/ml and 0.5 µg/ml of cinnamic acid, ferulic acid, and *p*-coumaric acid enriched potato dextrose agar.

Grosmannia penicillata and *O. bicolor* were grown in 6 cm Petri dishes containing 1.5% potato dextrose agar (PDA) supplemented with phenolic acids dissolved in DMSO to reach the final concentrations of 0.5, 5, and 50 µg/ml. A 5 mm agar plug containing an actively growing fungal colony was placed in the center of the Petri dish and incubated at 25˚C in darkness for 4 days. The growth of each fungus was marked every day. Each treatment was replicated five times. Images of the plates taken on the fourth day were analyzed using Image J (**Figure 2.5**).

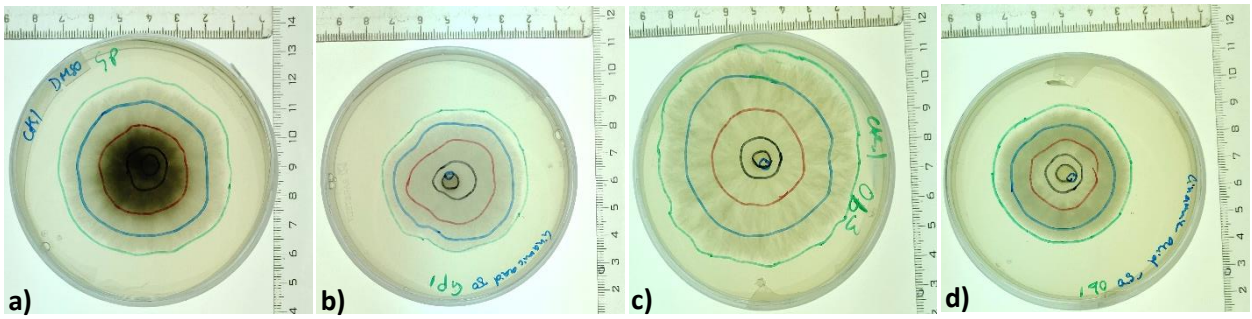

Figure 2.5: Growth of **a)** *G. penicillata* in potato dextrose agar (PDA) with no cinnamic acid, control **b)** *G. penicillata* in PDA with 50 µg/ml cinnamic acid **c)** *O. bicolor* in PDA with no cinnamic acid **d)** *O. bicolor* in PDA with 50 µg/ml cinnamic acid, after 4 days of inoculation.

2.9.2. Toxicity of phenolic acids to beetles

An assay was performed to assess the effect of different doses of cinnamic acid on the tunneling behavior of beetles. The performance of the adult beetles was ascertained by their change in weight and tunnel lengths made by beetles. Artificially prepared galleries were made using 10 cm long pneumatic tubes with an outer diameter of 6 mm, and an inner diameter of 4 mm (Sang-A Pneumatic, Daegu, Korea). These tubes were filled with spruce bark agar (SBA) made from 7% (w/v) of finely milled spruce inner bark powder spruce bark, mixed with 4% bactoagar (Roth) in water and autoclaved for 20 minutes at 121 °C. Different doses of cinnamic acid, 5 µg/ml and 50 µg/ml (in the same quantity of DMSO) and DMSO as control were added to the diet and mixed thoroughly. The concentrations were selected based on the relative abundance of cinnamic acid in the tree (3.1.3, Results) The diet and chemical mixture was drawn up using a disposable sterile syringe (Omnifix 30 ml, B. Braun Melsungen, Germany) to load it in the assay tube. Approximately 9 cm of the tube was filled with the diet leaving 1 cm empty to facilitate the insertion of the beetle into the tubes. The medium was allowed to cool down for 10- 15 minutes and one end of the open assay tubes was sealed with parafilm. The bioassay setup resembled the tunnels that beetles make in their natural system where the beetles can move either forward by tunneling or step back and stay at the starting point (**Figure 2.6**). Before introducing the beetles to the tubes, their weights were recorded. The beetles were placed carefully in the assay tube (1 beetle per tube) with its head facing towards the diet. The opened end of the tube was then sealed with

tissue culture tape to prevent beetles from escaping. The length of the feeding tunnel in each tube was recorded after 6, 24, and 48 hours.

Each treatment was replicated 20 times (10 male and 10 female beetles). Tubes from each treatment (10 tubes) were attached to a whiteboard (**Figure 2.6**). The whole setup was then placed in the climate chamber for 48 hours. After 48 hours, the beetles were taken out from the tubes and their final weights were recorded along with the mortality rate.

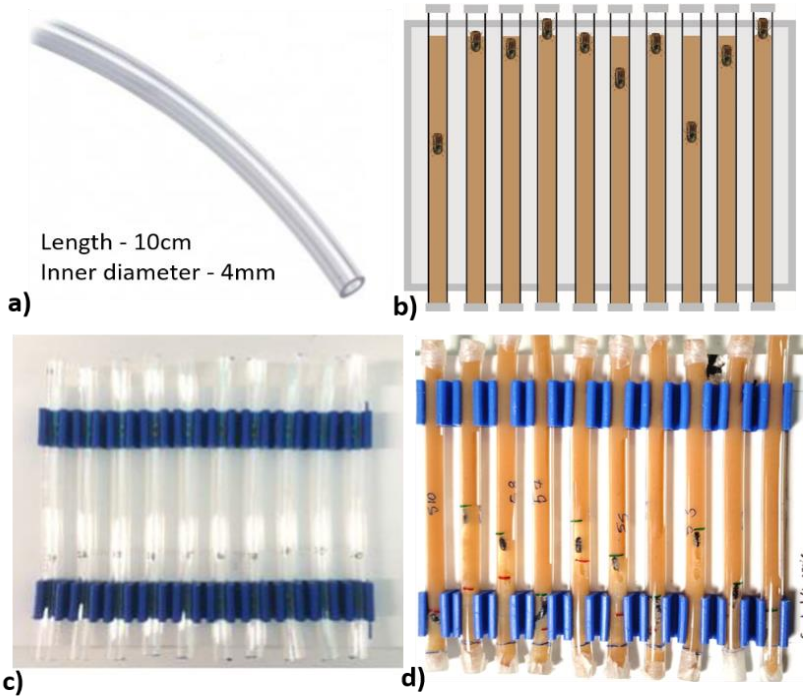

Figure 2.6: Bark beetle feeding assay setup: **a)** pneumatic tube, **b)** representation of the setup, **c)** the tubes attached to the board and numbered, **d)** beetles feeding on an artificial diet mixed with cinnamic acid and tubes marked to measure the tunnel length of the beetles.

2.10. Trap bioassay

The bioassay was performed to assess the choice of adult *I. typographus* to fungal biotransformation products of cinnamic acid products. This assay was performed in two ways, one with fungi grown on spruce bark agar (SBA) media with cinnamic acid and the other with volatile compounds that are known to be produced by the fungi from the previous GC-MS studies.

The olfactometer used in this bioassay was redesigned based on the one described in (Kandasamy et al. 2018) and (Kandasamy, 2019). It was designed for adult beetles to make their choice primarily through olfaction and not by contact cues. Glass Petri dishes with the dimensions 150x25 mm were used, and two 3D printed cups of 20 mm diameter and 220mm height were placed at approx. 4.5cm away from each other and 1.5 cm from the walls of Petri dish. Four holes were made on the side approx. 1cm from the base of Petri dish for the beetles to enter inside the cups. The agar plugs used in the experiment were prepared by adding 7% powdered spruce inner bark to 4% bactoagar. A cork borer (10 mm diameter) was used to make plugs from SBA, which were then inserted into circular cups. Four male or female adult beetles were placed inside each

arena and the olfactometer was placed inside a laminar flow cabinet in the dark. Each experiment was replicated at least 10 times with 4 beetles per replicate (**Figure 2.7**). The choice of beetles was determined periodically for up to four hours by counting the number of beetles trapped inside the cups at an interval of 2 hours and chemotaxis indices (CI) were calculated using the formula, CI= (No. of beetles in the treatment trap- no. of beetles in the control trap)/ total number of beetles.

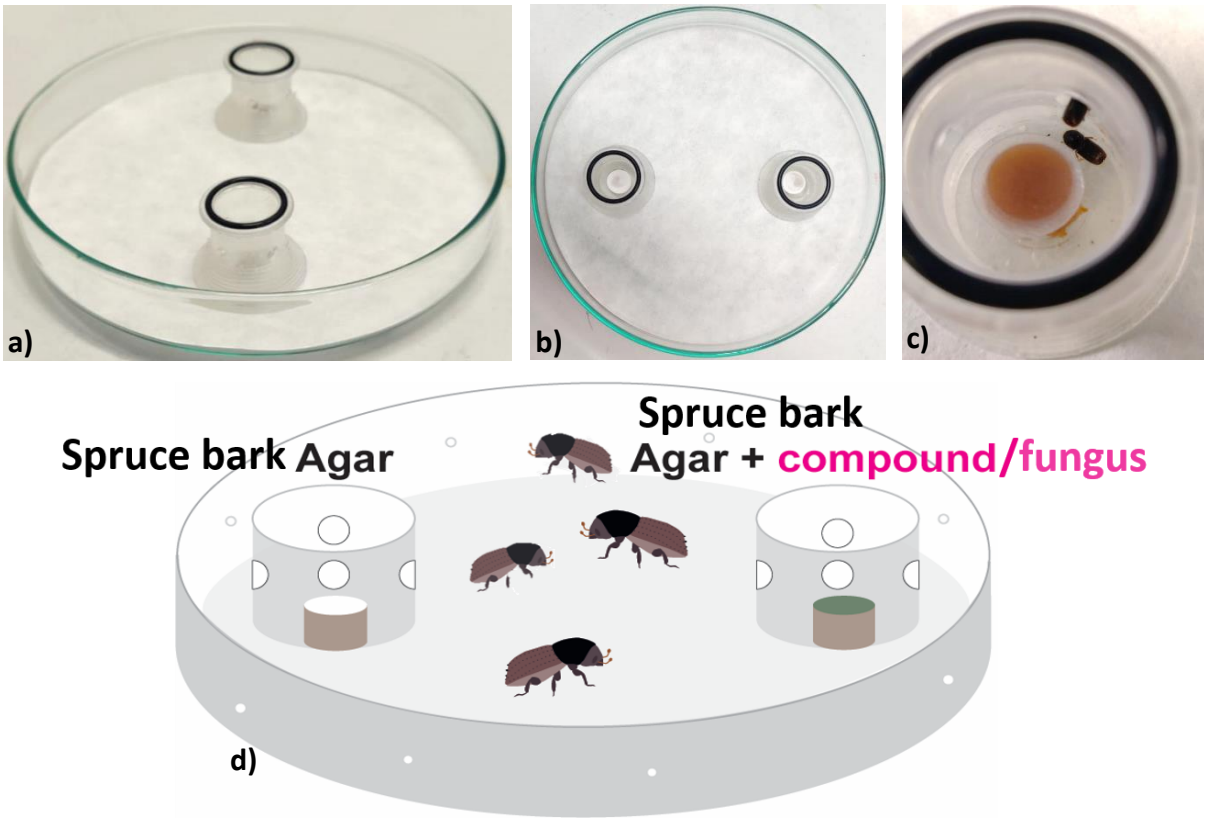

Figure 2.7: Olfactory-based trap choice bioassay setup **a)** side view, **b)** top view, **c)** Beetle entering the cup containing spruce bark agar (SBA) plug covered with a phenolic compound, **d)** Setup for olfactory-based trap choice assay with 4 beetles and two choices, control, and treatment. For the test with fungal blends, the fungi *G. penicillata* or *O. bicolor* were grown on SBA with or without cinnamic acid. For the test with compounds, styrene, benzaldehyde, and benzyl alcohol were added individually on the filter paper that was placed on the top of agar plugs.

2.10.1. Trap bioassay using fungi grown on media amended with phenolic acids

Cinnamic acid (50 µg/ml) was added to 7% spruce bark agar (SBA) and this concentration was sufficient to produce the entire bouquet of biotransformation products (2.9.1, Materials and Method). To determine the performance of bark beetles on cinnamic acid biotransformation products, both *G. penicillata* and *O. bicolor* were used individually, as their volatile profile was different in the presence of cinnamic acid. Controls were fungus grown in SBA without cinnamic acid and SBA with cinnamic acid (**Figure 2.7**).

2.10.2. Trap bioassay using volatile phenolics

For bioassays using phenolic acids, stock solutions were prepared by serial dilution of the pure styrene, benzaldehyde, and benzyl alcohol in mineral oil. 10 µl of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} (10%, 1%, 0.1%, 0.01%) dilutions were added on a filter paper (Grade1, Whatman filter paper, GE Healthcare Life Sciences) carefully placed on top of the 7% spruce bark agar plugs (10mm) in the treatment cups of the olfactometer. The control cups had the plug of SBA with only mineral oil on the filter paper. This experiment was to determine the overall response of the adult beetles to changes in dilutions of pure compounds (**Figure 2.7**).

2.11. Statistical analysis

All the statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software, La Jolla California USA, www.graphpad.com). For statistical analysis, the normal distribution of all the data was checked by the Shapiro Wilk test.

To test the growth of fungi on spruce, the average length and area of different fungi and control were compared using two-way ANOVA with 0.05 as the alpha value. Uncorrected Fisher's LSD was the *post hoc* test used to test differences between fungi.

Change in phytohormones and phenolic acid concentrations in the bark samples with fungal growth were tested using two-way ANOVA with 0.05 as the alpha value. Turkey's multiple comparison test was used to test differences among treatments. Quantification of the volatiles produced by the *G*. *penicillata* and *O. bicolor* in the bark was done using an unpaired two-tailed t-test with 0.05 as the alpha value.

The breakdown of cinnamic acid present in the media, production of styrene, benzaldehyde, and benzyl alcohol were statistical compared using an unpaired two-tailed t-test with a 0.05 alpha value.

The toxicity of different phenolic acids to fungi and their concentrations was tested using a two-way ANOVA test, followed by Tukey's multiple comparisons. For bark beetle toxicity assay, tunnel length and weight gain data were tested by the Shapiro- Wilk and Kolmogorov-Smirnov tests for normality. Data were analysed 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 change. Two-way ANOVA followed by Sidak's post hoc test was used for testing changes in tunnel lengths.

Response indices were calculated for the behavioural change of males and females in the olfactory-based trap bioassay. Wilcoxon signed-rank test was performed between the two choices given to the male and female beetles with 0.05 as the alpha value.

3. Results

3.1. Analysis of fungal growth in spruce logs

3.1.1. Growth of fungi in spruce logs

The virulence of different fungi was tested by measuring the length and area of the necrotic lesions in the phloem of the bark induced in response to artificial inoculation of fungi in spruce tree logs. The images of lesions were processed, and the length and area of lesions were measured using Image J.

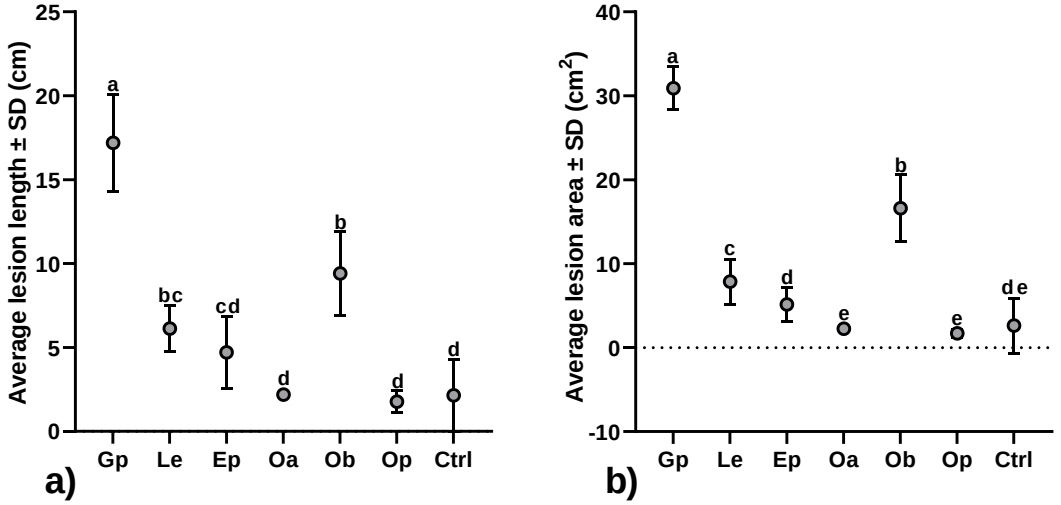

Figure 3.1: Average **a)** lesion length, **b)** lesion area in spruce logs in response to different fungal inoculations after 14 days. The average was measured using 10 samples (2 replicates from each of the 5 logs). The error bars represent the standard deviation. Different letters indicate significant differences among treatments. (Two-way ANOVA, Uncorrected Fishers LSD's *post hoc* test, P<0.05). Gp- *G. penicillata,* Le- *L. europhioides,* Ep- *E. polonica,* Oa- *O. ainoae,* Ob- *O. bicolor,* Op- *O. piceae* and Ctrl- control.

Treatment	F value / t value for lesion length	p value for lesion length	F value / t value for lesion area	p value for lesion area
Overall Fungi	79.14	****(<0.0001)	65.56	****(<0.0001)
Gp - control	17.02	****(<0.0001)	11.11	****(<0.0001)
Le - control	4.505	**(0.0099)	3.25	** (0.0041)
Ep - control	2.901	ns	1.352	ns
Oa - control	0.044	ns	0.2776	ns
Ob - control	8.218	***(0.0004)	5.503	****(<0.0001)
Op - control	0.432	ns	0.5810	ns
Gp - Ob	4.650	****(<0.0001)	6.408	***(0.0002)

Table 3.1: Results of Shapiro- Wilk normality for both average lesion lengths and area for different fungi. The difference between *G. penicillata* and *O. bicolor* was analyzed using two-tailed unpaired t-test. Gp- *G. penicillata*, Le- *L. europhioides*, Ep- *E. polonica*, Oa- *O. ainoae*, Ob- *O. bicolor*, Op- *O. piceae*, Ctrl-control.

The mean lesion length and area for *G. penicillata* (Gp), *O. bicolor* (Ob), and *L. europhioides* (Le) were significantly larger compared to the wounded control (**Table 3.1**). Among fungi, *G. penicillata* caused the longest lesions followed by *O. bicolor* than the other four fungi and the wounded control (**Figure 3.1**).

3.1.2. Quantification of phytohormones

Phytohormone levels were on average higher in fungus-infected phloem when compared to pre-control and post-controlsamples. The amount of jasmonic acid was among the highest of all phytohormone followed by jasmonic acid-isoleucine. The amount of COOH-jasmonic acid was among the lowest of all produced phytohormone.

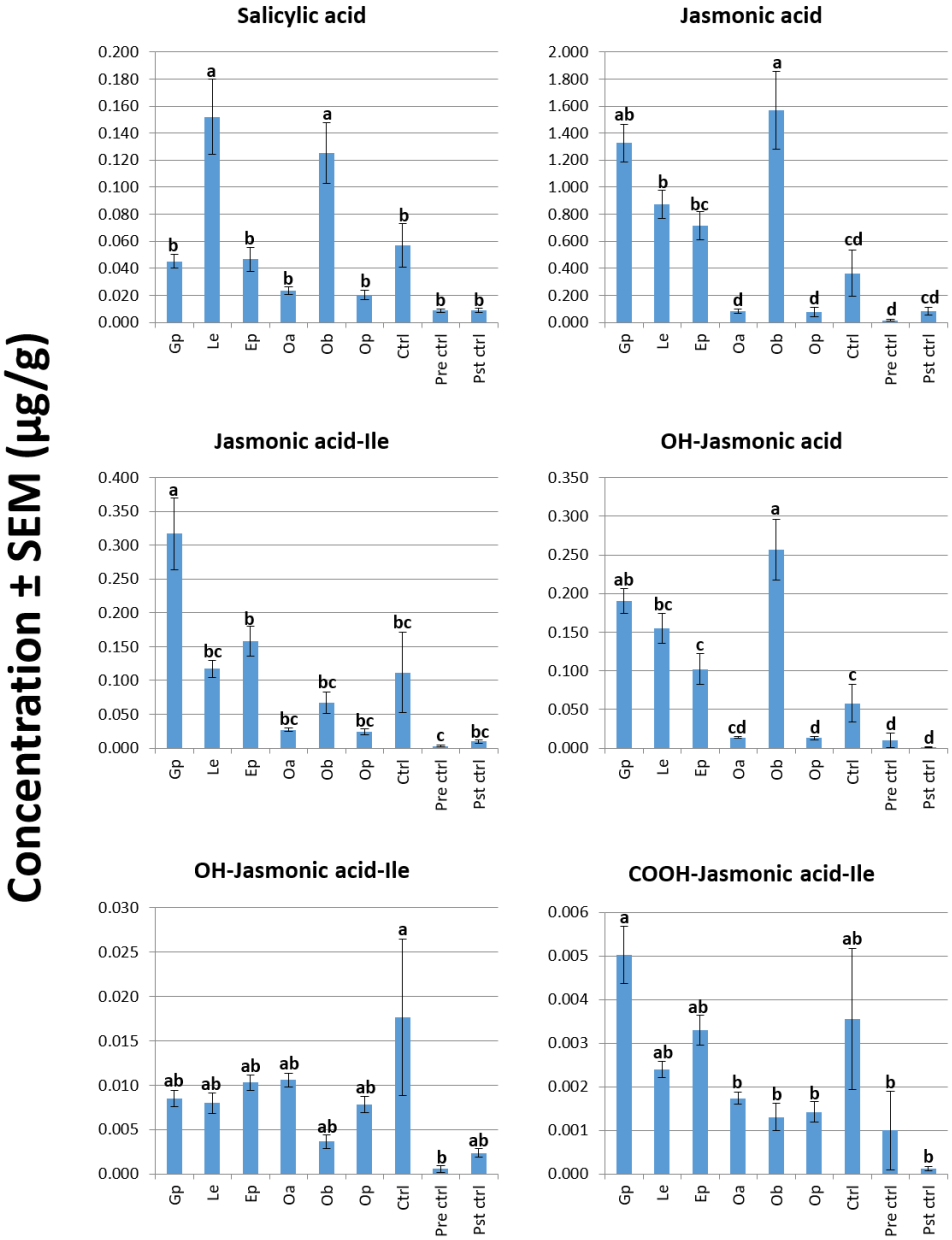

Figure 3.2: Quantification of phytohormones from spruce logs inoculated with different fungi and uninfected controls. Controls included samples removed before (Pre-ctrl) and after (Pst-ctrl) infection or wounding

treatment from undamaged logs, and a sample into which medium was inoculated without fungi (Ctrl). Different letters indicate significant differences among treatments. (Two-way ANOVA, Uncorrected Fishers LSD's *post hoc* test, $P<0.05$). Gp- *G. penicillata*, Le- *L. europhioides*, Ep- *E. polonica*, Oa- *O. ainoae*, Ob- *O. bicolor*, Op- *O. piceae*, Ctrl-control.

Salicylic acid was found to be in high concentration in the bark infected by *L. europhioides* and *O. bicolor* when compared to other fungi and all controls. When compared to the controls, *O. bicolor, G. penicillata,* and *L. europhioides* infected bark contained significantly higher concentrations of jasmonic acid. *G. penicillata*-infected bark contained higher concentrations of jasmonic acid-isoleucine when compared to all other fungi and all controls. *O. bicolor* and *G. penicillata* had a higher concentration of OH-jasmonic acid compared to all the controls. The concentration of OH-jasmonic acid-isoleucine was not significantly different in the fungal samples when compared to the control. *G. penicillata*-infected bark had a significantly high concentration of COOH-jasmonic acid- isoleucine when compared to the pre and post control. (**Figure 3.2**).

3.1.3. Quantification of phenolic acids

The chemical composition of spruce bark after fungal infection was determined using LC-MS. Phytohormones and phenolic acids were quantified from the bark samples of different treatments.

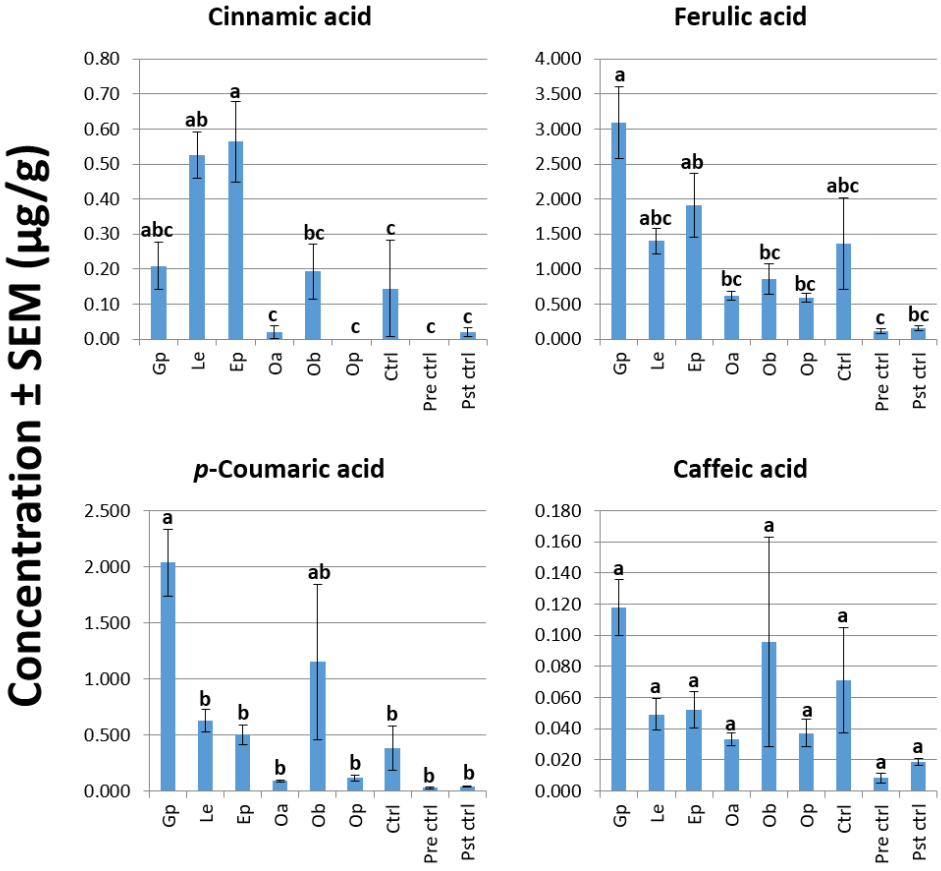

Figure 3.3: Quantification of phenolic acids in spruce logs after fungal infection. Controls included samples removed before (Pre-ctrl) and after (Pst-ctrl) infection or wounding treatment from undamaged logs, and a sample into which medium was inoculated without fungi (Ctrl). Different letters indicate significant differences

among treatments. (Two-way ANOVA, Uncorrected Fishers LSD's *post hoc* test, *P*<0.05). Gp- *G. penicillata*, Le- *L. europhioides*, Ep- *E. polonica*, Oa- *O. ainoae*, Ob- *O. bicolor*, Op- *O. piceae*, Ctrl-control, Pre ctrl- pre control, Pst ctrl- post control.

Ferulic acid was the phenolic acid produced in highest amounts. Cinnamic acid was in significantly higher concentrations in *L. europhioides* and *E. polonica* when compared to the control. *Grosmannia penicillata,* had significantly higher concentrations of ferulic acid when compared to *O. ainoae*, *O. bicolor*, *O. piceae*, pre and post-control but not when compared to *L. europhioides, E. polonica,* and control. The concentration of *p*-coumaric acid was significantly higher in *G. penicillata* when compared to control. Caffeic acid showed no significant difference between the fungi and all the controls. There was no significant difference between *G. penicillata* and *O. bicolor* phenolic acids (**Figure 3.3**) except for ferulic acid (p=0.0004, t=4.353, dF=18, Unpaired t-test). The concentration of caffeic acid was the lowest of the phenolic acids, and hence not considered for further experiments. (The remaining phenolics tested are represented in Supplementary, **Figure S2**).

3.1.4. Identification and quantification of volatiles from fungal-infected spruce logs using GC-MS and GC-FID

Fungal biotransformation of phenolic acids is likely to produce aromatic volatiles. Thus, we quantified aromatic compounds from fungal-infected spruce logs that might be metabolites of phenolic acids. The identification and quantification of fungal volatiles were performed using pure standards, a standard mixture of alkanes (retention time standard) and the NIST11 library to ensure the accuracy of the compounds analyzed. Retention time standards and pure standards were used to calculate retention indices of volatile by-products (**Figure 2.1**, Materials and Method) (**Table 3.2**).

Styrene was produced only by *G. penicillata* in quantifiable concentrations of 0.412 ng/mg of fresh bark. Both *G. penicillata* and *O. bicolor* produced benzaldehyde and benzyl alcohol, which were not observed in control samples. *G. penicillata* also produced benzaldehyde (*P*=0.0036, t=3.380, dF=17, Unpaired t test) and benzyl alcohol (p=0.0002, t=4.474, dF=20, Unpaired t test) significantly higher compared to *O. bicolor* (**Figure 3.4**).

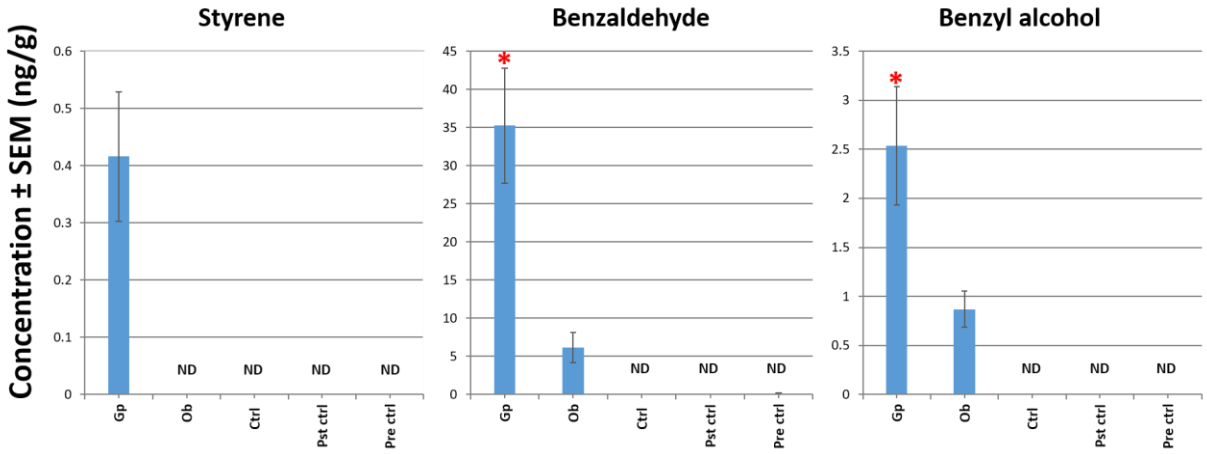

Figure 3.4: Aromatic volatiles produced by *G. penicillata* and *O. bicolor* growing on spruce logs. Benzaldehyde and benzyl alcohol were the major products of both the fungi. *Grosmannia penicillata* also produced styrene.

ND- not detected, *-(p<0.0001) (Unpaired tow tailed t-test). Gp- *G. penicillata*, Ob- *O. bicolor*, Ctrl-control, Pre ctrl- pre control, Pst ctrl- post control.

Compound	Retention time of compound (minute)	No. of carbon atoms of alkane before the compound	RI
Styrene	5.45	8	888
Benzaldehyde	7.07	9	960
Benzyl alcohol	9.25	10	1036

Table 3.2 –Retention indices calculated using the formula in (**Figure 2.1**, Material and Method) for different compound of interest using retention time of pure standards and number of carbon atoms of alkane compounds in the retention time standard (**Figure S1**, Supplementary).

3.2. Analysis of phenolic acid-amended medium colonized by fungi

3.2.1. Metabolism of cinnamic acid in media colonized by fungi

The concentration of the remaining cinnamic acid added in the synthetic agar medium after fungal growth was quantified using LC-MS. Interestingly both *G. penicillata* and *O. bicolor* completely degraded cinnamic acid added to the media. Cinnamic acid was identified only in cinnamic acid control plates with no fungal growth (**Figure 3.5**).

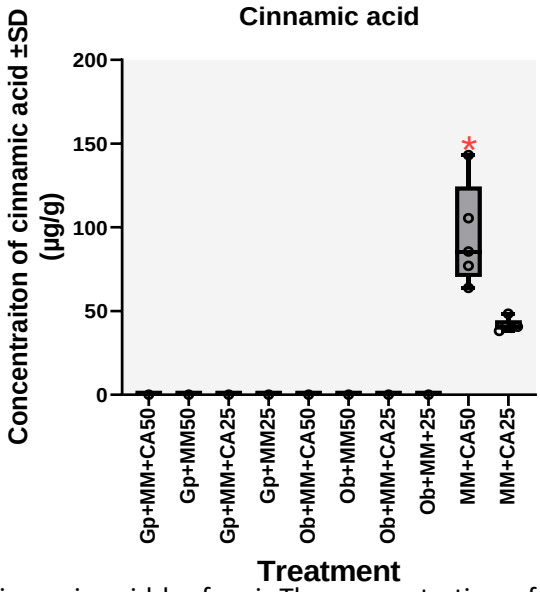

Figure 3.5: Degradation of cinnamic acid by fungi. The concentration of remaining cinnamic acid was analyzed in the samples after *G. penicillata* (Gp) and *O. bicolor* (Ob) were allowed to grow on minimal media amended with it. *- significance, Gp- *G. penicillata* and Ob- *O. bicolor*, MM- Minimal media, CA50- cinnamic acid 50 µg/ml, CA25- cinnamic acid 25 µg/ml.

3.2.2. Identification and quantification of fungal volatiles produced on synthetic medium containing cinnamic acid

3.2.2.1. TDU-GC-MS

Relative quantification and identification of the volatiles emitted from fungi grown on cinnamic acid amended medium were done using TDU-GC-MS. Styrene was detected in samples containing *G. penicillata* (**Table 2.3**, Materials and Method) (**Figure S3,** Supplementary), while other samples

showed little to no presence of styrene. On the other hand, benzaldehyde and benzyl alcohol were emitted from *O. bicolor* grown on cinnamic acid (**Figure S4,** Supplementary).

The peak area of styrene produced by *G. penicillata* in 25 µg/ml of cinnamic acid medium was significantly higher compared to the same fungus grown on 50 µg/ml of cinnamic acid (*P*=0.029, t=2.755, dF=8, Unpaired t test). There was no significant difference in the emission of benzaldehyde or benzyl alcohol between the two concentrations, although the 50 µg/ml concentration showed higher peak areas for both the volatiles (**Figure 3.6**).

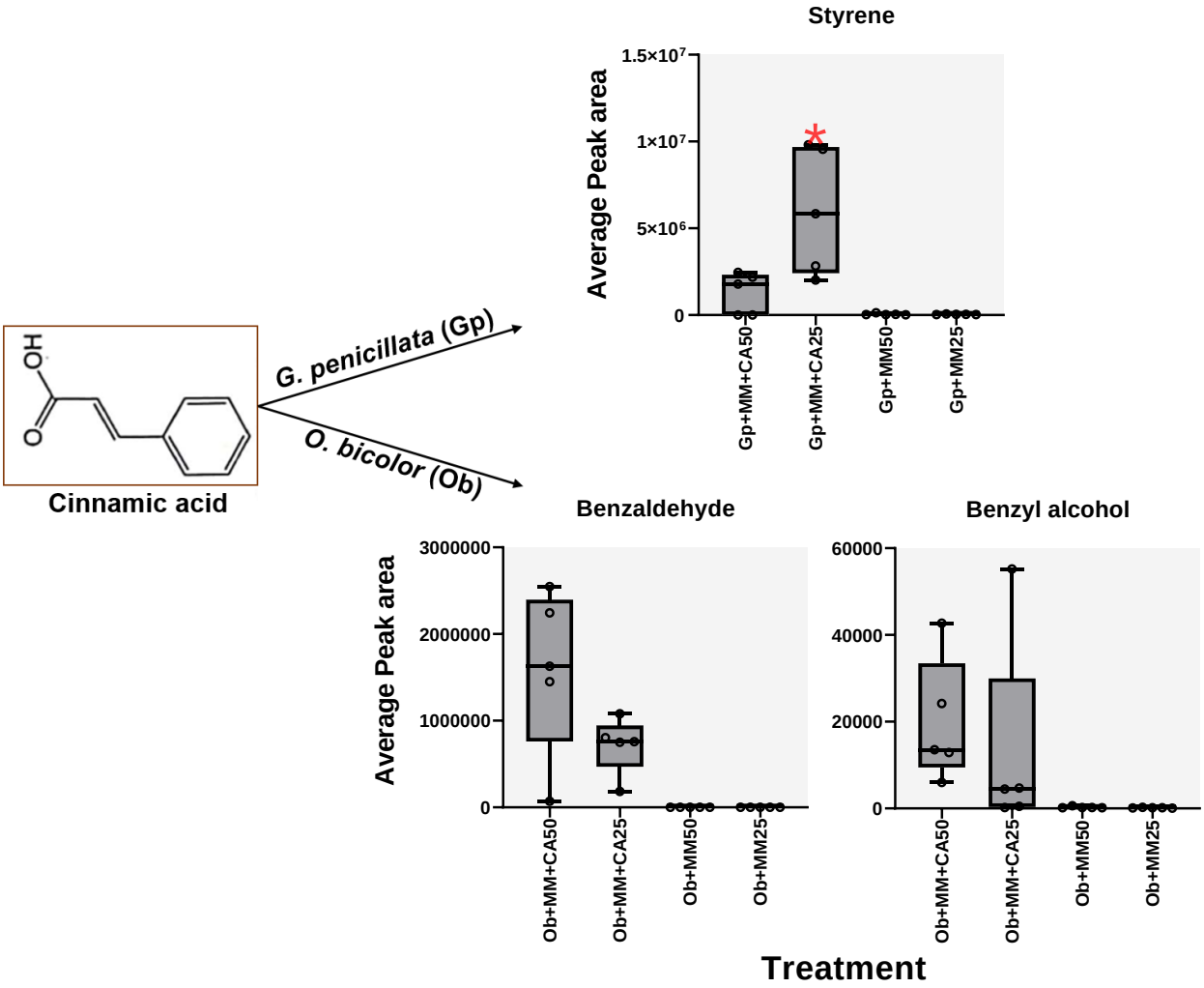

Figure 3.6: Headspace volatiles from *G. penicillata* and *O. bicolor* grown on cinnamic acid-amended medium measured using a TDU-GC-MS. Styrene was the only biotransformation product produced by *G. penicillata.* Benzaldehyde and benzyl alcohol were produced by *O. bicolor*. Gp- *G. penicillata* and Ob- *O. bicolor*, MM- Minimal media, CA50- cinnamic acid 50 µg/ml, CA25- cinnamic acid 25 µg/ml.

3.2.2.2. GC-FID/MS

The fungal volatiles extracted from the cinnamic acid-enriched medium colonized by different fungi were identified and quantified using GC-MS and GC-FID. Styrene and benzyl alcohol were quantified using GC-FID, whereas benzaldehyde was quantified using GC-MS.

A calibration curve was made using external standards (**Table 3.2**). The concentration for benzaldehyde was then calculated using the calibration curve (**Figure 3.7**).

Results were similar to the results of the headspace volatiles, with respect to the presence of styrene observed in *G. penicillata* and of benzaldehyde and benzyl alcohol in *O. bicolor*. A higher concentration of styrene was observed with 25 µg/ml of cinnamic acid compared to 50 µg/ml, though there was no significant difference observed. Benzaldehyde was found to be greater in 50 µg/ml than 25 µg/ml of cinnamic acid but this difference was not significant. Benzyl alcohol showed similar concentrations for both 25 and 50 µg/ml of cinnamic acid (**Figure 3.8**)**.**

Sl no.	Benzaldehyde (µg/ml)	Peak area
1	5	126201306
2	2.5	67170810
3	1.25	32860272
4	0.625	14670443
5	0.3125	7964737
6	0.15625	3246200
7	0.078125	1466116
8	0.039063	595155
9	0.019531	309970

Table 3.2: Dilutions of benzaldehyde and peak area from GC-MS at given dilutions.

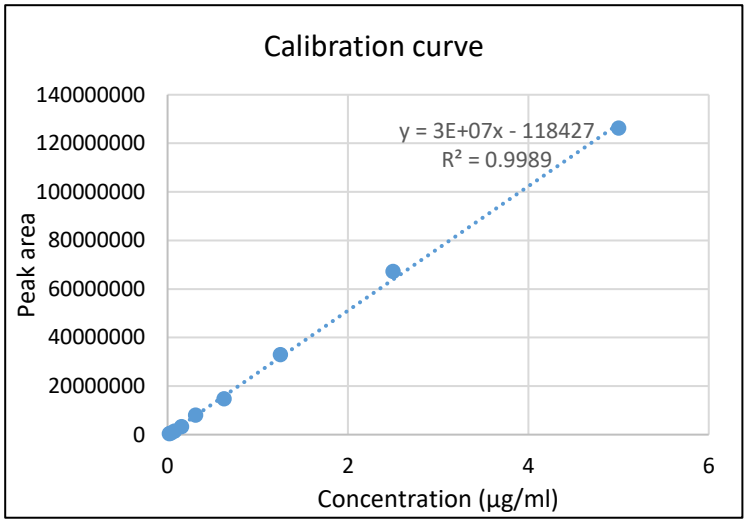

Figure 3.7: External calibration curve generated for quantifying benzaldehyde using GC-MS.

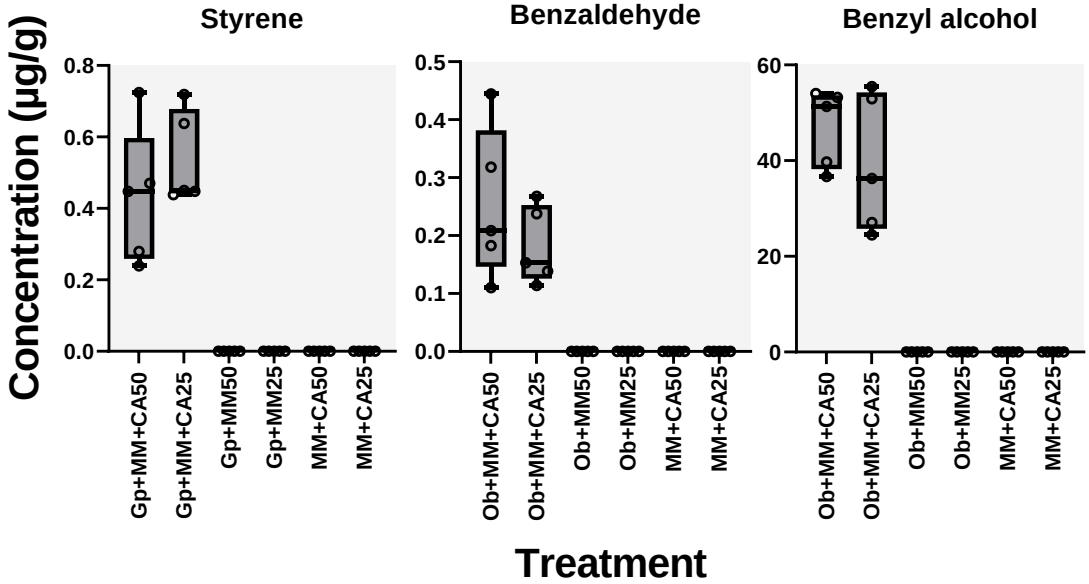

Figure 3.8: Metabolites produced from *G. penicillata* and *O. bicolor* grown on cinnamic acid-amended medium. Compounds found previously as volatiles were extracted from the medium and quantified using a GC-FID/MS and GC-FID. Styrene was the major biotransformation product produced by *G. penicillata.* Benzaldehyde and

benzyl alcohol were produced by *O. bicolor*. Gp- *G. penicillata* and Ob- *O. bicolor*, MM- Minimal media, CA50- cinnamic acid 50 µg/ml, CA25- cinnamic acid 25 µg/ml.

3.3. Toxicity of phenolic acids

3.3.1. Toxicity of phenolic acids to fungi

The growth of *G. penicillata* was significantly higher on PDA medium amended with ferulic acid as compared to medium amended with cinnamic acid (p=0.0074, Two-way ANOVA, Tukey's multiple comparison *post hoc* test) and *p*-coumaric acid (p=0.0024, Two-way ANOVA, Tukey's multiple comparison *post hoc* test) based on the surface area of growth after 4 days post-inoculation. There was no significant difference observed between cinnamic acid and p-coumaric acid. The growth of *O. bicolor* was not significantly different for the three phenolic acids. *Grosmannia penicillata* grew best in ferulic acid (mean=55.06 cm^2) followed by p-coumaric acid (mean= 38.52 cm^2) and grew in cinnamic acid the least (mean=30.40 cm^2). *O. bicolor* showed the least growth in cinnamic acid (mean =36.10 cm^2) (**Figure 3.9**).

Both *G. penicillata* and *O. bicolor* grew significantly less in 50 µg/ml of cinnamic acid (Two-way ANOVA, Šídák's multiple comparisons test *post hoc* test, P<0.05) (**Table 3.3**) compared to the control. However, *G. penicillata* had a significantly higher growth rate at 0.5 and 5 µg/ml of cinnamic acid amended medium compared to the unamended control medium. Their growth was not significantly affected by the change in concentrations of ferulic or *p*-coumaric acid. (**Table 3.3**). Among the two fungi, the growth of *O. bicolor* was significantly higher than *G. penicillata* for most of the concentrations except for 50 µg/ml, in which *G. penicillata* grew more than *O. bicolor* but not significantly (**Table 3.3**).

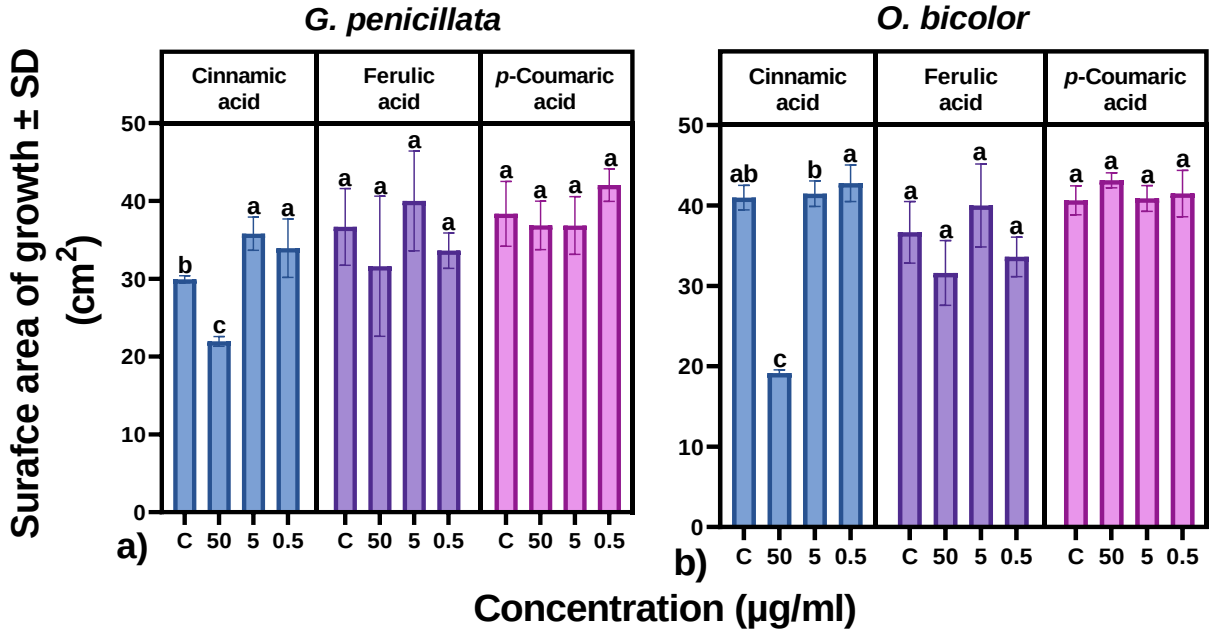

Figure 3.9: Growth of **a)** *G. penicillata* and **b)** *O. bicolor*, in different phenolic acids such as cinnamic acid, ferulic acid, and *p*-coumaric acid and control at different concentrations. Different lower-case letters indicate that values are significantly different from each other (p<0.05).

Factor	Cinnamic acid concentration (µg/ml)	Summary	*P* value
G. penicillata	50 vs. 5	****	<0.0001
	50 vs. 0.5	****	<0.0001
	50 vs. control	****	<0.0001
	5 vs. 0.5	ns	
	5 vs. control	***	0.0008
	0.5 vs. control	*	0.0281
O. bicolor	50 vs. 5	****	<0.0001
	50 vs. 0.5	****	<0.0001
	50 vs. control	****	<0.0001
	5 vs. 0.5	ns	
	5 vs. control	***	0.0008
	0.5 vs. control	*	0.0289
G. penicillata and O. bicolor	50 vs. 5	****	<0.0001
	50 vs. 0.5	****	<0.0001
	50	ns	
	5	**	0.005
	0.5	****	<0.0001
	control	****	(<0.0001)

Table 3.3: Statistical results of analysis of variance (ANOVA), on the growth of *G. penicillata* and *O. bicolor* on PDA medium amended with various concentrations of cinnamic acid and control medium. For comparison of fungal growth on control and different concentrations of cinnamic acid, two-way ANOVA with Dunnett's multiple comparisons test was performed. To compare the growths of *O. bicolor* and *G. penicillata,* two-way ANOVA and, Šídák's multiple comparisons test was used. ns – not significant.

3.3.2. Toxicity of cinnamic acid to adult beetle using feeding assay

The toxicity of cinnamic acid to the adult beetles was tested in artificial gallery tubes filled with phloem- based diet amended with various concentrations of cinnamic acid. The presence of cinnamic acid in the medium did not result in any mortality after 48 hours of feeding on the artificial diet.

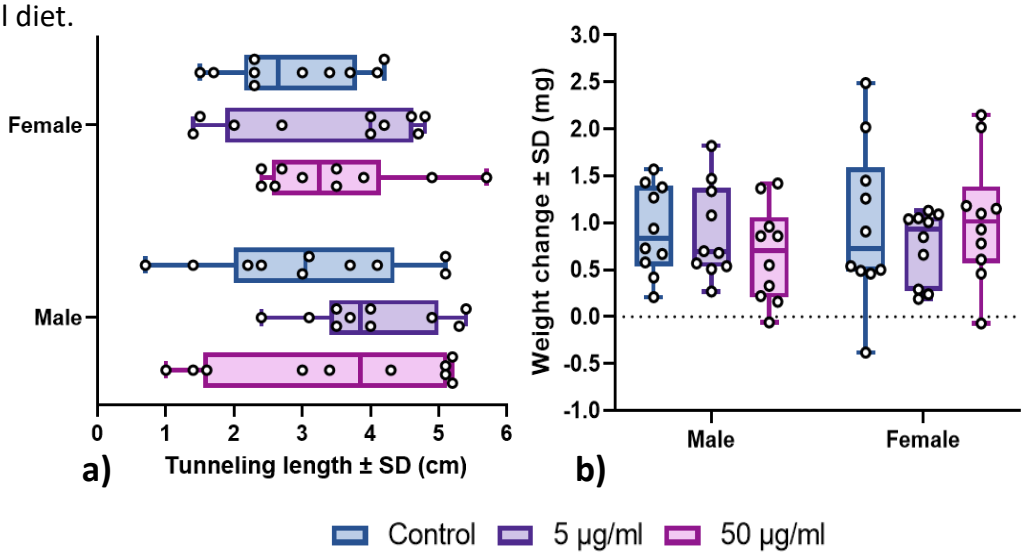

Figure 3.10: **a)** Tunnel length and **b)** weight gained the beetles after 48 hours in artificial gallery tubes, across various concentrations of cinnamic acid for males and females. For each concentration and sex there was no significant difference in tunneling length or weight gain.

Most of the beetles in all the experimental groups gained weight regardless of their sex and doses. Tunneling length increased with time in all the tested doses, especially at a lower dose (5 µg/ml) (**Figure 3.10**). Overall, the tunnelling length or weight gain of the adult beetles was not significantly affected by dose, time, and sex.

3.4. Behavioural response of adult bark beetles to fungal metabolites

3.4.1. Trap bioassays using live fungi grown on cinnamic acid amended and unamended media

Olfactory-based choice assays were conducted to understand the functional role of fungal biotransformation products. Overall *I. typographus* beetles were attracted to spruce bark agar (SBA)- colonized by fungi compared to only SBA. Male beetles showed significantly stronger attraction towards SBA colonized by both fungi compared to SBA alone within 4 hours of exposure. Female beetles showed similar attraction, but it was not significant. When the beetles were given a choice between SBA with cinnamic acid and SBA with cinnamic acid and fungi, males and females showed a significant preference for SBA with cinnamic acid and *G. penicillata* (W=55, *P*=0.0020, Wilcoxon's test, Males), (W=28, *P*=0.0156, Wilcoxon's test, Females) . Whereas, for *O. bicolor* only males showed a significant attraction to SBA with cinnamic acid and *O. bicolor* (W=36, *P*=0.0078, Wilcoxon's test).

Interestingly, when the beetles were given a choice between SBA with fungi and SBA with cinnamic acid and fungi, males significantly preferred *G. penicillata* grown on SBA with cinnamic acid (W=21, *P*=0.0313, Wilcoxon's test). On the contrary, adult males significantly preferred *O. bicolor* grown on unamended SBA over *O. bicolor* grown on cinnamic acid amended SBA (W=36, *P*=0.0352, Wilcoxon's test). The females showed indifferent response towards SBA with cinnamic acid and fungi (**Figure 3.11**).

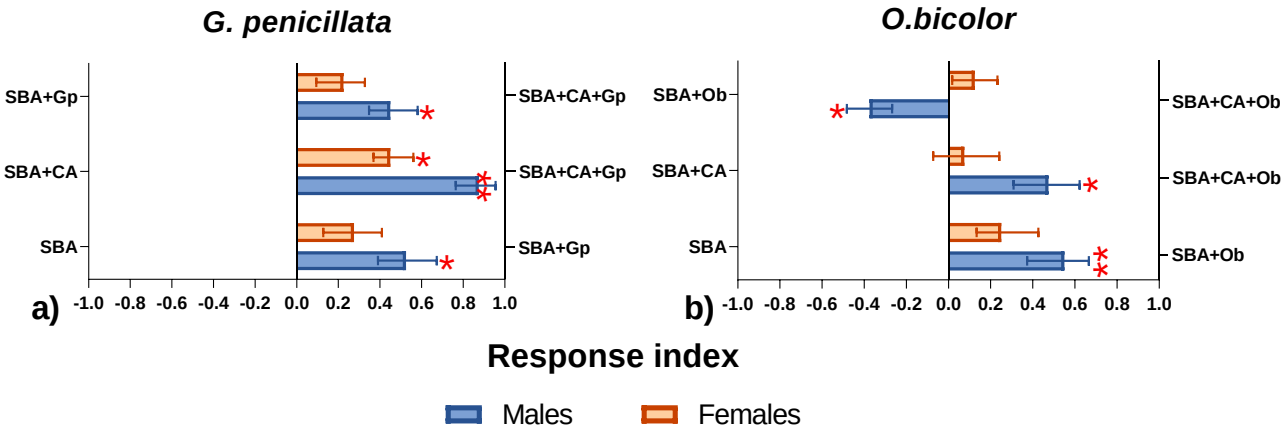

Figure 3.11: Trap bioassays for male and female bark beetles given a binary choice between spruce bark agar (SBA), SBA amended with cinnamic acid (CA) (50 µg/ml) or fungi, or SBA with CA and fungi. A chemotaxis response index was calculated for the choice between all the given combinations with **a)** *G. penicillata* (Gp) and **b)** *O. bicolor* (Ob). RI = 1, complete attraction, RI = -1, complete avoidance, RI = 0, no response. RI for each treatment was determined using at least 40 beetles with four beetles per replicate and the deviation of RI against zero was tested using Wilcoxon's test. Asterisks represent a significant difference * -p<0.05, ** -p<0.01. SBA- spruce bark agar, CA- cinnamic acid, Gp - *G. penicillata* and Ob- *O. bicolor*.

3.4.2. Trap bioassay using pure synthetic fungal biotransformation products

To determine the role of individual fungal biotransformation products of cinnamic acid on adult beetles, olfactory-based choice bioassays were performed for male and female adult beetles separately using pure compounds. A chemotaxis response index was calculated for males and females across different dilutions of styrene, benzaldehyde, and benzyl alcohol in mineral oil. Beetles were exposed to 10^{-1} (10%), 10^{-2} (1%), 10^{-3} (0.1%), 10^{-4} (0.01%) dilutions of tested compounds. Bioassays with styrene showed an overall positive attraction towards styrene by both males and females at most dilutions. However, only males showed a significantly stronger attraction to styrene at 10^{-2} (1%) dilution (W=45, *P*=0.0039, Wilcoxon's test) (**Figure 3.12, a**).

On contrary, benzaldehyde did not elicit any response in both sexes across all dilutions (W=2, *P*=0.875, Wilcoxon's test). Although both males and females were attracted to benzaldehyde at 10^{-2} (1%) dilution, the attraction was not significant. (**Figure 3.12, b**).Similar results were obtained for benzyl alcohol where no significant choice was made by males and females across all dilutions (**Figure 3.12, c**).

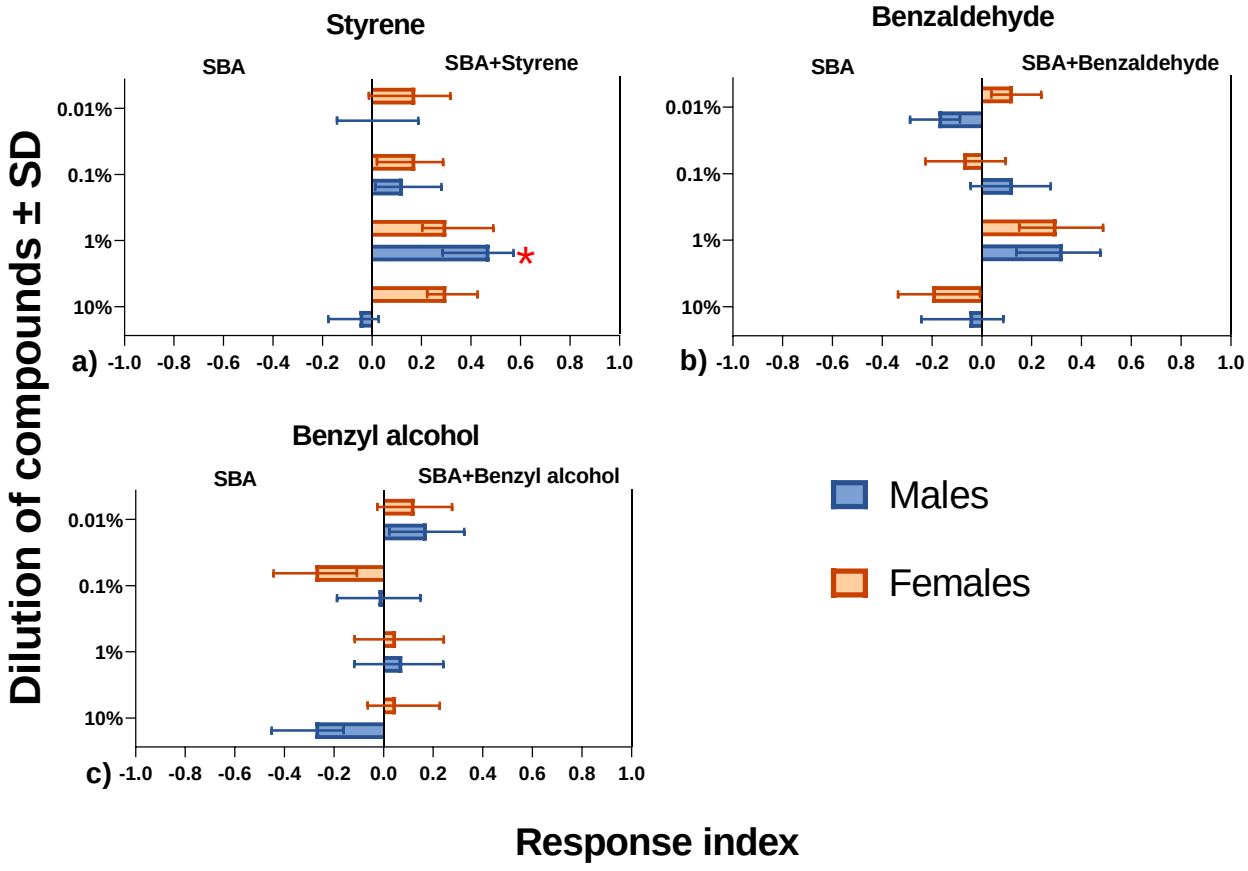

Figure 3.12: Choice of male and female bark beetles to synthetic fungal biotransformation products. Response indices for bark beetles were calculated when insects were given a choice between mineral oil and **a)** styrene, **b)** benzaldehyde and **c)** benzyl alcohol at different dilutions of 0.01%, 0.1%, 1%, 10% made using mineral oil. RI = 1, full attraction, RI = -1, full avoidance, RI = 0, no response. RI for each treatment was determined using at least 40 beetles with four beetles per replicate and the deviation of RI against zero was tested using Wilcoxon's test. * Represent a significant difference between only SBA and SBA with compound, * -p<0.05. SBA- Spruce bark agar.

4. Discussion

Insect microbe interactions often depend on the chemical communications that occur between these organisms. Such communications are vital for the reproduction and survival of both parties (Zhao et al., 2019). Ectosymbiotic ophiostomatoid fungi are among the plethora of microbial symbionts that bark beetles interact with. These fungi can play an important role in increasing the growth and survival of bark beetles in spruce tree bark by providing vital nutrients, producing of semiochemicals, and detoxifying host defenses (Six, 2012). There are multiple species of ophiostomatoid fungi associated with the tree-killing European spruce bark beetle *Ips typographus* (Kirisits, 2004).

In this thesis we tested the hypothesis that volatiles produced by fungal biotransformation of host tree, *P. abies* phenolic acids could act as semiochemicals and influence the behavior of bark beetles *I. typographus*. We used analytical methods to identify and quantify secondary metabolites such as phenolic acids and the fungal biotransformation products of cinnamic acid. To understand the effect of phenolic acids on the fungi and beetles, we performed toxicity tests at different concentrations of phenolic acids. The behavioral response of the adult beetles to fungal volatile organic compounds (VOCs) was tested using olfactory-based trap bioassays.

4.1. *Grosmannia penicillata* and *Ophiostoma bicolor* are virulent fungal symbionts of *Ips typographus*

Most bark beetles colonizing conifers have symbiotic relationships with specialized fungi that they inoculate into the host tree phloem and sapwood. Several studies show that fungal associates of bark beetles vary in their pathogenicity to the host tree and various factors such as microbial species, tree chemistry, and tree genotype determine the success of fungal infection (Krokene & Solheim, 1998) (Linnakoski et al., 2012). The spruce bark beetle, *I. typographus* is associated with multiple fungal symbionts from genera like *Grosmannia, Ophiostoma, Leptographium,* and *Endoconidiophora* (Lieutier, 2004; Zhao et al., 2019). The necrotic lesions that are induced by fungal growth in the phloem and sapwood of the spruce tree are caused by an active tree defense response. These tissues are saturated with chemical defense compounds like terpenoid oleoresins and phenolic compounds to prevent microbial growth (Zhao et al., 2019) (Franceschi et al., 2005). It is known from previous studies that lesion length and area are correlated to the virulence of the fungal infection (Solheim, 1992) (Krokene & Solheim, 1998).

Pathogenicity tests in a fresh log of Norway spruce clearly showed that *G. penicillata* was highly virulent and outperformed all the other fungi. *Ophiostoma piceae* was the least virulent of the six fungi that were tested, owing to its saprophytic lifestyle (Krokene & Solheim, 1998). These results corroborate with some findings of Krokene and Solheim (1998) and Kirisits (2004) using similar tests. Their results demonstrated that *G. penicillata* and *E. polonica* were highly virulent, whereas *O. bicolor* and *L. europhioides* were only moderately virulent and *O. piceae* was least virulent, as the lesion length was comparable to that of the wounded control. Our results instead showed that *O. bicolor* and *L. europhioides* were more virulent when compared to *E. polonica*, contradicting the early findings.

Most previous studies on fungus-tree-bark beetle interactions in Europe have been focused on *E. polonica*, which because of its high virulence was deemed as an important fungal associate of the spruce bark beetles (Paal, 2015). However, the work in this thesis showed that fungi such as *O. bicolor* and *L. europhioides* are, more pathogenic despite being, largely neglected in ecological studies related to bark beetles. Therefore, it is likely that other bark beetle-associated fungi can also fulfill roles similar to *E. polonica* such as metabolism of toxic secondary metabolites produced by the host three during bark beetle colonization and brood production. Hence, we speculate that different spruce bark beetle-associated fungi may form a rather redundant and interchangeable community. Individual species might be more or less important to beetles in different regions or during different population phases (Zhao et al., 2019).

4.2. *Grosmannia penicillata* and *Ophiostoma bicolor* degrade cinnamic acid at a faster rate than other fungi

Plants synthesize complex mixtures of secondary metabolites. Contrary to primary metabolites, they are not necessary for tissue growth and development. Secondary metabolites play multiple important roles such as attracting pollinators, protecting plants from abiotic stressors, acquisition of nutrition from the soil and defending plants against herbivores, and pathogens (Metsämuuronen & Sirén, 2019) (Pang et al., 2021). Fungal growth elicits a defense response in Norway spruce, including the biosynthesis of terpenes and phenolic compounds (Zhao et al., 2019). The defense response of the host tree initially starts with the induction of defense hormones such as jasmonic acid and its derivatives. The accumulation of these defense hormones actives a signaling cascade downstream, leading to the production of phenolic acids and other metabolites (Wasternack et al., 2006).

Higher levels of jasmonic acid and OH-jasmonic acid were found in phloem inoculated with *O. bicolor*, *G. penicillata* and *L. europhioides* compared to mock-inoculated bark. These fungi were the most virulent causing the longest lesions among fungi used in this study. As we observed an up-regulation of defense hormones for *O. bicolor* and *G. penicillata* infected bark, we expected to see an increase in the concentration of phenolic acids and other secondary metabolites in the bark. However, the levels of cinnamic acid were lower than expected, especially when compared to other phenolic acids, despite the long necrotic lesions and elevated jasmonate levels in *O. bicolor* and *G. penicillata* infected bark. It is possible that virulent fungi metabolize cinnamic acid faster than other phenolic acids and more efficiently than the other fungi. Cinnamic acid is an upstream product that leads to the formation of lignin and is also the precursor for the production of other phenolics. Degradation of cinnamic acid could exhaust the tree of resources that would otherwise be redirected to induce defenses against the fungi and the bark beetles (Aoun, 2017) (Paal, 2015). This hypothesis was further strengthened by the presence of styrene, benzaldehyde, and benzyl alcohol in the bark lesions of *G. penicillata* and *O. bicolor*. These volatiles products are speculated to be produced by the biotransformation of cinnamic acid (Zhao et al., 2019). Previous research (Hammerbacher et al., 2013) demonstrated similar metabolism of stilbenes and flavonoids in Norway spruce phloem by *E. polonica* at a higher rate than the trees could produce them. These results further suggest that *G. penicillata* and *O. bicolor* can be as effective as *E. polonica* in degrading spruce-derived phenolic compounds.

4.3. Cinnamic acid is metabolized into styrene, benzaldehyde and benzyl alcohol by fungi

Phenolic compounds comprise structurally and functionally diverse groups of aromatic hydrocarbon rings, and contain at least one hydroxyl group. The majority of phenolic compounds in plants are synthesized via the phenylpropanoid pathway (Iriti & Faoro, 2009). Many of these compounds are phytoalexins, antimicrobial agents synthesized naturally in wood plant species in response to microbial attack (Ganthaler et al., 2017; Schultz & Nicholas, 2000). The phenylpropanoid pathway generates an array of secondary metabolites and their concentration and composition in a plant species vary (Vogt, 2010). An important upstream intermediate of phenylpropanoid metabolism is cinnamic acid. Cinnamic acid and its derivatives have been reported to exert antimicrobial effects against multiple microorganisms (F et al., 2000) (Yilmaz et al., 2018).

Interestingly many fungi degrade secondary metabolites like cinnamic acid to produce other compounds that are less toxic to them. The fungus *Phomopsis liquidambari* completely degrades cinnamic acid from its host plant *Bischofia polycarpa* to CO_2 and H_2O reducing the biological activity of this medical plant (Liu et al., 2021). Some fungi are known to breakdown secondary metabolites to produce volatiles intermediates that are less toxic to them (Hammerbacher et al., 2013) (Kandasamy et al., 2019).

In our study, biotransformation of cinnamic acid by *G. penicillata* resulted in a high amount of styrene in the fungal headspace and in the extracts of the fungal medium. The production of styrene by fungi is not unknown in nature. Several fungi such as *Aspergillus*, *Penicillium, Saccharomyces,* and *Trichoderma* can metabolize cinnamic acid, by non-oxidative decarboxylation to produce styrene (Clifford et al., 1969) (R. J. M. Lubbers et al., 2019) (Milstein et al., 1983) (Pinches & Apps, 2007) (Lafeuille et al., 2009) (Plumridge et al., 2010). In our study, in addition to styrene, benzaldehyde was observed, but only in bark samples infected with *G. penicillata*. Interestingly, *G. penicillata* did not produce benzaldehyde when grown on cinnamic acid enriched agar. This discrepancy could be due to the instability of styrene under the experimental conditions. Styrene is a highly volatile compound with a vapor pressure of 0.67 kPa at 20°C, whereas benzaldehyde is less volatile with a vapor pressure of 0.13kPa at 20°C, making styrene more likely to evaporate before collection easier to lose styrene before it is collected. Another explanation for this could be that conversion of styrene to benzaldehyde takes place in the bark from different pathways. Conversion of styrene produced by biotransformation of cinnamic acid to benzaldehyde has been suggested before *for Aspergillus niger*, but the enzymatic process is not known (R. J. M. Lubbers et al., 2019).

In the case of *O. bicolor*, benzaldehyde was was the volatile produced in highest amounts followed by benzyl alcohol. It was interesting that *O. bicolor* did not produce any styrene both in spruce logs and in media amended with cinnamic acid. This could suggest that *O. bicolor* utilizes a different pathway to metabolize cinnamic acid. A similar conversion of cinnamic acid to benzaldehyde has been observed in the fungus *Phanerochaete chrysosporium* (Jensen et al., 1994). Similarly, the conversion of cinnamic acid to benzaldehyde and benzyl alcohol was observed in the fungus *Bjerkandera adusta*, (Lapadatescu et al., 2000) (R. Lubbers et al., 2019).

The ability of a microorganism to metabolize an antimicrobial compound like cinnamic acid could provide an advantage leading to faster growth and inhibition of competitors with toxic metabolites. Certain *Trichoderma spp*. have been reported to produce volatiles possessing an inhibitory effect against other fungi (Dennis & Webster, 1971) although the antimicrobial properties of styrene have not been established (Pinches & Apps, 2007).

Cinnamic acid is a central intermediate in the biosynthesis of both lignin and polyphenols. Therefore any changes in the concentration of cinnamic acid might affect the overall turnover rate of plant defense compounds. It is interesting to speculate that biotransformation of cinnamic acid might be one of the adaptations of fungi to prevent plant tissues from producing defense chemicals such as polyphenols and reduce lignification of cells which may facilitate colonization success (Mandal et al., 2010). (**Figure S2**, Supplementary).

4.4. Cinnamic acid is fungistatic to blue-stain fungi

We tested the antifungal properties of different phenolic acids against *G. penicillata* and *O. bicolor*. Both fungi grew well on ferulic and *p*-coumaric acid-enriched media at all tested concentrations, as there was no significant difference between their growth at different doses when compared to the control. On the other hand, on cinnamic acid, both fungi showed growth inhibition at 50 µg/ml when compared to other concentrations. Interestingly, growth of *G. penicillata* was stimulated at 5 and 0.5 µg/ml and there was no significant difference in the growth of *O. bicolor* between control and 5 or 0.5 µg/ml of cinnamic acid.

Previous studies that tested the growth inhibition of *Fusarium oxysporum* and *Botrytis cinerea* showed that inhibition caused by ferulic acid and *p*-coumaric acid, was greater than the inhibition caused by cinnamic acid (Lattanzio et al., 1994), the opposite pattern to that observed here. Although cinnamic acid had been reported to be more toxic to fungi such as *Sclerotinia sclerotiorum* and *Penicillium digitatim*, compared to *p*-coumaric acid, the same was not observed against ferulic acid (Lattanzio et al., 1994).

4.5. Cinnamic acid is not toxic to adult bark beetles

Contrary to the toxicity of cinnamic acid to fungi, we observed no toxic effects on adult bark beetles. In the tunneling assay, beetles were provided with an artificial diet enriched with different concentrations of cinnamic acid for up to 48 hours and no mortality of adult bark beetles was observed, even at the highest dose (50 µg/ml) used. Previous studies have shown that tunneling assay in monoterpenes, oxygenated monoterpenes, and in phenolic enriched media also did not cause any mortality in *I. typographus* (Faccoli et al., 2005) (Faccoli & Schlyter, 2007). This could be because some secondary metabolites that show toxicity to insects do it through routes other than tunneling, such as inhalation, feeding or ingestion, and dermal contact. Although cinnamic acid did not stimulate tunneling in adult *I. typographus*, previous studies have shown that outer bark extracts of conifers stimulated tunneling in other bark beetle species such as *Ips paraconfusus*, *Dendroctonus frontalis, Dendroctonus ponderosa, Scolytus multistriatus* and *Scolytus rugulosus* (Elkinton et al., 1981) (Doskotch et al., 1970) (Raffa & Berryman, 1982) (Thomas et al.). Perhaps the stimulation of tunneling in these beetles was caused by other phenolics or a combination of different phenolics and other nutrients.

Beetle tunnel length significantly increased after each observation made at 6, 24, 48 hours in all treatments. Furthermore, beetles feeding on all concentrations of cinnamic acid added in the diet gained weight similar to the control group. Phenolic acids and other phenolic compounds are important dietary antioxidants; the presence of these compounds in high concentrations is linked with a reduction of the nutritional value of food. Phenolics are thought to crosslink with proteins precipitating them inside insect guts and making them inaccessible for digestion (Ananthakrishnan, 1997) (Sambangi & Rani, 2016). Overall, high concentrations of cinnamic acid showed no significant effect on weight gain in adult *I. typographus*, but their effect on larvae is still unknown. Similarly, when *Hyphantria cunea* larvae were presented with an artificial diet amended with cinnamic acid, no significant effect on the larval growth was observed (Mo et al., 2020). This suggests that cinnamic acid may not be toxic to beetles at any life stage (Jiang et al., 2021).

4.6. Adult *Ips typographus* are attracted to fungal biotransformation products of cinnamic acid produced by *Grosmannia penicillata*

One of the major focuses of this thesis was to observe the behavior of adult *I. typographus* when exposed to volatile compounds produced by the associated symbiotic fungi grown on a spruce bark diet. Specifically, the choice of the beetles in response to fungal biotransformation products of cinnamic acid was evaluated. It is speculated that volatile chemical cues from symbiotic fungi could attract bark beetles, especially if both partners mutually benefit from this interaction (Bentz et al., 2010). To understand the influence of fungal volatiles on the preference of bark beetles an olfactory-based trap bioassay was used (**Figure 2.7**, Materials and method). The chemical cues from different fungi in short-range reception were used to evaluate the behavior of adult *I. typographus.* Previously, the headspace volatile profile of *G. penicillata and O. bicolor* grown on potato dextrose agar were found to be dominated by aliphatic and aromatic alcohols and these volatiles in a specific ratio attracted adult beetles (Kandasamy, 2019).

The influence of the volatile blend produced by the fungal biotransformation of cinnamic acid on the choice made by bark beetles was investigated. A volatile blend of *G. penicillata* and *O.bicolor* growing on only SBA was highly attractive to both male and female adult beetles. The trap bioassays collectively showed that volatiles associated with specific fungi alone are sufficient for bark beetles to recognize and be attracted towards the fungi. However, when the diet was amended with cinnamic acid the same was not true for both the fungi. The attraction of *I. typographus* was specific towards volatile blends produced by one specific fungus, *G. penicillata* in the presence of cinnamic acid. Colonization of *G. penicillata* on diet containing cinnamic acid was highly attractive to adult males and females, whereas inoculation of *O. bicolor* on the same diet resulted in strong aversion. These results could be an indication that volatile cues from fungi may assist bark beetles to orient towards specific microorganisms. Similar observations have been previously reported for some ambrosia beetles and other insects (Hulcr et al., 2011) (Witzgall et al., 2012) (Luna et al., 2014). Taken together this study highlighted the important role of host-derived fungal volatiles in adult beetle's preferences and how this depended on the fungal species and on the biotransformation products emitted by them (Kandasamy et al., 2019; Kandasamy et al., 2016; Schiebe et al., 2012).

4.7. Adult *Ips typographus* males are attracted to styrene

Volatiles identified from the fungi grown on a cinnamic acid-enriched diet were tested as pure compounds. Trap bioassays using pure compounds showed that females are slightly attracted to styrene at all dilutions and males are attracted at certain specific concentrations. Interestingly, styrene was significantly attractive to males at a dilution of 1% (10^{-2}), although males showed an indifferent response to styrene at lower dilutions (**Figure 3.12**, Material and method). The response of *I. typographus* both benzaldehyde and benzyl alcohol inconclusive. None of the responses were significant enough to state that beetles showed an attraction or aversion to either of these compounds.

These behavioral responses of the beetles towards pure compounds corresponded with the results of our bioassays with fungal blends. In the case of *G. penicillata*, both adult males and females were highly attracted to fungus grown on cinnamic acid diet. We speculate that styrene could be one of the major compounds in the fungal blend that leads to beetle attraction towards *G. penicillata*, making it an important volatile for the beetles to detect symbiotic fungi. Furthermore, adult *I. typographus* possesses olfactory sensory neurons (OSN) that primarily detect styrene produced by fungi ((Schiebe et al., 2019) (Kandasamy, 2019). Since styrene is produced mostly by fungi, the presence of styrene OSN in bark beetles indicates the importance of this volatile in the interaction of beetles with potentially beneficial fungi. Attraction towards *O. bicolor* growing in media with cinnamic acid was not strong. Although the beetles did show an attraction towards *O. bicolor* itself, this could be because fungus produces volatile compounds other than benzaldehyde and benzyl alcohol from spruce bark that could be attractive to them.

Volatile phenolics have been reported in other microbes (Huang et al., 1993) (Adeboye et al., 2015) including the compounds detected here, styrene, benzaldehyde and benzyl alcohol. Insects have shown specific responses towards these compounds that were different to those observed for *I. typographus* in this study. For example, the pine weevil, *Hylobius abietis* showed a strong aversion towards styrene that was produced by the fungus *Penicillium expansum* (Azeem et al., 2013). Similar aversion to styrene was observed in the banana black weevil *Cosmopolites sordidus* (Lozano-Soria et al., 2020). Benzaldehyde was attractive to the pest weevil *Sitona humeralis* (Lohonyai et al., 2019) but was not attractive to the green leaf bug, *Apolygus lucorum (Sun et al., 2014).* Volatiles such as benzaldehyde and benzyl alcohol have also been reported to strongly enhance the detection of sex pheromones in insects like the *Spodoptera litura* moth (Fang et al., 2018). A similar effect was observed in *Blastophaga psenes*, as the reduction of benzyl alcohol in a blend of volatiles resulted in the loss of attraction to their host plant *Ficus carica* (Proffit et al., 2020). These results collectively show that volatiles such as styrene, benzaldehyde, and benzyl alcohol elicit different kinds of behavioral responses in insects which likely depends on the ecological role of the source microbe or other organism in the life of the insect.

5. Conclusion

It has been speculated that insects may exploit volatile chemical signals associated with mutualistic fungi to indicate the nutritional state of their host-fungus complex. For example, for *I. typographus* and other bark beetles the attractive blend released by mutualistic fungi from the host tree comprises mainly volatile alcohols and esters produced from amino acids, which could indicate the presence of essential amino acids, which are vital for insect development. Styrene, benzaldehyde, and benzyl alcohol may also be nutritional signals since they are produced by fungi using cinnamic acid which is derived from phenylalanine (R. J. M. Lubbers et al., 2019) (Lapadatescu et al., 2000), an essential amino acid.

Volatiles could also function to indicate the presence of dietary antioxidants, as in *Drosophila* where microbial byproducts of host hydroxycinnamic acids are reported to have this function (Dweck et al., 2015). Since both benzaldehyde and benzyl alcohol also have antioxidant properties (Ullah et al., 2015) (Lee & Shibamoto, 2001), bark beetles could be using these compounds to also locate food rich in antioxidants. Thus, different microbial volatiles could have different meanings as olfactory signals for insects in different systems.

Previous studies on insect attraction to microbial volatiles have usually investigated the response to a single volatile rather than to blends. In this thesis, we demonstrated that the attraction of bark beetles to particular fungi is due to the blend of fungal volatiles and could not always be duplicated by single compounds. Further research on fungal volatile's should include assays performed with full blends (Proffit et al., 2020).

6. Outlook and future prospects

This thesis provides considerable evidence that bark beetle-associated virulent fungi like *Grosmannia penicillata* and *Ophiostoma bicolor* produce volatile organic compounds by biotransformation of tree secondary metabolites like cinnamic acid. As cinnamic acid is fungistatic to these fungi, but non-toxic to beetles, future experiments could focus on investigating the relevance of fungal biotransformation of phenolic acids. As the effect of cinnamic acid was only tested on fully developed adult beetles, experiments in the future should test the effects of phenolic acids on other developmental stages. Since *I. typographus* was strongly attracted to the volatile blends produced by the fungi growing on cinnamic acid-enriched medium, it would be interesting to assess the response of beetles to fungal blends produced by biotransformation of multiple phenolic acids. Moreover, it would also be interesting to test if the fungal volatiles provide cues to beetles about the nutritional quality of the tree, especially the presence of antioxidants and essential amino acids. More research can also be done on the effect of other phenolic compounds on the development of beetles. Phenolics are abundant secondary metabolites in Norway spruce trees but there is still much too little information about their interactions with beetles.

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Supplementary

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Catalog No. : 31080 **Lot No.:** A0169733

Description : Qualitative Retention Time Index Std

Qualitative Retention Time Index Std 100-200µg/mL, Hexane, 1mL/ampul

Container Size : 2 mL **Pkg Amt:** > 1 mL

Expiration Date : April 30, 2028 **Storage:** 10°C or colder

Handling: Sonicate prior to use. **Ship:** Ambient

diluted to 5µg/mL ⇒ 5µg/mL ⇒ 1µL per injection wt

CERTIFIED VALUES

Elution Order	Compound	Grav. Conc. (weight/volume)	Expanded Uncertainty (95% C.L.; K=2)	
1	n-Heptane (C7) **CAS #** 142-82-5 (Lot SHBL9221) **Purity** 99%	100.0 µg/mL	+/- 0.7091 µg/mL +/- 2.5145 µg/mL +/- 3.0031 µg/mL	Gravimetric Unstressed Stressed
2	n-Octane (C8) **CAS #** 111-65-9 (Lot SHBM4827) **Purity** 99%	100.0 µg/mL	+/- 0.7091 µg/mL +/- 2.5145 µg/mL +/- 3.0031 µg/mL	Gravimetric Unstressed Stressed
3	n-Nonane (C9) **CAS #** 111-84-2 (Lot SHBK7143) **Purity** 99%	100.3 µg/mL	+/- 0.7114 µg/mL +/- 2.5228 µg/mL +/- 3.0131 µg/mL	Gravimetric Unstressed Stressed
4	n-Decane (C10) **CAS #** 124-18-5 (Lot SHBL4313) **Purity** 99%	100.0 µg/mL	+/- 0.7091 µg/mL +/- 2.5145 µg/mL +/- 3.0031 µg/mL	Gravimetric Unstressed Stressed
5	n-Undecane (C11) **CAS #** 1120-21-4 (Lot SHBL4418) **Purity** 99%	100.0 µg/mL	+/- 0.7091 µg/mL +/- 2.5145 µg/mL +/- 3.0031 µg/mL	Gravimetric Unstressed Stressed
6	n-Dodecane (C12) **CAS #** 112-40-3 (Lot SHBK0925) **Purity** 99%	100.3 µg/mL	+/- 0.7114 µg/mL +/- 2.5228 µg/mL +/- 3.0131 µg/mL	Gravimetric Unstressed Stressed
7	n-Tridecane (C13) **CAS #** 629-50-5 (Lot MKCM6532) **Purity** 99%	100.7 µg/mL	+/- 0.7138 µg/mL +/- 2.5312 µg/mL +/- 3.0231 µg/mL	Gravimetric Unstressed Stressed

8	n-Tetradecane (C14) **CAS #** 629-59-4 **Purity** 99%	(Lot STBJ3715)	100.7 µg/mL	+/- 0.7138 +/- 2.5312 +/- 3.0231	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
9	n-Pentadecane (C15) **CAS #** 629-62-9 **Purity** 99%	(Lot MKCG9920)	200.3 µg/mL	+/- 1.1899 +/- 4.9772 +/- 5.9660	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
10	n-Hexadecane (C16) **CAS #** 544-76-3 **Purity** 99%	(Lot SHBL8588)	100.7 µg/mL	+/- 0.7138 +/- 2.5312 +/- 3.0231	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
11	n-Heptadecane (C17) **CAS #** 629-78-7 **Purity** 99%	(Lot MKCD4523)	99.7 µg/mL	+/- 0.7067 +/- 2.5061 +/- 2.9931	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
12	n-Octadecane (C18) **CAS #** 593-45-3 **Purity** 97%	(Lot VZKOJ)	99.3 µg/mL	+/- 0.7039 +/- 2.4959 +/- 2.9810	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
13	n-Nonadecane (C19) **CAS #** 629-92-5 **Purity** 99%	(Lot 6700.05)	100.0 µg/mL	+/- 0.7091 +/- 2.5145 +/- 3.0031	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
14	n-Eicosane (C20) **CAS #** 112-95-8 **Purity** 99%	(Lot MKCF7888)	100.0 µg/mL	+/- 0.7091 +/- 2.5145 +/- 3.0031	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
15	n-Heneicosane (C21) **CAS #** 629-94-7 **Purity** 99%	(Lot MKBZ8320V)	100.0 µg/mL	+/- 0.7091 +/- 2.5145 +/- 3.0031	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
16	n-Docosane (C22) **CAS #** 629-97-0 **Purity** 99%	(Lot MKCH2086)	100.7 µg/mL	+/- 0.7138 +/- 2.5312 +/- 3.0231	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
17	n-Tricosane (C23) **CAS #** 638-67-5 **Purity** 99%	(Lot 281054/1695)	100.7 µg/mL	+/- 0.7138 +/- 2.5312 +/- 3.0231	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
18	n-Tetracosane (C24) **CAS #** 646-31-1 **Purity** 99%	(Lot MKCJ8741)	100.0 µg/mL	+/- 0.7091 +/- 2.5145 +/- 3.0031	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
19	n-Pentacosane (C25) **CAS #** 629-99-2 **Purity** 98%	(Lot 0000074511)	100.3 µg/mL	+/- 0.7111 +/- 2.5217 +/- 3.0117	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
20	n-Hexacosane (C26) **CAS #** 630-01-3 **Purity** 99%	(Lot MKCD4540)	100.7 µg/mL	+/- 0.7138 +/- 2.5312 +/- 3.0231	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
21	n-Heptacosane (C27) **CAS #** 593-49-7 **Purity** 99%	(Lot BCBM6600V)	101.0 µg/mL	+/- 0.7162 +/- 2.5396 +/- 3.0331	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
22	n-Octacosane (C28) **CAS #** 630-02-4 **Purity** 99%	(Lot BCCB6836)	100.3 µg/mL	+/- 0.7114 +/- 2.5228 +/- 3.0131	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
23	n-Nonacosane (C29) **CAS #** 630-03-5 **Purity** 99%	(Lot F7SIM)	101.0 µg/mL	+/- 0.7162 +/- 2.5396 +/- 3.0331	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed

24	n-Triacontane (C30) **CAS #** 638-68-6 **Purity** 98%	(Lot MKCJ4572)	201.6 µg/mL	+/- 1.1972 +/- 5.0075 +/- 6.0023	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
25	n-Hentriacontane (C31) **CAS #** 630-04-6 **Purity** 98%	(Lot FIM01)	200.2 µg/mL	+/- 1.1894 +/- 4.9750 +/- 5.9634	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
26	n-Dotriacontane (C32) **CAS #** 544-85-4 **Purity** 99%	(Lot BCBW0661)	201.3 µg/mL	+/- 1.1959 +/- 5.0020 +/- 5.9958	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed
27	n-Tritriacontane (C33) **CAS #** 630-05-7 **Purity** 99%	(Lot BCBX4890)	200.0 µg/mL	+/- 1.1879 +/- 4.9689 +/- 5.9561	µg/mL µg/mL µg/mL	Gravimetric Unstressed Stressed

Solvent: Hexane
CAS # 110-54-3
Purity 99%

Column:
30m x 0.25mm x 0.25µm
Rtx-5 (cat.#10223)

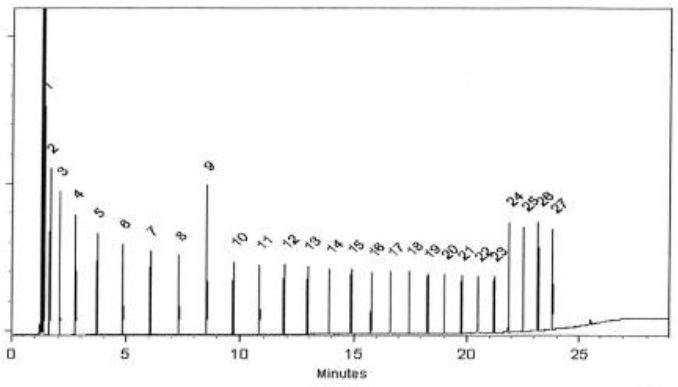
Carrier Gas:
hydrogen-constant flow 1.8 mL/min.

Temp. Program:
80°C (hold 0.1 min.) to 330°C
@ 9.6°C/min. (hold 2.86 min.)

Inj. Temp:
250°C

Det. Temp:
340°C

Det. Type:
FID



This chromatogram represents a general set of testing conditions chosen for product acceptance. For optimal results in your lab, conditions should be adjusted for your specific instrument, method, and application.

Walker Workman - Operations Technician I

Date Mixed: 03-Mar-2021 **Balance:** B442140311

Alexis Shelow - Operations Tech I

Date Passed: 08-Mar-2021 REVIEWED

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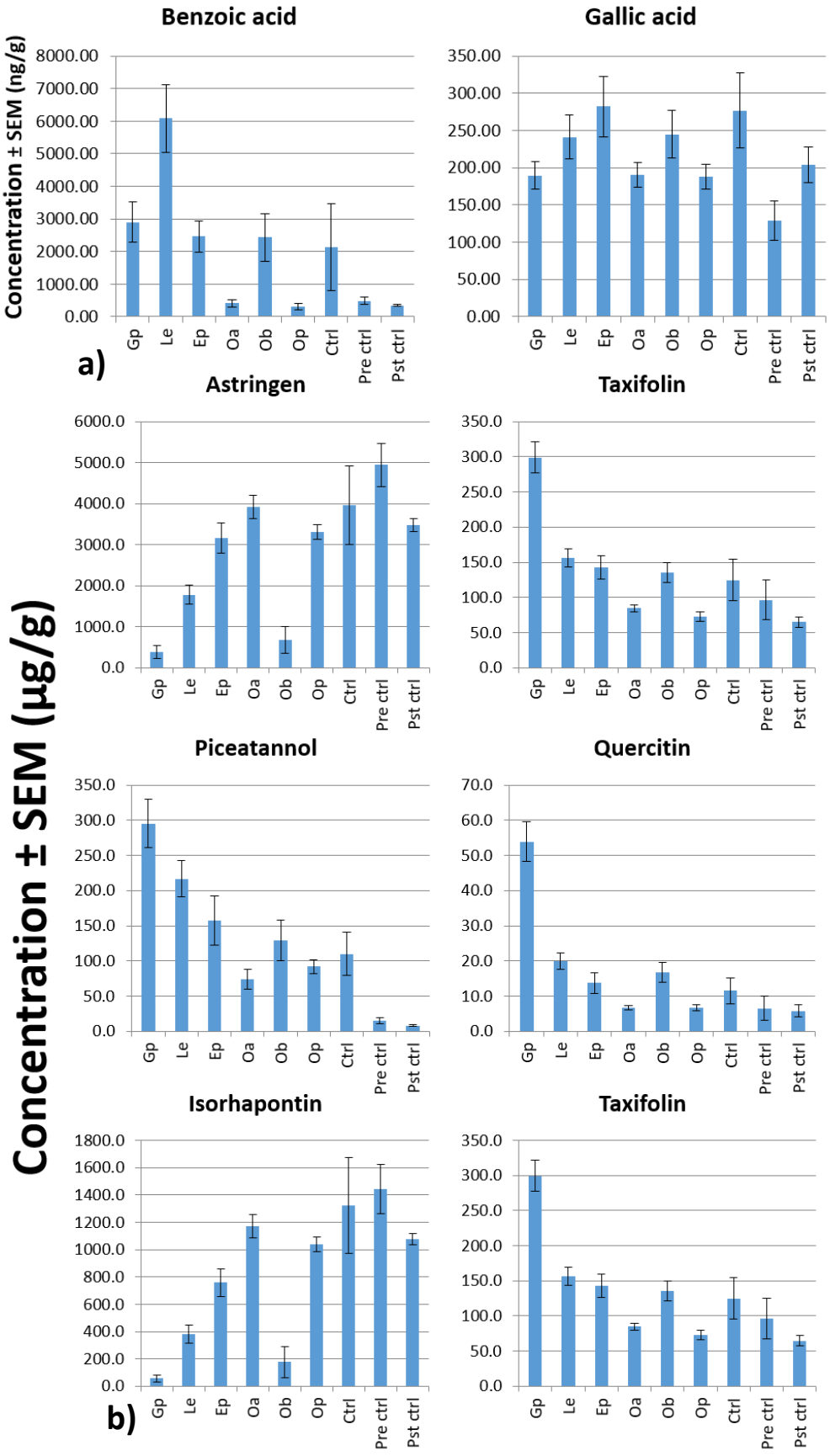
Figure S1: Details about the qualitative retention time index standards used for gas chromatography.


Benzoic acid
Gallic acid
Concentration ± SEM (ng/g)
8000.00
7000.00
6000.00
5000.00
4000.00
3000.00
2000.00
1000.00
0.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
Gp
Le
Ep
Oa
Ob
Op
Ctrl
Pre ctrl
Pst ctrl
a)
Astringen
Taxifolin
Concentration ± SEM (µg/g)
6000.0
5000.0
4000.0
3000.0
2000.0
1000.0
0.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
Piceatannol
Quercitin
70.0
60.0
50.0
40.0
30.0
20.0
10.0
Isorhapontin
Taxifolin
1800.0
1600.0
1400.0
1200.0
1000.0
800.0
600.0
400.0
200.0
b)

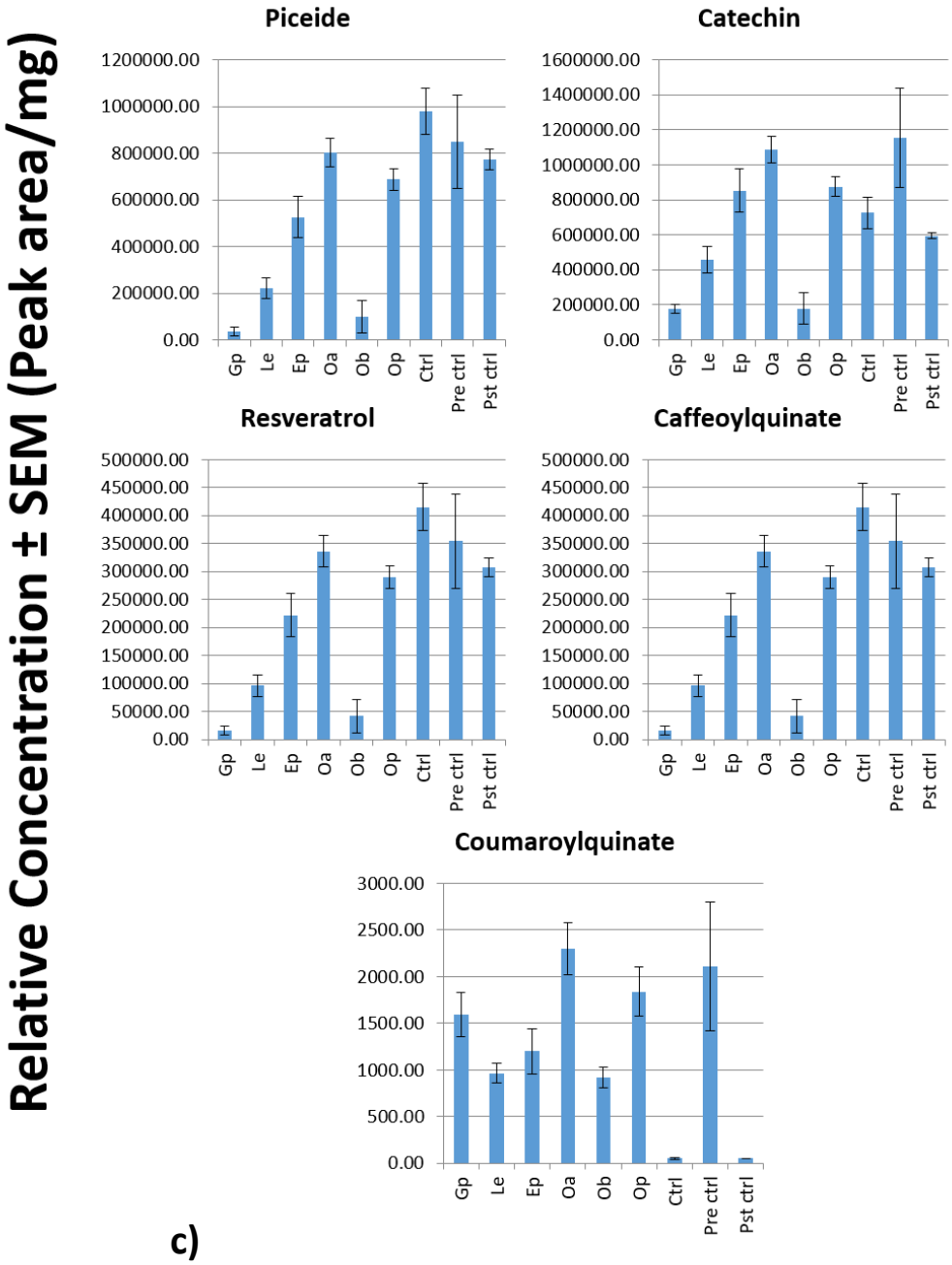


Figure S2: Quantification of other spruce polyphenols in the bark phloem. Absolute quantification of, **a)** Phenolic acids in ng/g, **b)** Flavonoids and stilbenes in µg/g. Relative quantification of **c)** Flavonoids and stilbenes. Gp- *G. penicillata*, Le- *L. europhioides*, Ep- *E. polonica*, Oa- *O. ainoae*, Ob- *O. bicolor*, Op- *O. piceae*, Ctrl-control, Pre ctrl- pre control, Pst ctrl- post control LC-MS results for other phenolic compounds in the bark infected by fungi.

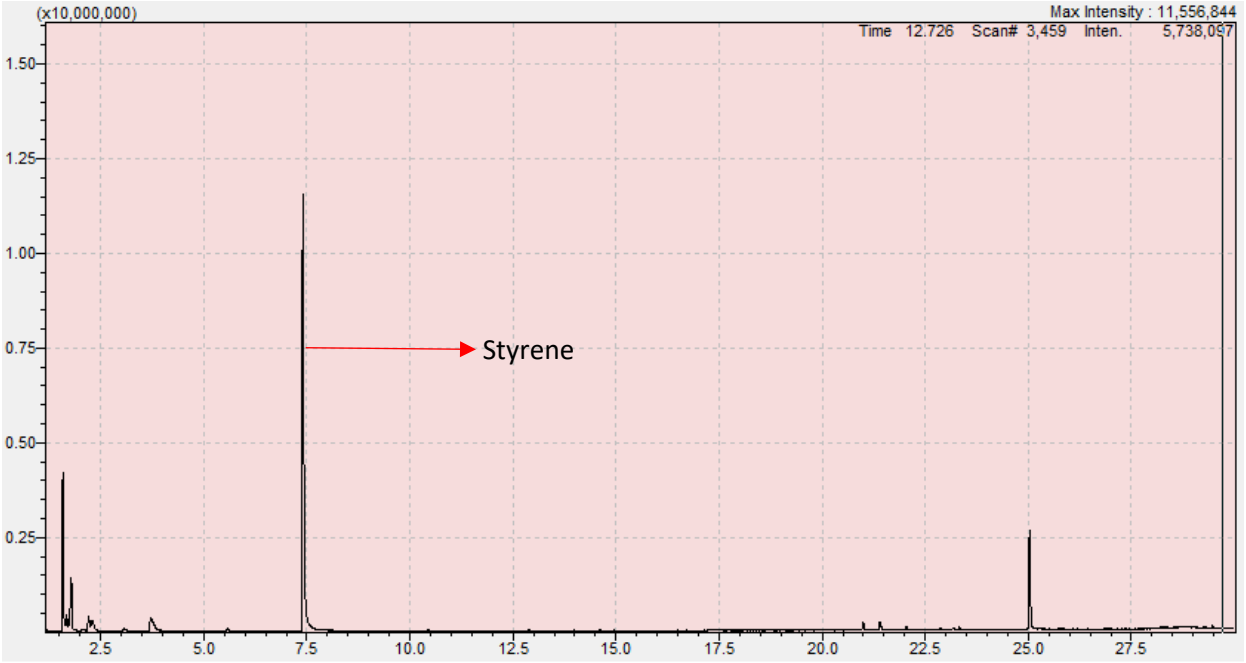


Figure S3: TDU-GC-MS chromatogram representing styrene peak observed in petri plates with *G. penicillata* grown in minimal media with 25 µg/ml cinnamic acid.

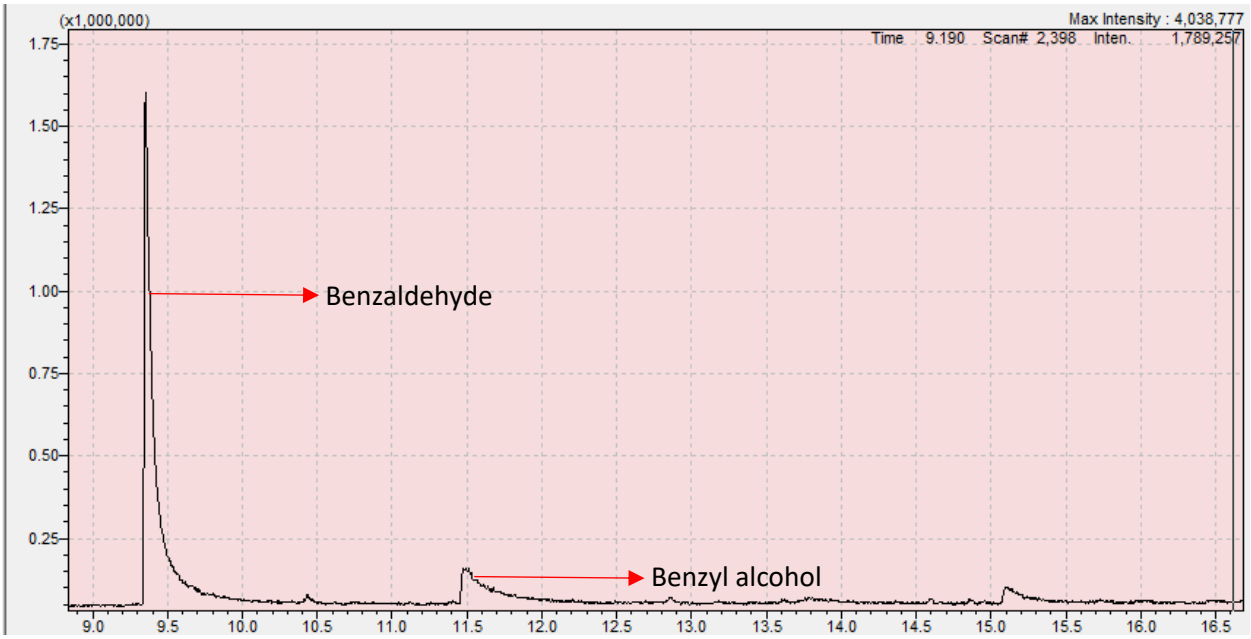


Figure S4: TDU-GC-MS chromatogram representing benzaldehyde and benzyl alcohol peak observed in petri plates with *O. bicolor* grown in minimal media with 25 µg/ml cinnamic acid.

Acknowledgment

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Declaration of Authorship

I hereby confirm that this thesis has been composed and written solely by myself. I certify that to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. This thesis was not submitted in any form for another degree or diploma at any university and has not been published.

Date: 29.08.2022 Akanksha Jain