Fungal endophytes mediate tree-insect interactions

Dissertation

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by Christin Walther née Uhe (Master of Science)

born on 28.01.1989 in Beckendorf-Neindorf now Oschersleben (Bode), Germany

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1. INTRODUCTION

Plants and insects constantly interact with each other under natural conditions. Over the years research on plant-insect interactions has tried to elucidate the molecular and chemical patterns behind these interactions. Given that plants and insects have coexisted for more than 350 million years, a variety of beneficial, neutral or negative interactions have developed (Gatehouse *et al*. 2002, Mithöfer *et al*. 2009). Studies investigating plant-insect interactions have mainly focused on herbaceous plants and here mainly crop plants, even though 33 % of the landmass of our planet is covered with forest (FAO 2015). The size, architecture and longevity of trees may result in a multitude of different interactions with a plethora of insects that are beneficial as well as detrimental for the host tree (Lämke & Unsicker 2018). This multitude of interactions and the resulting arms-race between insects and plants is suggested as the main driver of expansion of various plant defense strategies. However, trees are not only a host for various insects and vertebrates, but also for microorganisms. Each tree hosts an enormous diversity of microorganisms below- and aboveground that might influence the host plant chemistry either as mutualists, pathogens, or neutralists. Fossil records show that the interaction between fungi and plants is approximately 110 million years older than plant-insect interactions and started at least 460 million years ago (Redecker *et al.* 2000), indicating that an evolutionary arms race did not happen solely between plants and insects. These findings highlight the importance of microorganisms as potential drivers of plant-insect interactions.

1.1 Plant-insect interactions

Organisms unavoidably encounter interactions with other organisms, either within the same species or with other species. These interactions can be beneficial for only one partner (e.g., predation), negative for both partners (e.g., competition), or positive for both (e.g., symbiosis). These types of interactions result in strong interdependencies between organisms, since they directly affect the fitness of individuals and likely result in co-evolution. On the other hand, interactions that result in a neutral outcome for at least one of the involved organisms (e.g., commensalism, amensalism, and neutralism) are less likely to promote co-evolutionary processes. During the last 350 million years, interactions between plants and insects have evolved that are beneficial (e.g., pollination mediated by insects or zoochory) or deleterious (e.g., insect herbivory, insect gall-formation, or seed predation) (Gatehouse *et al*. 2002, Mithöfer *et al.* 2009). The deleterious interactions between plants and insects have led to the diversification of a plethora of specialized plant compounds. The awareness that these specialized compounds are not just waste products of plant metabolism, but instead contribute to

plant defense against herbivores and pathogens was one of the key steps to disentangle factors driving plant-insect interactions (Dyer *et al.* 2018, Fraenkel 1959).

Plant defense strategies

A large variety of different defense compounds as well as defense strategies have evolved over time. Plant defense strategies can be categorized according to various characteristics: temporal occurrence (constitutive or induced), effect on the attacking herbivore (direct or indirect), and functions (e.g., morphological, chemical, or biochemical defenses). Constitutive strategies include the permanent presence of defenses, like morphological and structural features (e.g., thorns, prickles, thick cell walls or cuticles) and constitutively produced chemical defense compounds (e.g., alkaloids and phenolics) (Hartmann & Ober 2000, Kaplan *et al.* 2008, Mithöfer *et al.* 2009). In contrast, induced defenses are activated upon herbivory or pathogen attack, e.g., the release of volatile organic compounds (VOCs). Activation requires recognition of herbivore- or pathogen-associated molecular patterns (HAMPs or PAMPs), such as chemical compounds from oral secretions from attacking insects or oviposition fluids (Basu *et al.* 2018, Mithöfer & Boland 2008).

Both constitutive and induced defenses can be further classified into direct or indirect defenses. In direct defense, the insect herbivore is directly affected by morphological, chemical, or biochemical defenses. For instance, plant-produced specialized compounds are either deterrent or reduce the performance of the organism. A typical example of direct defense compounds are the constitutively produced pyrrolizidine alkaloids (PAs), which are nitrogen-containing specialized compounds and are known to lower the performance especially of generalist insect herbivores or deter potential attackers (Hartmann & Ober 2000, Kaur 2020). Indirect defenses employ a third trophic level like parasitoids and predators that are attracted by plants and attack the herbivore. For instance, constitutive defenses like extrafloral nectars, food bodies, and domatia can be additionally induced to attract natural enemies (Aljbory & Chen 2018 and references therein). The most prominent example of indirect defenses are herbivore-induced plant volatiles (HIPVs) to attract natural enemies of herbivorous insects, as was shown for nitrogenous compounds released by black poplar upon herbivory (Clavijo Mccormick *et al.* 2014a, Clavijo Mccormick *et al.* 2014b).

HIPVs are by far the most important indirect defenses, as they are widespread and many possible recipients are reached. However, VOCs can also act directly to repel phytophagous insects (Irmisch *et al.* 2014a), or can further function as signal within the plant to adjust their defense response for an upcoming herbivore attack (Heil & Silva Bueno 2007, Mithöfer *et al.* 2009). Plants can emit a vast number of volatiles consisting of different volatile classes like green leaf volatiles

(C_6 fatty-acid-derived), aromatic compounds, terpenoids, and nitrogen-containing compounds (Arimura *et al.* 2009, Irmisch *et al.* 2014b, Maffei *et al.* 2011). Among VOCs, terpenoids are the largest and most diverse compound class (Tholl 2015). All terpenoids are derived from the five-carbon building blocks, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which are produced either by the mevalonate pathway or by the 2-C-methylerythritol-4-phosphate (MEP) pathway. A head to tail condensation of the five-carbon units results in products of different chain lengths: geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15), and geranylgeranyl diphosphate (GGPP, C20). Using these precursors as substrates, various terpene synthases (TPS) produce numerous different terpenoids (Degenhardt *et al.* 2009, Irmisch *et al.* 2014b). According to the importance of terpenoids in plant-insect interactions, a vast amount of research has been done to elucidate the biosynthesis of various terpenoids including the identification and characterization of terpene synthases (Degenhardt *et al.* 2009).

Insect ecology

Approximately half of the estimated known insect species feed on living plant material (Bernays 1992, Gullan & Cranston 2014, Speight *et al.* 1999). As primary producers, plants are the starting points in trophic networks generating nutrients through photosynthesis and making these nutrients then accessible for other organisms, like herbivores, decomposers and mutualists (Price *et al.* 2011). Each plant and plant organ thus provides different resources but also constrains the access to those resources by allelochemicals, resulting in different ecological niches for insects. This, in turn, leads to the diversification of different feeding guilds and specialization grades of insect herbivores. Therefore, herbivorous insects can be classified in various ways, according to their host range (monophagous, oligophagous, and polyphagous), their feeding guild (chewing versus piercing-sucking), the plant organ they feed on (e.g., leaves, stems, bark, seeds), and how they manipulate or process plant material (e.g., leaf rollers, gall formers, shredders) (Bernays 1992, Price *et al.* 2011, Strong *et al.* 1984). It is assumed that plant defense strategies are a key mechanism that leads to the diversification of insect herbivores. The Brassicaceae, for example, possess a two-component defense system, in which a non-toxic precursor gets hydrolyzed to form toxic isothiocyanates. Even though the isothiocyanates themselves are effective against both generalist and specialist herbivores, larvae of *Pieris rapae* can circumvent this defense. By manipulating the hydrolysis reaction, they inhibit the formation of isothiocyanates in favor of the production of nitriles, which can be easily excreted (Wittstock *et al.* 2004).

In general, studies on plant-insect interactions have mainly focused on herbaceous and economically important crop plants, whereas research on tree-insect interaction is scarce. Due to their various defense strategies, their architecture and their long lifetime, during which trees experience various environmental conditions, they represent a spatially and temporally heterogeneous environment that can harbor species-rich communities of herbivorous insects. The fact that trees can be colonized repeatedly during their lifetime even increases the species richness of herbivorous insect communities (Lämke & Unsicker 2018).

1.2 Plant-fungus interactions

Every single plant organ (roots, stem, bark, leaves, and flowers) can be inhabited by different microorganisms, such as bacteria, fungi, archaea, algae, and protists, either on the plant surface or within the plant tissue (Baldrian 2017). These interactions can be beneficial (e.g., in mycorrhiza), or negative (e.g., pathogenic fungi), or may shift from initially beneficial to later pathogenic. In my thesis, I focused on fungi that inhabit the inner leaf tissues without causing apparent disease symptoms. Fungi, as well as bacteria, that live inside a plant tissue and undergo a quiescent stage, for at least a part of their life cycle, are called endophytes (Petrini 1991).

It is estimated that the global leaf surface of woody plants is several times bigger than the land area (Sieber 2021). The leaf surface as well as the inside of the leaf tissue are colonized by a variety of microorganisms, with fungi accounting for a large proportion of leaf colonizers (Sieber 2021, Yan *et al.* 2019). Fungal endophytic hyphae and spores were found in 460 million-year-old fossilized plant tissue, indicating that the co-evolution between plants and fungi started as soon as plants first appeared on earth (Redecker *et al.* 2000). The definition of endophytes has changed over the years and now includes bacteria as well as endophytes with an epiphytic phase and latent pathogens (Yan *et al.* 2019). Since fungal organisms have been found inside all analyzed plant tissues so far without causing apparent disease symptoms, these have evoked curiosity since the 1970s about their potential role in an ecological context as well as a new source of novel bioactive compounds (Yan *et al.* 2019).

Transmission and colonization of endophytic fungi in plants

Plants are surrounded by a plethora of microorganisms. Many of the endophytic fungi originate from the surrounding soil, air, or from insects or other animals that serve as vectors (Rodriguez *et al.* 2009, Sasse *et al.* 2018). In general, there are two kinds of transmission patterns. Vertically transmitted endophytes are transferred via plant seeds to the next plant generation and systemically colonize the intercellular spaces of newly formed shoots (Gagic *et al.* 2018). This strategy is used by endophytic

species of the *Clavicipitaceae*, including e.g., *Epichloë* sp., *Claviceps* sp., and *Balsania* sp., which exclusively form symbioses with grass, rush, and sedge hosts (Bacon & White 2000, Saikkonen *et al.* 2002). In contrast, the so called nonclavicipitaceous endophytes are predominantly transmitted horizontally via spores and/or hyphal fragmentation by wind, rain, and/or herbivores as vectors from plant to plant (Wiewiora *et al.* 2015). When endophytic spores germinate, the hyphae can enter the leaf tissue through penetration, stomata or other leaf openings and undergo a quiescent state after infection (Rodriguez *et al.* 2009, Sieber 2007). However, successful colonization depends on various factors like plant tissue type and genotype, environmental conditions and previous colonization events in the host plant (Bonito *et al.* 2014, Carroll & Carroll 1978, Hardoim *et al.* 2015, Yan *et al.* 2019). It has been shown that the colonization of plants with beneficial microbes triggers a plant immune response comparable to that against pathogens. Signal molecules emitted by the microbes are perceived by the plant and activate the immune system in two different defense systems. First, the recognition of microbe-associated molecular patterns (MAMPs) via cell surface-localized pattern recognition receptors (PRRs) in the plant can lead to MAMP-triggered immunity (MTI) (Lopez-Gomez *et al.* 2012, Yan *et al.* 2019, Zamioudis & Pieterse 2012). Second, the plant identifies effectors (molecules produced by microorganism) and then activates the effector-triggered immunity (ETI) (Mendoza-Mendoza *et al.* 2018, Yan *et al.* 2019, Zamioudis & Pieterse 2012). It is known that beneficial soil-borne microbes manipulate the effector-triggered susceptibility state and therefore overcome defense responses and successfully colonize plant tissues (Zamioudis & Pieterse 2012 and references therein). Whether this is true for endophytic fungi colonizing leaf tissue needs to be investigated, as the molecular mechanisms that lead to an endophytic colonization in plant tissues are not fully understood.

Effects of endophytic fungi on plants

Plants harboring endophytes enjoy various benefits as endophytes may promote plant growth (Jaber & Enkerli 2016, Khan *et al.* 2016), increase plant resistance to abiotic stresses like drought (Bano *et al.* 2012, Khan *et al.* 2015), metals (Wang *et al.* 2016), and temperature fluctuations, as well as protect plants against pathogen and herbivore attacks (Arnold *et al.* 2003, Jallow *et al.* 2004). Endophytic fungi can even produce VOCs that inhibit growth of other fungi as a direct defense or as allelochemicals for competitors of their host plant (Macias-Rubalcava *et al.* 2010). A vast amount of literature on plant-derived VOCs exists, beginning with the characterization of a plant's volatile profile and the changes in this profile due to different abiotic and biotic factors, the determination of the bioactivity of individual compounds, and the elucidation of the biosynthetic pathways via certain enzymes, e.g., terpene synthases. However, the discovery of endophytic organisms inside plant tissues has raised the

curiosity about the capabilities and functions of these microorganisms including the possibility that they also produce VOCs. Literature about VOCs isolated from endophytic fungi has increased over time, and currently 300 fungal VOCs have been characterized so far (Lemfack *et al.* 2014), with some of them already tested for their bioactivity potential. Yet, the biosynthesis of the VOCs and the mechanisms how endophytes produce VOCs inside leaf tissue is poorly understood. Furthermore, it has been shown that endophytic fungi are able to produce phytohormones that might influence leaf senescence and leaf fall (Nassar *et al.* 2005). So far, only a few studies have investigated the molecular mechanism of endophyte-mediated changes in plant metabolism. Hence, our understanding of the mechanisms by which endophytes lead to better plant fitness is still in its infancy (Chagas *et al.* 2018, Yan *et al.* 2019).

A mutualistic endophyte-plant interaction is often shown, especially for grass-associated endophytic fungi, which are mostly transmitted vertically. However, endophyte species can also include latent pathogens, commensals, temporal residents or latent saprotrophs, resulting in no beneficial effect for the host plant (Davis & Shaw 2008, Suryanarayanan 2013). Further it is speculated that the type of plant-endophyte interaction may switch according to environmental conditions, which favor the endophytes and/or are unfavorable for the host plant (Sieber 2007). For example, it has been shown that endophytes reduce photosynthetic rate (Mejía *et al.* 2014) and facilitate diseases (Adame-Alvarez *et al.* 2014, Busby *et al.* 2016). Studies investigating the role of a certain endophytic fungus usually are carried out under controlled conditions favorable for the host plant and more studies under field-realistic conditions are needed to reveal the role of a certain endophytic species (Hardoim *et al.* 2015). However, the lifestyle of most endophytic fungi associated with trees remains unknown (Hardoim *et al.* 2015).

Endophytic fungi – a novel source for bioactive compounds

Endophytic microorganisms are a rich source of potential agents for drug discovery. The bioactive metabolite classes present include alkaloids, polyketides, terpenoids, flavonoids, quinones, peptides, phenolic compounds, and steroids (Uzma *et al.* 2019, Zaferanloo *et al.* 2012). The first reported fungal metabolites that have an insecticidal purpose were alkaloids (Kaur 2020). Although, it was once believed that alkaloids only occur in plants, mainly in angiosperms (Waller & Nowacki 2012), four types of endophytic alkaloids are now known: ergot, indole-diterpene, pyrrolizidine, and peramine alkaloids (Bastías *et al.* 2017a). Chemically they represent a diverse group of nitrogen-containing compounds that do not share a common biosynthetic pathway (Saunders *et al.* 1992). They are synthesized from the amino acids lysine, phenylalanine, tyrosine, tryptophan, ornithine, histidine, or from purines and

pyrimidines (Karban & Agrawal 2002, Walters 2011). The quantity and quality of alkaloids depends on the plant physiological state, such as the age of the plant and its stage of development (Kaur 2020).

As already mentioned, endophytic fungi are also able to produce VOCs. Around 300 fungal volatiles have been described, including aliphatic alcohols, ketones, aldehydes, terpenoids, benzenoids and cycloalkanes (Effmert *et al.* 2012, Lemfack *et al.* 2014, Roy & Banerjee 2019). Many fungal VOCs are known to have antimicrobial activity, to induce the growth of host plants, or to shape plant communities, but these can also affect insect behavior (Daisy *et al.* 2002, Ezra & Strobel 2003, Macias-Rubalcava *et al.* 2010, Roy & Banerjee 2019, Strobel *et al.* 2004, Ting *et al.* 2010). In contrast to plants, terpenoids in fungi are exclusively produced by the mevalonate pathway (Schmidt-Dannert 2015). However, for now only a few terpene synthases have been identified and characterized from endophytic fungi compared to enzymes known from plants and bacteria (Shaw *et al.* 2015, Wu *et al.* 2016).

In general, endophyte-plant interactions and their chemistry are best described for vertically transmitted grass-associated clavicipitaceous endophytes. However, a tree crown provides an enormous amount of space with different niches for fungal colonization, as there are vertical and horizontal gradients in abiotic conditions, which results in a diverse leaf chemistry environment within one tree crown (Lämke & Unsicker 2018). Thus, a tree crown can offer space for different endophytic fungi to colonize. Furthermore, horizontally transmitted non-clavicipitaceous endophytes, as they occur in trees, outnumber the amount of grass-associated clavicipitaceous endophytes (Rodriguez *et al.* 2009). These considerations together with their complex lifestyle behavior make tree-associated endophytes a fascinating and multifaceted system to study.

1.3 Effects of endophytic fungi on plant-insect interactions

The arms race between plants and herbivorous insects has resulted in a plethora of different plant defenses and an equivalent number of counter-defense strategies by herbivores (Schoonhoven *et al.* 2005, Speed *et al.* 2015). The discovery of endophytic microorganisms however raises doubt that the co-evolutionary process is a consequence of only two interacting species (Bastías *et al.* 2017a, Saikkonen *et al.* 2013). Especially for grass-associated endophytes a defense mutualism between the plant and the endophyte has often been shown, while for tree-associated endophytes this role is more inconsistent (Christian *et al.* 2020, Herre *et al.* 1999, Van Bael *et al.* 2009). Endophytic fungi can influence plant-insect interactions directly by acting on insects, or indirectly by modulating the defense signaling and chemistry of the host plant. However, most research has been done on bioactive

compounds of endophytic fungi with a strong focus on alkaloids produced by the clavicipitaceous fungi of grasses (Hardoim *et al.* 2015). It has been shown in several systems that endophytic fungi reduce pathogen and herbivore damage in a wide range of host plants (Arnold *et al.* 2003, Christian *et al.* 2017a, Christian *et al.* 2020, Cosme *et al.* 2016, Estrada *et al.* 2013, Mejía *et al.* 2014). Already in 1978 Carroll and Carroll suggested that endophytes influence the defense system of trees. However, literature on the underlying mechanisms is scarce and it is often unclear whether defense compounds are produced by the plant or by the endophytes (Soliman *et al.* 2013), and which compounds are induced by the interaction between the plant and the endophytes (Christian *et al.* 2020, Hartley *et al.* 2015, Mejía *et al.* 2008). Furthermore, most of the studies, which address the molecular mechanisms behind insect-endophyte-plant interactions were done under laboratory conditions but rarely under field conditions. In contrast, field studies investigating the effect of endophytes on the herbivory load lack information about the plant chemistry. Considering the fact that all plants harbor endophytic microorganisms, investigating their impact on phytochemistry and plant-insect interactions is still an emerging research field, particularly in woody plants. Most of the knowledge on the chemical ecology of plant-insect interactions has been collected on economically important crop plant species and this is where most of the studies on plant-endophyte interactions and the influence of endophytes have also been done. As trees harbor a species-rich insect as well as endophyte community, research on endophytes of woody plants and their impact on associated insects is both complex and captivating.

1.4 Study organisms

To investigate the effects of endophytic microorganisms on plant chemistry and plant-insect interactions, I chose young black poplar trees (*Populus nigra*), the endophytic fungus *Cladosporium* sp., and the insect herbivores *Lymantria dispar*, *Amata mogadorensis* and *Chrysomela tremulae*.

Black poplar

The deciduous tree species black poplar (*Populus nigra*) belongs to the Salicaceae family and is distributed throughout the northern hemisphere (Isebrands & Richardson 2014), where it is adapted to flooding events and nutrient-poor soils in riverbanks and floodplain forests (Karrenberg *et al.* 2002). In Europe, black poplar is a key species for riparian ecosystems, where it plays an important role especially in the initial phase of the development of riparian forests (Smulders *et al.* 2008). As a woody model organism it is suitable because it grows fast and can be clonally propagated via stem cuttings (Regier *et al.* 2009). Further, the genome of a related poplar species, *Populus trichocarpa*, has been fully sequenced (Tuskan *et al.* 2006), leading to an increased usage of poplar species to study molecular mechanisms of trees (Regier *et al.* 2009).

Poplar trees are also ecologically relevant as a host for a plethora of organisms. In Europe, approximately 525 arthropods are assumed to be associated with tress of the genus *Populus* (Philippe & Bohlmann 2007). As these trees are attacked by a large variety of different herbivores and pathogens, poplars have developed an arsenal of various chemical and biochemical defense mechanisms (Philippe & Bohlmann 2007). A group of chemical defense compounds that is specific for the Salicaceae family members are the salicinoids. This group of phenolic compounds consists of more than 20 different chemical structures that act as direct defenses against herbivores, especially generalist insect herbivores and mammals (Boeckler *et al.* 2011, Boeckler *et al.* 2016, Hemming & Lindroth 1995, Lindroth 1991). Another defense strategy against insects is the emission of VOCs. Upon feeding damage, poplar trees release a diverse blend of VOCs containing compounds that attract parasitoids as an indirect defense (Clavijo Mccormick *et al.* 2014a) or directly repel herbivorous insects (Irmisch *et al.* 2014a). The volatile blend of poplars consists of approximately 70 different VOCs upon feeding damage, including various mono-, sesqui- and homo-terpenoids, aromatic compounds, and nitrogenous compounds (Clavijo Mccormick *et al.* 2014b, Danner *et al.* 2011, Irmisch *et al.* 2013). Poplar trees also produce flavan-3-ols, which are effective against fungal pathogens, like catechin and proanthocyanidin B1 (Ullah *et al.* 2017). The vast variety of plant-insect and plant-microbe interactions and the longevity of these trees make black poplar an ideal research organism for studying chemical ecology.

Poplar-associated fungi

Extensive research on the pathogenic microbiome was done for poplar species, with hundreds of fungal and bacterial pathogens being reported (Duplessis *et al.* 2009). For example, the widely spread poplar rust *Melampsora larici-populina* causes high biomass losses in poplar plantations (Gérard *et al.* 2006, Wan *et al.* 2013). Even though literature about endophytic microorganisms has increased during the last years, it mainly focused on bacterial endophytes. Only a few studies investigated the community of endophytic fungi colonizing poplars so far. However, they showed that poplar trees regularly harbor species of *Alternaria, Epicoccum, Aureobasidium* and *Cladosporium* (Martín-García *et al.* 2011).

Fungi of the genus *Cladosporium* are cosmopolitan being found in natural and anthropogenic habitats (e.g., on plants, in the soil, or on food and textiles) (Bensch *et al.* 2012, Ellis 1971, Ellis 1976). Conidia of *Cladosporium* sp. are small and mostly spread by air (Bensch *et al.* 2012, Farr *et al.* 1989, Flannigan 2001) over long distances (Bensch *et al.* 2012). Species of this genus are known as plant pathogens (Schubert 2005), hyperparasites on other fungi (Heuchert *et al.* 2005), and epiphytes in the

phyllosphere (Inácio *et al.* 2002, Islam & Hasin 2000, Jager *et al.* 2001, Levetin & Dorsey 2006, Stohr & Dighton 2004), but are also frequently isolated as endophytes (Bensch *et al.* 2012, Brown 1998, Riesen 1985). As endophytes, Huang *et al*. (2018) showed that hyphae of *Cladosporium* enter the inside of the leaf tissue of *Populus trichocarpa* via open stomata incidentally. No penetration through the cuticle was observed. In addition, species of *Cladosporium* were found to produce antifungal toxins (Bensch *et al.* 2012, Wang *et al.* 2013). Due to their widespread distribution and large environmental impact, this genus is of interest for researchers from variant scientific disciplines.

Poplar-associated insects

In Europe, poplar trees encounter approximately 525 different herbivorous insect species (Mattson *et al.* 2001), including defoliators, shoot feeders, and stem borers (Philippe & Bohlmann 2007). Usually poplar trees are able to stand the loss of larger quantities of leaves (Philippe & Bohlmann 2007), but during a mass outbreak, herbivorous insects can defoliate whole branches or young trees.

Caterpillars of the moth *A. mogadorensis* and *L. dispar* (the gypsy moth) are generalists that feed on many species, including black poplar trees (Wink & Schneider 1990). The gypsy moth is a common threat to a variety of tree species and is considered one of the most destructive herbivores of the world (GISD 2021) as it feeds on approximately 40 different plant families, with a preference for deciduous tree species like oak, poplar, and birch (Robinson *et al.* 2010). Like, *A. mogadorensis*, *L. dispar* also belongs to the Erebidae family and is native to Europe, while it is invasive in North America, where it was first introduced in Massachusetts in 1869 (Hoover 2000). The larvae represent the most mobile phase and they are able to disperse up to 50 km. With the production of silk threads, they can be blown away via wind, which is called ballooning. As the females are unable to fly, the larvae choose the oviposition site for the next generation (Wilson 2021). *L. dispar* usually produces one generation per year, but during favorable environmental conditions they can produce a second generation within the same year, increasing the stress on their host plants.

The leaf beetle *C. tremulae* is closely related to the poplar leaf beetle *C. populi*. It is a specialist on *Populus* and *Salix* species and a known pest species in forestry (Urban 2006). It occurs in most parts of Europe, as well as in America (Urban 2006). In this species, both adults and larvae feed on young leaves and reduce the growth rate of host plants (Leplé *et al.* 1995). The larvae of the poplar leaf beetle use the specialized metabolite salicin for their own protection against predators. By converting salicin to salicylaldehyde, they produce a bioactive volatile, which is deterrent to predators and reduces microbial infections (Strauss *et al.* 2014).

All three of these herbivore species may occur on back poplar trees at the same time, mainly at the end of the season (Fabisch *et al.* 2019). As they all are chewing insects, they can drastically reduce leaf foliage by removing large areas from the leaves.

1.5 Aim of the thesis

Endophytic microorganisms have gained much attention due to their potential role as mediators of plant-herbivore interactions, and their role as defense mutualists is often discussed. Most of the studies however have focused on grass endophytes, which are known to produce compounds with bioactive properties against herbivorous insects. Despite the omnipresence of fungal endophytes in forest ecosystems, their role on tree-insect interactions, either direct or indirect, is understudied.

The aim of my thesis was to elucidate the role of endophytic fungi in tree-insect interactions in the model system black poplar (*Populus nigra*). To determine the potential role of endophytic fungi in the overall plant volatile blend, I analyzed the volatile profile of several endophytic fungi isolated from mature black poplar trees and characterized terpene synthases from the endophyte *Cladosporium* sp.. Further, we used this species to study the influence of an endophytic fungus on plant chemistry either directly or indirectly in face of herbivory. Lastly, we analyzed the ecological consequences of *Cladosporium* sp. colonization for single herbivores and arthropod communities in the lab and under field conditions, respectively.

2. OVERVIEW OF MANUSCRIPTS

2.1 Manuscript I

Friend or foe?

The role of leaf-inhabiting fungal pathogens and endophytes in tree-insect interactions

Franziska Eberl, Christin Uhe and Sybille B. Unsicker

Published in *Fungal Ecology* (2019), 38, 104-112, doi:10.1016/j.funeco.2018.04.003

Summary

While studies on microbes and how they affect the interactions between herbaceous plants and insect herbivores are common in the literature, research on tripartite interactions between woody plants, insect herbivores and microbes is still in its infancy. This review summarizes current knowledge on the direct and indirect effects of pathogenic and endophytic fungi on trees and on tree-insect interactions. We focused on factors influencing fungal colonization and the phytochemical response from the host plant, as well as the consequences for attacking herbivorous insects. Further, we highlighted the use of different experimental conditions, such as the field, common gardens or the laboratory, and the different levels of observation used, such as the population, single trees or single leaves. Lastly, we recommended different methodological and experimental approaches, combining laboratory and field experiments for future research.

2.2 Manuscript II

Volatile emission and biosynthesis in endophytic fungi colonizing black poplar leaves

Christin Walther, Pamela Baumann, Katrin Luck, Beate Rothe, Peter H. W. Biedermann, Jonathan Gershenzon, Tobias G. Köllner, and Sybille B. Unsicker

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Summary

In this study, fungal endophytes were isolated and identified from leaves of mature black poplar (*Populus nigra*) trees and the volatile bouquet from each individual endophyte was qualitatively analyzed. Volatile organic compounds were detected from the endophytic fungi grown on agar medium that are also known to be produced by the host plant itself, e.g., 2-phenylethanol, 3-methyl-1-butanol and various sesquiterpenes. Further, we were able to isolate and characterize two sesquiterpene synthases from a species of *Cladosporium*, which produced the sesquiterpene (*E*)-β-caryophyllene among others. Since some of the compounds produced by the endophytic fungi are known to play a role in plant-insect interactions, the question arises whether these fungal volatiles influence direct and indirect plant defenses. Our research demonstrates that the capability of endophytes to make their own volatiles should be considered in future studies on plant-insect interactions.

2.3 Manuscript III

A fungal endophyte modifies leaf phytochemistry and shapes insect communities in poplar

Christin Walther, Beate Rothe, Michael Reichelt, Pamela Medina van Berkum, Jonathan Gershenzon, and Sybille B. Unsicker

In preparation

Summary

This study investigates the role of the endophyte *Cladosporium* sp. as a defense mutualist in black poplar (*Populus nigra*) against various herbivorous insect species. Larvae of the generalist lepidopteran *Lymantria dispar* preferred control over endophyte-inoculated leaf tissue in an *in planta* assay. The presence of the endophyte led to higher concentrations of constitutive as well as induced defense compounds after herbivory. Furthermore, the alkaloid stachydrine, which is produced by the endophyte, was found in leaves of inoculated plants. In a preference assay with stachydrine coated leaf discs, this compound deterred the generalist larvae *Amata mogadorensis* and attracted the specialist beetle *Chrysomela tremulae*. Under natural conditions, we showed that the endophyte also modulates the composition of the insect community. These findings reveal that rather than being a universal defense mutualist in plant-insect interactions, the studied endophyte *Cladosporium* sp. has a species-specific effect on herbivores.

3. MANUSCRIPT I

Angaben zum Eigenanteil

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Autoren: Franziska Eberl, Christin Uhe and Sybille B. Unsicker

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Friend or foe? The role of leaf-inhabiting fungal pathogens and endophytes in tree-insect interactions

Franziska Eberl, Christin Uhe, Sybille B. Unsicker*

Max Planck Institute for Chemical Ecology, Department of Biochemistry, Hans-Knöll Str. 8, 07745, Jena, Germany

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ABSTRACT

Trees are large organisms that structure forest ecosystems by providing an environment for an enormous diversity of animal, microbial and plant species. As these species use trees as their common hosts, many are likely to interact with each other directly or indirectly. From studies on herbaceous plant species we know that microbes can affect the interaction of plants with herbivorous insects, for example *via* changes in plant metabolite profiles. The consequences of fungal colonization for tree-insect interactions are, however, barely known, despite the importance of these ecological communities. In this review we explore the interaction of leaf-inhabiting pathogenic and endophytic fungi with trees and the consequences for tree-living insect herbivores. We discuss molecular, physiological, chemical, biochemical and ecological aspects of tree-fungus interactions and summarize the current knowledge on the direct and indirect effects of tree-inhabiting fungi on insect herbivores.

Our mechanistic understanding of the tripartite interaction of trees with leaf-inhabiting fungi and insect herbivores is still in its infancy. We are currently facing substantial drawbacks in experimental methodology that prevent us from revealing the effect of one single fungal species on a particular insect herbivore species and *vice versa*. Future studies applying a versatile toolbox of modern molecular, chemical analytical and ecological techniques in combined laboratory and field experiments will unequivocally lead to a better understanding of fungus-tree-insect interactions.

1. Introduction

Trees are dominant components of many terrestrial ecosystems and they host an astounding diversity of herbivorous insect and microbial species. All tissues in trees (roots, stem, bark, leaves and flowers) can be colonized by fungi (Baldrian, 2017) but the actual diversity of arboricolous fungal species worldwide can only be estimated (reviewed by Hawksworth, 2001). Fungus-tree interactions range from mutualistic to antagonistic (Faeth and Hammon, 1997; Stierle and Stierle, 2015) and it is difficult to strictly classify tree-inhabiting fungi into pathogens or endophytes, since the latter can sometimes switch their lifestyle from one to the other (reviewed by Sieber, 2007).

Most of our knowledge on plant-fungus interactions comes from studies on fungal pathogens in the model plant *Arabidopsis thaliana* and other herbaceous plant species that have mostly been investigated within an agricultural context (Ahuja et al., 2012). Pathogenic fungi can drastically modify plant signaling (Derksen et al., 2013), and plant metabolism (Glazebrook, 2005) with varying consequences for insect herbivores (Biere and Bennett, 2013; Franco et al., 2017; Raman and Suryanarayanan, 2017). To what extent endophytes can affect plant-insect interactions is still barely known, as these fungi often go unnoticed due to their symptomless lifestyle. The molecular mechanisms of fungal colonization in woody species have so far only been investigated in a few pathosytems (e.g. the interaction of poplar trees with the leaf rust fungi) (Duplessis et al., 2009; Hacquard et al., 2011) but our mechanistic understanding of endophyte-tree interactions is meager. Even fewer studies looked at the consequences of pathogen and endophyte infections in trees for the performance and the behavior of insect herbivores (Table 1). Here, we review the recent literature on the interaction of trees with fungi and insect herbivores in the foliage. Among the fungi, we differentiate between biotrophic and necrotrophic pathogens and endophytes *sensu lato*. We first give a general introduction to the fungus-tree interaction by looking at molecular, physiological, biochemical, chemical, and ecological aspects (Fig. 1). In the second half of our manuscript, we

* Corresponding author.
E-mail address: sunsicker@ice.mpg.de (S.B. Unsicker).

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Table 1
Selected studies on fungus-tree-insect interactions, classified by fungal life strategy and tree species. Parameters investigated in the studies are categorized into behavior (feeding or olfactory choice, leaf area consumed, oviposition), performance (larval or pupal weight, growth rate, development time, food conversion, survival, reproduction, embryo development, population size) and abundance (species richness, density, number of individuals). The effect on the insect was either positive (+), negative (−) or none (0). Experiment locations were either field/common garden (F) or laboratory conditions (L). The methodical levels are divided into population, individual tree, single leaf and molecular level (including chemical analyses), as depicted in Fig. 1. Taxonomic classification can be found in Table S1.

Fungal life strategy	Tree species	Fungal species	Insect species	Insect feeding guild	Parameter	Effect on insect	Location	Level	Reference
biotroph	*Populus nigra*	*Melampsora larici-populina*	*Lymantria dispar*	chewing	behavior	+	L	tree, molecular	(Eberl et al., 2017)
	Salix viminalis	*Melampsora epitea*	*Phratora vulgatissima*	chewing	behavior	−	L	tree	(Peacock et al., 2003)
	Salix x cuspidata	*Melampsora allii-fragilis*	*Plagiodera versicolor*	chewing/ skeletonizing	performance	−	L	leaf	(Simon and Hilker, 2003)
	Salix x cuspidata	*Melampsora allii-fragilis*	*Plagiodera versicolor*	chewing	behavior	−	L	tree, leaf	(Simon and Hilker, 2005)
	Quercus spp.	*Erysiphe alphitoides*	*Cynipidae*	galling	abundance	−	F	population	(Zargaran et al., 2012)
	Quercus robur	*Erysiphe alphitoides*	arthropod community	all	abundance	+/− [a]	F	population	(Tack et al., 2012)
			Tischeria ekebladella	mining	behavior	+	F	leaf	
			Acronicta psi	chewing	performance	−	F	leaf	
	Betula pubescens	*Melampsoridium betulinum*	*Epirrita autumnata*	chewing	performance	-/0 [b]	F	population, leaf	(Lappalainen et al., 1995)
	Betula pubescens	*Melampsoridium betulinum*	*Epirrita autumnata*	chewing	performance	0	F	leaf	(Ahlholm et al., 2002)
			Deporaus betulae	rolling	abundance	0	F	tree	
			Eriophyes rudis	galling	abundance	0	F	tree	
			Arge sp.	chewing	performance	0	F	leaf	
			Priophorus pallipes	chewing	performance	0	F	leaf	
			Dineura pullior	skeletonizing	performance	−	F	leaf	
	Cinnamomum yabunikkei	*Melanopsichium onumae*	arthropod community	spore & gall feeding	abundance	+	F	population	(Funamoto and Sugiura, 2017)
	Acacia dealbata	*Uromycladium spp.*	arthropod community	all	abundance	+	F	population	(Bashford, 2002)
necrotroph	*Populus spp.*	*Drepanopeziza populi*	arthropod community	all	behavior	−	F	tree	(Busby et al., 2015)
					abundance	−	F	tree	
	Quercus rubra	*Phytophthora plurivora*	*Lymantria dispar*	chewing	behavior	+	F + L	population, leaf	(Milanović et al., 2015)
					performance	+	F + L	leaf, molecular	
	Betula pendula	*Marssonia betulae*	*Euceraphis betulae*	piercing-sucking	abundance	+	F	population	(Johnson et al., 2003)
					behavior	+	F	leaf	
					performance	+	F	leaf, molecular	
	Pinus nigra	*Sphaeropsis sapinea*	*Neodiprion sertifer*	chewing	performance	-/0 [c]	F	tree	(Eyles et al., 2007)
endophyte	*Populus tremula*	*Aureobasidium sp.*	*Phratora vitellinae*	chewing	behavior	−	F	tree	(Albrectsen et al., 2010)[d]
	Quercus garryana	*Discula quercina*	*Besbicus mirabilis*	galling	performance	−	F	leaf	(Wilson and Carroll, 1997)
			Bassettia ligni	galling	performance	0	F	leaf	
	Quercus emoryi	*Asteromella sp.*	*Cameraria sp.*	mining	performance	0	F	leaf	(Faeth and Hammon, 1997)
		Plectophomella sp.	*Cameraria sp.*	mining	performance	0/- [e]	F	leaf	
	Betula pubescens	*Fusicladium sp.*	*Epirrita autumnata*	chewing	performance	- [f]	F	leaf	(Ahlholm et al., 2002)
			Deporaus betulae	rolling	abundance	−	F	tree	
			Eriophyes rudis	galling	abundance	0	F	tree	
			Arge sp.	chewing	performance	0	F	leaf	
			Priophorus pallipes	chewing	performance	0	F	leaf	
			Dineura pullior	skeletonizing	performance	0 [f]	F	leaf	
		Melanconium sp.	*Epirrita autumnata*	chewing	performance	0	F	leaf	
			Deporaus betulae	rolling	abundance	0	F	tree	
			Eriophyes rudis	galling	abundance	0	F	tree	
			Arge sp.	chewing	performance	0	F	leaf	
			Priophorus pallipes	chewing	performance	0	F	leaf	
			Dineura pullior	skeletonizing	performance	+	F	leaf	
	Embothrium coccineum	various species	arthropod community	all	behavior	−	F	population, tree	(Gonzalez-Teuber, 2016)
	Cordia alliodora	unknown	*Atta colombica*	cutting	behavior	−	L	tree	(Bittleston et al., 2011)
	Arbutus unedo	*Talaromyces pinophilus*	*Acyrthosiphon pisum*	piercing-sucking	performance	−	L	molecular [g]	(Vinale et al., 2017)
	Picea rubens	unknown	*Choristoneura fumiferana*	chewing	performance	−	L	molecular [h]	(Sumarah et al., 2010)

(*continued on next page*)

Table 1 (*continued*)

Fungal life strategy	Tree species	Fungal species	Insect species	Insect feeding guild	Parameter	Effect on insect	Location	Level	Reference
	Picea glauca	*Phialocephala scopiformis*	*Choristoneura fumiferana*	chewing	performance	–	L	leaf, molecular	(Miller et al., 2008)
	Picea glauca	*Phialocephala sp.*	*Choristoneura fumiferana*	chewing	performance	–	L	molecular [h]	(Sumarah et al., 2008)
			Lambdina fiscellaria	chewing	performance	–	L	molecular [h]	
			Zeiraphera canadensis	chewing	performance	–	L	molecular [h]	
	Pseudotsuga menziesii	*Rhabdocline parkeri*	*Contarinia spp.*	galling	performance	–	F	population, tree	(Carroll, 1995)

[a] Effect on community composition.
[b] Negative effect on pupal weight, no effect on development time.
[c] Effect depends on fertilization level.
[d] Suggested correlation between fungal abundance and tree susceptibility to herbivores.
[e] No effect on survival and pupal mass, but negative effect on developmental time.
[f] Inconsistency within different years or locations of study.
[g] Direct application on aphids feeding on *Vicia faba* leaf discs.
[h] Using artificial diet.

summarize the current knowledge on direct and indirect effects of tree-inhabiting fungi on herbivorous insects. Finally, we make suggestions for future research directions.

2. Fungus-tree interactions

2.1. Pathogenic fungi

Signs of pathogen infection in leaves are often readily visible in the form of classical disease symptoms like rust, blight or leaf spots. Pathogens invade leaves *via* vectors such as phloem feeding insects (Kluth et al., 2002), through wounds and stomata or with the help of appressoria and pegs that are able to break the leaf epidermis (Dean et al., 2012). Once the pathogen has entered the plant, it takes up nutrients from its host using one of two possible strategies. These different strategies are also generally used to classify plant pathogens (Glazebrook, 2005): biotrophic pathogens require a living host and deprive nutrients from intact plant cells by specialized structures like haustoria, which are well characterized in the rust fungus *Uromyces fabae* (Voegele and Mendgen, 2003). Necrotrophic pathogens, however, kill the host cells and take up nutrients that become available during or after cell death. Pathogens that pass through both lifestyles, depending on environmental conditions or their life stages, are called hemibiotrophic pathogens. Here, a biotrophic phase in the early infection stage usually precedes a necrotrophic phase as the infection continues (Ferreira et al., 2006).

Fungal diseases are widespread in plant systems, although a successful infection requires the synergy of a susceptible host, a virulent pathogen and favorable environmental conditions (Ferreira et al., 2006). Plants can recognize a pathogen attack either by general pathogen-associated molecular patterns, provoking pattern-triggered immunity (Naidoo et al., 2014), or by a specific interaction involving the gene-for-gene theory (Flor, 1971; Ferreira et al., 2006), also termed as effector-triggered immunity (Naidoo et al., 2014). In the latter interaction, the plant receptor (product of *R* (resistance) gene) binds to the product of the *avr* (avirulent) gene from the pathogen. If both the *R* gene of the plant and the corresponding *avr* gene in the pathogen are present, it will be considered an incompatible interaction: the plant defense mechanisms will be triggered early and no infection will be established (Dangl and Jones, 2001; Ferreira et al., 2006). If one of the two

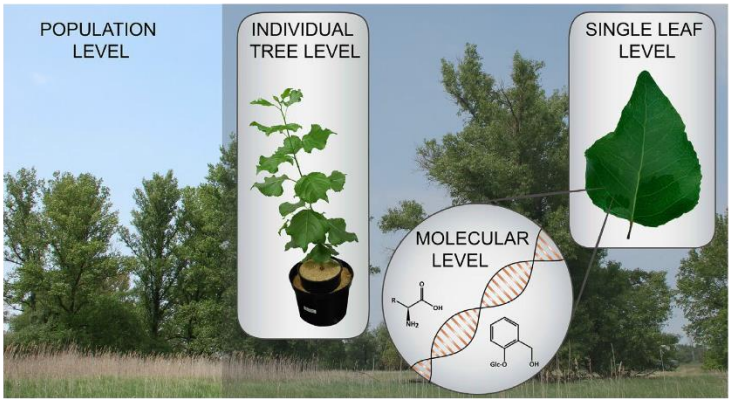

Fig. 1. Different levels of observation used in the studies focusing on fungus-tree-insect interactions. Populations are considered in field and common garden surveys, other studies work on the level of individual trees, in the field as well as in laboratory studies. Setups using single leaves – attached or detached – are common in both field and laboratory studies, for example, in choice tests or performance assays. Analyses at the molecular level, such as gene expression, phytohormones, primary and secondary compounds, are rare but crucial in understanding the interaction between trees, fungi and insects.

components is missing, or not functioning, a compatible interaction occurs, thus leading to successful disease development (Ferreira et al., 2006). In poplar trees (*Populus sp.*) the nucleotide-binding-site leucine-rich-repeat (NBS-LRR) family, an important class of *R* genes, is larger than in other angiosperm genomes. For example, *Arabidopsis* possesses 178 NBS-LRR genes, whereas *Populus trichocarpa* has 402 NBS-LRR genes (Duplessis et al., 2009). Within the *P. trichocarpa* genome, this gene family is one of the largest families, making up about 1% of the total number of genes (Duplessis et al., 2009). This suggests that early pathogen recognition may be more important for long-lived, large-sized trees than for smaller annual plants.

At early steps of pathogen recognition, plants start a complex signaling cascade involving, among other factors, defense hormones and systemic signaling. Here, biotrophic pathogens usually induce salicylic acid (SA), while necrotrophic fungi activate the jasmonic acid (JA)/ethylene pathway (Glazebrook, 2005; Derksen et al., 2013). The knowledge on these molecular mechanisms mostly comes from studies of herbaceous plants, and still needs to be validated in woody plant species. JA has been reported to play an important role in tree defense against insect herbivores and pathogens (Kozlowski et al., 1999; Lawrence et al., 2006; Krokene et al., 2008; Semiz et al., 2012; Arnerup et al., 2013; Boeckler et al., 2013). However, even within the same tree genus, differences in response to artificial JA treatment can be observed (Cooper and Rieske, 2008). By contrast, the role of SA in trees is not as clear as for JA, even though some studies indicate the involvement of SA in tree defense against pathogens (Xu et al., 2009; Naidoo et al., 2013; Xue et al., 2013; Eberl et al., 2017). Germain and Séguin (2011) summarized contradictory studies about SA-involvement in poplar defense and proposed that this hormone functions in an age-dependent manner. These examples just depicted suggest that there is much more diversity in regulatory mechanisms of anti-pathogen defenses among tree species than has been observed so far in herbaceous plants.

Plant defense mechanisms can be either constitutive (preformed) or induced after pathogen infection. Pathogen-induced defenses include cell wall reinforcement by lignification or callose deposition, leading to a more robust and rigid leaf morphology. Another defense, specifically against biotrophic pathogens, is the so called “hypersensitive response”, which includes the production of reactive oxygen species (ROS, “oxidative burst”) with signaling function, and presumably additional direct effects on the pathogen. The hypersensitive response ultimately ends in programmed cell death at the site of infection, limiting water and nutrient access for the pathogen (Glazebrook, 2005). As a more general defense response the biosynthesis of secondary metabolites (e.g. phytoalexins) and proteins is induced (Eyles et al., 2010). Even though the molecular mechanisms are not well understood in woody species, the consequences at the phenotypic level, such as the hypersensitive response or protein biosynthesis, are frequently reported.

The pathogen will try to evade recognition by the host and continuously counteract the defense mechanisms, making the infection process an ongoing battle between fungus and plant. The production of the sugar alcohol mannitol, an acyclic polyol, for example was reported in biotrophic rust fungi (Voegele et al., 2005) as well as the necrotrophic fungus *Alternaria* (Jennings et al., 2002). Its antioxidant properties might help the pathogen to quench ROS produced by its host plant. Some pathogens also release enzymes that target the host plant cell wall and hydrolyze the long-chain carbon polymers (Ferreira et al., 2006). Biotrophic fungi are even able to shift source-sink relationships within the host, so that infected tissue becomes a sink to which carbon sources are transported (Ferreira et al., 2006; Voegele and Mendgen, 2011). Necrotrophic fungi can harm the plant in a more aggressive way by releasing phytotoxic compounds (Evidente and Motta, 2001). The red-band needle blight-causing fungus *Dothistroma pini* for example produces a red-colored toxin with a similar anthraquinone structure as aflatoxin (Bradshaw, 2004). It is activated by light and leads to the production of damaging oxygen radicals. Alkaloids, which are well known from grass diseases involving ergot alkaloids (Miedaner and Geiger, 2015), also play a role in tree-pathogen interactions. The conifer pathogen *Sphaeropsis sapinea* produces sapinopyridione which causes yellowing and dieback of young cypress trees (Evidente et al., 2006). Plant defense mechanisms have been intensivley studied, but the activity of pathogen-derived responses should not be underestimated and needs further attention in future studies.

2.2. Endophytic fungi

Tree endophytes that mainly consist of Ascomycota and Basidiomycota (Petrini, 1986) belong to the nonclavicipitaceous group of endophytes (Rodriguez et al., 2009) and occur in above-ground plant tissues and in roots, distinguishable from mycorrhiza by lacking external hyphae (Mandyam and Jumpponen, 2005; Yuan et al., 2007). Like fungal plant pathogens hyphae of germinating endophytic spores can penetrate directly through leaf cuticle, stomata or other leaf openings, but unlike pathogen infection, endophytes undertake a quiescent state after infection (Sieber, 2007). Apart from random distribution of fungal spores e.g. *via* wind dispersal, the occurrence and distribution of endophytes is assumed to be affected by the genetic structure of the host plant (Hata and Futai, 1996; Collado et al., 1999; Ahlholm et al., 2002; Helander et al., 2006) and/or large- and small-scale climate conditions (Carroll and Carroll, 1978; Arnold and Herre, 2003; Arnold and Lutzoni, 2007). Unterseher et al. (2007) showed that the fungal endophyte distribution in trees also varies within the tree crown e.g. depending on whether leaves are sun-exposed or not. Furthermore, this study revealed that the endophyte species *Apiognomonia errabunda* exhibits distinct seasonal patterns in occurrence, with high abundance in young leaves in spring and very low abundance in older leaves in autumn. The authors of this study also suggest a relationship between the distribution of fungal endophytes and the accumulation of antifungal secondary metabolites in the tree throughout the growing season (Unterseher et al., 2007). Our knowledge of which specific factors determine the distribution and occurrence of fungal endophytes in treetops is limited, specifically with respect to species-specific requirements. It has been shown that some fungal endophytes benefit their host by enhancing plant growth (Khan et al., 2016). Bullington and Larkin (2015) speculated that early colonization with beneficial fungal endophytes allows better plant growth and thus promotes plant defense. For instance, re-inoculation of *Preussia* sp. in the host tree *Boswellia sacra* leads to enhanced plant growth and accumulation of photosynthetic pigments (Khan et al., 2016). In another study, the plant growth promoting siderophore ferrirubin was isolated from *Talaromyces pinophilus,* an endophytic fungus colonizing the strawberry tree (*Arbutus unedo*, Vinale et al., 2017). Endophytic yeast fungi isolated from the stems of poplar trees were shown to produce plant hormones which directly increase plant growth (Doty, 2011). In general our knowledge of the consequences of endophyte infection for tree performance and tree defense is scarce and limited to only a few studies on single-species interactions. Under natural conditions, however, the foliage of trees is colonized by numerous microbial endophytes at the same time. Investigating whether the consequences of complex multi-species interactions for tree performance and defense can be predicted from simple single-species interactions should be the focus of studies in the future.

Not much is known about the molecular mechanisms in tree-endophyte interactions. The anti-cancer drug and fungicide taxol, harvested from *Taxus* trees, has been found to be synthesized *in vitro* by various endophytes, e.g. *Paraconiothyrium*, hosted in *Taxus* plants (reviewed by Zhou et al., 2010). Soliman et al. (2013) also showed that the endophytic fungi affect plant taxol yield by eliciting transcription of plant taxol biosynthesis genes. These findings may also explain the high variation of taxol levels regularly found in *Taxus* trees and show that endophytes can stimulate their host to produce compounds that are active against plant pathogens. Some *in vitro* experiments have shown that fungi synthesize secondary metabolites only under certain conditions e.g. nitrogen limitation (reviewed by Sumarah and Miller, 2009). However, the results from *in vitro* experiments are very artificial and do not allow us to predict the fungal metabolite production *in planta* (Fan et al., 2017).

A study by Kusumoto and Matsumura (2012) showed that exogenous phytohormone application on *Quercus serrata* leaves results in an induced tree defense response, which in turn substantially alters the leaf endophyte species composition. SA and 1-aminocyclopropane-1-carboxylic acid (precursor of ethylene) treatment lead to a decrease in the abundance of the most dominant endophyte and thus allowed less dominant fungal species to occupy the niche that becomes available thereafter. Endophytic fungi are also able to produce phytohormones themselves, and it has been suggested that these endophyte-derived phytohormones can influence leaf senescence and leaf fall (Nassar et al., 2005; Suryanarayanan, 2013). Furthermore, endophytic fungi are able to produce volatile organic compounds (VOCs) that can function as direct defenses against other endophytic fungi and may even act as allelochemicals for competitors of their host trees, as shown by Macias-Rubalcava et al. (2010) with the endophytic fungus *Muscodor yucatanensis* isolated from the tropical tree species *Bursera simaruba*. Based on the results of the aforementioned studies, it is conceivable that endophytic fungi not only influence the microbial composition in the foliage of their host trees but also affect the interaction of trees with insects and other plants in the community *via* their impact on phytohormone and/or VOC production. There have only been a few studies on endophyte-mediated changes in plant secondary metabolism, and even fewer studies have investigated the molecular mechanisms of tree-endophyte interactions. It has been suggested, that the recognition of endophytes by their host plants triggers a cascade of signal transduction that leads to a change in the plant metabolic state similar to plant-pathogen interactions (Yuan et al., 2007; Van Bael et al., 2017). Unraveling these signaling cascades following endophytic colonization should be addressed in future research, and will help to understand the phenotypic patterns observed.

3. Direct and indirect effects of fungi on tree-insect interactions

The effect of plant-inhabiting fungi on herbivorous insects is either direct, from the fungus to the insect, or indirect *via* fungus-inflicted changes of the host plant. Direct effects can be provoked by ingestion of fungal tissue or by chemical compounds produced by the fungus. Insects can directly benefit from feeding on fungal tissue as it can provide nitrogen (Martin and Kukor, 1984; Gange, 1996), hydrolytic enzymes, choline and sterols (Martin, 1979), or B-vitamins (Martin, 1979; Voegele and Mendgen, 2011). On the other hand, both pathogenic and endophytic fungi can produce chemical compounds with toxic effects for insects (Bradshaw, 2004; Evidente et al., 2006; Miller et al., 2008; Sumarah et al., 2008; Kusari et al., 2012). Indirect, plant-mediated effects can be shifts of nutrient partitioning (reallocation, sink-source relationships (Ferreira et al., 2006)), increasing of defense responses (induction of systemic resistance, priming (Pastor et al., 2013)) or inhibiting defense responses (antagonistic phytohormone crosstalk (Eberl et al., 2017)).

It is hard to distinguish whether fungus-mediated effects on herbivorous insects are of direct or indirect nature. Many studies rely on correlations including all organisms at the same time, and do not consider the actual mechanism. Another difficulty clearly is that biotrophic pathosystems cannot be investigated without the host plant and, therefore, always include indirect effects, as *in vitro* cultivation is not established yet. However, *in vitro* cultivation (possible for necrotrophs) is highly artificial, providing environmental and nutritional conditions far away from the natural situation *in planta*. By applying modern molecular tools, such as transcriptomics or microdissection in future studies, we will be able to better distinguish between plant and fungal genes and thus better dissect direct and indirect effects of tree-inhabiting fungi on insect herbivores.

3.1. Tree pathogen effects on herbivorous insects

The effects of tree-pathogenic fungi on insect herbivore performance and behavior reported in the literature are inconsistent. A few studies report positive responses of herbivorous insects to pathogen infection (Johnson et al., 2003; Milanović et al., 2015; Eberl et al., 2017), while others document detrimental effects (Eyles et al., 2007; Zargaran et al., 2012; Busby et al., 2015) or no effect (Kellogg et al., 2005). General conclusions on the effect of tree-pathogens on insect herbivore performance and behavior cannot be drawn. The outcome of such multi-trophic interactions for insects seems to be dependent on many factors, and the interplay of these factors is not yet well understood. First, the identity of the fungus and the insect plays an important role, as seen in birch (Ahlholm et al., 2002) and in oak, where the composition of the arthropod community changed in response to mildew infection (Tack et al., 2012). Similarly, the species and genotype of the host tree, which both determine the susceptibility to biotic stressors, is an important predictor of the impact on herbivores (Busby et al., 2015). Furthermore, environmental conditions strongly influence the effect of tree-pathogen infection on insect herbivores. In Austrian pine (*Pinus nigra*), for example, the soil fertility level determines whether infection with the necrotrophic fungus *S. sapinea* has a slightly positive (high nitrogen), no (medium nitrogen) or a negative (low nitrogen) effect on the survival of sawfly (*Neodiprion sertifer*) larvae (Eyles et al., 2007).

Focusing on the fungal infection, there are also variables influencing trophic interactions. In the well-studied willow-rust system the behavior of the willow leaf beetle *Plagiodera versicolora* depends on the spatial distance of the beetle to the pathogen infection site (Simon and Hilker, 2005) as well as on the time since the onset of infection (Peacock et al., 2003; Simon and Hilker, 2005). In *Botrytis*-infected grapevine leaves, the importance of the disease severity was also shown, by monitoring the choice and oviposition of a lepidopteran moth (Rizvi and Raman, 2015).

A central problem for deducing generalities from tree-pathogen-insect studies are differences in experiment methodology and the varying level of observation (Fig. 1, Table 1). Several studies have been conducted under field conditions at the level of tree populations (Bashford, 2002; Zargaran et al., 2012; Funamoto and Sugiura, 2017), whereas other studies focused on detached single leaves from trees grown under laboratory conditions (Simon and Hilker, 2003; Rizvi and Raman, 2015) or collected from the field (Lappalainen et al., 1995; Ahlholm et al., 2002; Tack et al., 2012; Milanović et al., 2015). Some studies also performed experiments with different pathogen and insect species in individual trees

(Peacock et al., 2003; Kellogg et al., 2005; Simon and Hilker, 2005; Eyles et al., 2007; Busby et al., 2015; Eberl et al., 2017). On the insect side, mostly behavioral responses (Mondy et al., 1998; Peacock et al., 2003; Simon and Hilker, 2005; Moskowitz and Haramaty, 2012; Busby et al., 2015; Rizvi and Raman, 2015) and performance (Ahlholm et al., 2002; Simon and Hilker, 2003; Mondy and Corio-Costet, 2004; Kellogg et al., 2005; Eyles et al., 2007) in response to pathogen infection in trees have been assessed. Other studies have observed the arthropod community structure, i.e. species richness and abundance, as a consequence of pathogen infection in trees (Tack et al., 2012; Zargaran et al., 2012; Busby et al., 2015; Funamoto and Sugiura, 2017). Currently, there is still little information on the molecular mechanisms for tree-pathogen-insect interactions, such as phytohormone signaling (Xu et al., 2009; Naidoo et al., 2013), gene expression patterns (Duplessis et al., 2011; Büchel et al., 2012), or general phytochemical patterns including primary as well as secondary metabolites.

Studies that combine behavioral or performance assays with a molecular approach to explain the ecological patterns seen in these multi-trophic interactions are even rarer. Johnson et al. (2003) observed a positive correlation between the abundance of aphids (*Euceraphis betulae*) and the necrotrophic pathogen *Marssonia betulae* occurring in trees of a natural birch population. In addition, choice and performance tests supported the positive interaction between these two antagonists. Finally, the enhanced aphid performance could be explained by elevated levels of free amino acids that were found in infected birch leaves. More recently, Eberl et al. (2017) reported reduced emission of plant volatiles, an indirect anti-herbivore defense, in rust-infected and herbivore-attacked poplar trees. The diminished emission could be explained by an antagonistic phytohormonal crosstalk and a downregulation of biosynthetic genes. Furthermore, an olfactometer experiment revealed that gypsy moth (*Lymantria dispar*) caterpillars were more attracted to the odor of their food plant *Populus nigra* when it was infected with the leaf rust fungus *Melampsora larici-populina* than to the odor of non-infected control trees. The caterpillars were also attracted to the smell of rust spores alone, showing even a direct interaction between insects and fungus (Eberl et al., 2017). A clear plant-mediated effect was observed in oak (Milanović et al., 2015). In this study, the pathogen *Phytophthora plurivora* infected the roots below ground, whereas the herbivores *L. dispar* fed on aboveground tissue of the same tree. Here, the larvae performed better on leaves of infected trees due to a higher protein content compared to uninfected trees.

These examples illustrate that numerous factors, such as species identity, temporal and spatial patterns as well as environmental conditions, determine the effect of a plant pathogen on plant-feeding insects. To disentangle the role of individual factors, future studies should start manipulating one factor at a time while keeping the other factors as stable as possible. In this way, we can understand the impact of single factors and have a basis to extend studies manipulating two or more factors, to also reveal interactive effects.

3.2. Tree endophyte effects on herbivorous insects

The literature on the role of endophytic fungi in plant-insect interactions is dominated by studies on grass endophytes (reviewed by Hartley and Gange, 2009 and Saikkonen et al., 2010), where some are known to produce alkaloid-based defensive compounds with deterring effects against insect herbivores (Faeth and Bultman, 2002; Shymanovich et al., 2015; Bastias et al., 2017; Fuchs et al., 2017). Due to the asymptomatic nature of endophytic fungi in plants, they are frequently denoted as mutualists (Stone and Petrini, 1997). Horizontally transmitted endophytes, as they occur in herbaceous and woody plant species (Arnold and Herre, 2003), are thought to be less mutualistic (reviewed by Herre et al., 1999, Van Bael et al., 2009). This may be due the fact that latent pathogens and dormant saprotrophs also fall into this group (Bahnweg et al., 2005; Davis and Shaw, 2008; Suryanarayanan, 2013).

Carroll and Carroll (1978) were the first to suggest that endophytic fungi benefit coniferous trees by making the needles less palatable for herbivorous insects. A study with Spanish elm seedlings (*Cordia alliodora*) showed that leaf cutter ants (*Atta colombica*), when given a choice, prefer to cut leaves from trees with low over high endophyte density (Bittleston et al., 2011), suggesting that high loads of endophytes in tree leaves generate costs to the ants. *Phialocephala scopiformis* isolated from evergreen white spruce (*Picea glauca*) is probably the best studied endophytic fungus in the context of tree-insect interactions. This fungal species produces the yellow pigment rugulosin, known to negatively affect the performance of herbivorous insects such as the spruce budworm (*Choristoneura fumiferana*) and the hemlock looper (*Lambdina fiscellaria*) (Miller et al., 2002, 2008; Sumarah et al., 2008, 2010; Sumarah and Miller, 2009). Furthermore, numerous other compounds isolated from endophytes in conifer needles were shown to have toxic effects on the spruce budworm, suggesting that these fungi play a crucial role in tree defense against insect herbivores (reviewed by Sumarah and Miller, 2009). More recently, Vinale et al. (2017) showed that the survival of the pea aphid *Acyrthosiphon pisum* was negatively affected by the bioactive metabolite 3-O-methylfunicone isolated from *T. pinophilus*, an endophytic fungus of the strawberry tree (*A. unedo*). This result suggests that chemicals isolated from tree-inhabiting endophytic fungi may also play a role in protecting crop species against their insect pests in the future.

Studies investigating the consequences of horizontally transmitted endophytic fungi for plant–insect interactions under natural conditions are rare, particularly in woody plants. A few studies have shown that the density of tree endophytic fungi is negatively correlated with insect herbivore damage (Albrectsen et al., 2010; Gonzalez-Teuber, 2016) which suggests that trees benefit from fungal endophyte colonization. Unfortunately, the molecular mechanisms behind the endophyte-mediated tree-insect interaction are still scarcely understood. As described in the previous section, studies with endophytes were also performed at different levels of observation, complicating general conclusions about the role of endophytes in tree defense (Fig. 1, Table 1). For example, some studies were conducted at population (Carroll, 1995; Gonzalez-Teuber, 2016) and tree level (Carroll, 1995; Ahlholm et al., 2002; Albrectsen et al., 2010; Bittleston et al., 2011; Gonzalez-Teuber, 2016), while others were carried out in single leaves (Faeth and Hammon, 1997; Wilson and Carroll, 1997; Ahlholm et al., 2002; Miller et al., 2008) and at the molecular level, where studies on rugulosin producing endophytes dominate (Miller et al., 2008; Sumarah et al. 2008, 2010; Vinale et al., 2017).

Furthermore, the role of tree associated endophytes as defense mutualists is inconsistent, as compared to that of grass-endophytes. Specifically, the same endophyte can negatively affect one herbivore species while positively affecting another, as was shown for *Chaetomium cochliodes* found in creeping thistle (*Cirsium arvense*) (Gange et al., 2012). In contrast to the contradictory results on the role of endophytic fungi for insect herbivore performance, however, there is strong evidence for a role of endophytes in anti-pathogen defense (Arnold et al., 2003; Herre et al., 2007). If anti-microbial defense is a feature common to endophytic fungi, we would speculate that endophytic fungi indirectly affect herbivorous insects by negatively affecting endosymbionts in insects.

4. Concluding remarks

Leaf-colonizing fungi can produce chemical compounds or

modify the host plant metabolism and thus directly and indirectly affect the interaction of plants with insect herbivores. So far, the majority of studies investigating the tripartite interaction between fungi, plants and insects have been performed in short-lived annuals within an agricultural context, and mostly with plant pathogens, but rarely endophytes. The consequences of fungal colonization for the interaction of long-lived woody perennials with insect herbivores are barely known and our mechanistic understanding of the role of pathogenic and endophytic fungi for tree-insect interactions is incomplete. Neither pathogenic fungi nor endophytic fungi in trees have shown consistently negative or positive effects on insect herbivores in the studies reviewed for this manuscript (Table 1). Descriptive studies have shown that arboricolous fungi can shape the diversity and composition of insect herbivore species but the underlying molecular mechanisms for these community level effects are barely understood. So far, the taxonomic identification of endophytic fungi in trees has been restricted to easily cultivable species and thus numerous uncultivable species have been overlooked (Arnold, 2007; Hyde and Soytong, 2008; Unterseher et al., 2011). In the future, high-throughput sequencing techniques should be applied to detect less abundant and uncultivable taxa (Rajala et al., 2013). Furthermore, novel *in vitro* culturing techniques should be developed to reveal fungal metabolite profiles and biosynthetic pathways, also of so far non-cultivable species (e.g. biotrophic pathogens). Non-targeted metabolomics and proteomics of fungi *in vitro* and *in planta* will help to reveal fungus-derived compounds and fungus-inflicted metabolite changes in trees. A detailed knowledge of the biosynthetic pathways and metabolite profiles of single fungal species in trees will then allow us to study the consequences of fungal colonization for insect herbivores at the molecular level. Methodologically this is certainly challenging as it requires sterile trees to be infected with a single fungal species to start off with. Experiments with only one fungal species at a time are automatically restricted to young trees propagated from sterile tissue cultures under laboratory conditions, as trees under natural conditions are usually colonized by microbes. Although single-species interactions with immature trees do not reflect real-world scenarios, we argue that very controlled studies are essential to gain comprehensive mechanistic knowledge of the role of fungi in tree-insect interactions. To validate the results from laboratory studies, performing descriptive and experimental studies under natural field conditions are recommended.

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Supplementary data

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4. MANUSCRIPT II

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Autoren: Christin Walther, Pamela Baumann, Katrin Luck, Beate Rothe, Peter H. W. Biedermann, Jonathan Gershenzon, Tobias G. Köllner, and Sybille B. Unsicker

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Christin Walther (geb. Uhe)	30	75	75	75	0
Pamela Baumann	0	5	5	3	0
Katrin Luck	0	5	5	0	0
Beate Rothe	0	0	5	0	0
Peter H. W. Biedermann	0	0	5	0	0
Tobias G. Köllner	30	15	0	5	0
Jonathan Gershenzon	0	0	0	7	100
Sybille B. Unsicker	40	0	5	10	0

BEILSTEIN JOURNAL OF ORGANIC CHEMISTRY

Volatile emission and biosynthesis in endophytic fungi colonizing black poplar leaves

Christin Walther[1], Pamela Baumann[1,2], Katrin Luck[1], Beate Rothe[1], Peter H. W. Biedermann[1,2], Jonathan Gershenzon[1], Tobias G. Köllner[1] and Sybille B. Unsicker[*1]

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Address:
[1]Department of Biochemistry, Max Planck Institute for Chemical Ecology, Hans-Knöll Str. 8, 07745 Jena, Germany and [2]Chair of Forest Entomology and Protection, Institute of Forest Sciences, University of Freiburg, Fohrenbühl 27, 79252 Stegen-Wittental, Germany

Email:
Sybille B. Unsicker[*] - sunsicker@ice.mpg.de

* Corresponding author

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Abstract

Plant volatiles play a major role in plant–insect interactions as defense compounds or attractants for insect herbivores. Recent studies have shown that endophytic fungi are also able to produce volatiles and this raises the question of whether these fungal volatiles influence plant–insect interactions. Here, we qualitatively investigated the volatiles released from 13 endophytic fungal species isolated from leaves of mature black poplar (*Populus nigra*) trees. The volatile blends of these endophytes grown on agar medium consist of typical fungal compounds, including aliphatic alcohols, ketones and esters, the aromatic alcohol 2-phenylethanol and various sesquiterpenes. Some of the compounds were previously reported as constituents of the poplar volatile blend. For one endophyte, a species of *Cladosporium*, we isolated and characterized two sesquiterpene synthases that can produce a number of mono- and sesquiterpenes like (*E*)-β-ocimene and (*E*)-β-caryophyllene, compounds that are dominant components of the herbivore-induced volatile bouquet of black poplar trees. As several of the fungus-derived volatiles like 2-phenylethanol, 3-methyl-1-butanol and the sesquiterpene (*E*)-β-caryophyllene, are known to play a role in direct and indirect plant defense, the emission of volatiles from endophytic microbial species should be considered in future studies investigating tree-insect interactions.

Introduction

Plant volatile organic compounds (VOCs) can mediate plant–insect, plant–microbe, and plant–plant interactions [1-4]. The constitutive and herbivore-induced volatile blends of plants consist of different compound classes, including green leaf volatiles, benzenoids, terpenoids, and nitrogen-containing compounds [5-7]. Among these, terpenoids represent the largest and most diverse group of compounds. In poplar trees, large amounts of terpenoids can be emitted constitutively [8,9] and

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facilitate protection against thermal and oxidative stresses [10]. In addition, terpenoids are also produced in response to biological stresses such as herbivory [9,11] and can fulfill different functions in plant–insect interactions. For instance, together with other volatiles, some terpenoids are known to attract natural enemies of insect herbivores [2,12,13] or attract insects as shown for the sesquiterpene (*E*)-β-caryophyllene (**1**) [14,15]. Another sesquiterpene, (*E*)-β-farnesene (**2**), an aphid alarm pheromone, is also produced by plant species like *Arabidopsis thaliana* [16]. Besides terpenoids, other plant VOCs are also known to mediate plant–insect interactions. For instance, 2-phenylethanol (**3**) is a typical attractant for pollinators, but is also involved in direct and indirect plant defense [17-19].

Endophytic microorganisms are fungi or bacteria that live asymptomatically within healthy plant tissue (e.g., leaves, flowers and roots) for at least a part of their life cycle [20]. Endophyte colonization is widespread in the plant kingdom, but their role in plant–insect interactions is under debate [21]. Currently, most of our knowledge on the role of endophytes in plant defense responses comes from studies with fungal grass endophytes (clavicipitaceous endophytes) that are often mutualistic for the plant. The ecological significance of nonclavicipitaceous endophytes, which occur also in trees, is more ambiguous and only poorly understood [22-24].

Endophytic fungi themselves can produce VOCs. Currently, around 300 fungal VOCs have been characterized, including aliphatic alcohols, ketones, aldehydes, acids and esters, terpenoids, benzenoids, naphthalene derivatives, and cycloalkanes [25-27]. Endophytic fungal VOCs are frequently described to exhibit antimicrobial activity; however, they are also known to induce the growth and vigor of the host plant and to shape plant community structure [27-31]. Furthermore, volatiles released from endophytic fungi can also affect insect behavior. Daisy et al. isolated the endophytic fungus *Muscodor vitigenus* and characterized the volatile blend in culture [32]. Naphthalene, an insect deterrent that is used, e.g., in mothballs [33], was the most dominant compound in the fungal volatile blend and showed a repellent effect on the wheat stem sawfly *Cephus cinctus* in a Y-tube olfactometer experiment. However, the literature on endophytic volatiles and how they influence insect behavior is scarce, especially for the endophytes of trees despite the omnipresence of fungal endophytes in forest ecosystems [34] and their potential impact on plant–insect interactions [35-38].

Among the known endophytic volatiles, sesquiterpenes have gained much attention in recent years as they can play an important role in plant–plant, plant–microbe, and microbe–microbe interactions [39,40]. Weikl et al., for instance, analyzed the volatile emission of *Alternaria alternata* and *Fusarium oxysporum* in culture and showed that both species are able to produce sesquiterpenes like (*E*)-β-farnesene (**2**), α- and β-chamigrene (**4**), and germacrene D [41]. In general, terpenes are derived from the five-carbon intermediates dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), which are both produced by the mevalonate pathway in fungi [42]. The condensation of DMAPP with varying numbers of IPP residues results in products of various chain lengths: geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15), and geranylgeranyl diphosphate (GGPP, C20). Terpene synthases (TPS) then convert the precursors GPP, FPP, and GGPP into the different terpene skeletons [42-44]. However, our knowledge on terpene synthases of endophytic fungi is scarce, specifically in comparison to the vast knowledge on these enzymes in plants and bacteria [44,45].

Typical monoterpenes like limonene and linalool (**5**), sesquiterpenes like α-farnesene, chamigrene (**4**), aromatic alcohols like 2-phenylethanol (**3**), and aliphatic alcohols like 3-methyl-1-butanol (**6**) are also found in the headspace of endophytic fungi grown in culture [46-52]. Those studies have shown that volatile blends produced by some endophytic fungi qualitatively overlap with the VOC bouquets produced by numerous plant species [53-56] including black poplar (*Populus nigra*) [57-59]. Thus, the question arises whether endophytes found in plants contribute significantly to the overall plant volatile blend by expression of their own *TPS* genes and how these fungal volatiles influence plant–insect interactions. Identification of fungal *TPS* genes is a useful tool to assess the impact of fungal terpene emission on plant volatile composition and on plant–insect interactions.

In this study, we isolated and identified endophytic fungi from leaves of a natural population of mature black poplar trees. From these fungi, we qualitatively investigated the volatiles emitted in culture and compared the blend with that emitted from black poplar trees. In addition, we used transcriptome analysis and heterologous expression to identify and characterize terpene synthases in one of the endophyte species isolated. These fungal TPSs may contribute to the volatile blend of black poplar foliage and the compounds emitted may play a role in poplar plant–insect interactions.

Results

Endophytic fungi isolated from old-growth black poplar trees

We identified 12 endophyte species from nine different genera by sequencing the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA cistron. Two species were identi-

fied from the genus *Alternaria*, three from *Didymella*, two from *Aureobasidium*, and one each from *Arthrinium*, *Cladosporium*, *Fusarium*, *Sordaria*, and *Stemphylium* (Table 1). One unidentified species was also included in the volatile analysis. All the identified fungi belong to the Ascomycota, the largest fungal phylum.

Endophytic fungi emit typical plant VOCs

Altogether, we detected 77 volatile compounds in the headspaces of the 13 different endophytic species grown on agar medium. With 34 different compounds, the unidentified fungus was the endophyte emitting the most complex volatile blend. In contrast, in the headspace of both *Stemphylium* sp. and *Cladosporium* sp., only two volatile compounds were detected (Table 2). All endophytic fungi, except *Cladosporium* sp., produced aliphatic or aromatic alcohols like 2-methyl-1-propanol (**7**), 3-methyl-1-butanol (**6**) or 2-phenylethanol (**3**). Of 77 detected volatile compounds, 50 compounds are sesquiterpenes. Furthermore, seven out of 13 fungi produced sesquiterpenes. In general, the analyzed endophytic fungi have a species-specific volatile bouquet, and none of the endophytic species shared the same combination of volatile compounds. We had previously detected a number of these fungal volatiles in our volatile analyses of poplar leaves, including two alcohols 3-methyl-1-butanol (**6**) and 2-phenylethanol (**3**) and the two sesquiterpenes (*E*)-β-caryophyllene (**1**) and α-muurolene (**8**) (Table 2, Figure 1) [7,9,57-59].

Cladosporium sp. contains two sesquiterpene synthases that produce typical poplar volatile compounds in in vitro assays

The poplar fungal endophyte *Cladosporium* sp. emitted (*E*)-β-caryophyllene (**1**) in culture (Table 2, Figure 1). As this sesquiterpene is also a characteristic VOC in the constitutive and herbivore-induced blends of black poplar [57-59], we wanted to identify and characterize the responsible fungal terpene synthase, as this enzyme could contribute to the overall (*E*)-β-caryophyllene emission from the tree.

To identify terpene synthase genes potentially involved in volatile terpene formation in *Cladosporium,* we sequenced the transcriptome and performed a de novo assembly of the obtained reads. A TBLASTN analysis with *Aspergillus terreus* aristolochene synthase (pdb 20A6) as query and the de novo

Table 1: Fungal endophytes identified from leaves of mature black poplar (*Populus nigra*) trees.[a]

Species	Family	Best hit and accession number	Identity (%)
Alternaria infectoria	Pleosporaceae	*Alternaria infectoria* KX394561.1	100
Alternaria sp. 1	Pleosporaceae	*Alternaria* sp. KY788045.1	99
Stemphylium sp.	Pleosporaceae	*Stemphylium* sp. KX400960.1	99
Aureobasidium sp. 1	Dothioraceae	*Aureobasidium pullulans* KX869960.1	100
Aureobasidium sp. 2	Dothioraceae	*Aureobasidium pullulans* KT352844.1	97
Didymella glomerata	Didymellaceae	*Didymella glomerata* KY788126.1	99
Didymella sp. 1	Didymellaceae	*Didymella glomerata* KY788126.1	100
Didymella sp. 2	Didymellaceae	*Didymella glomerata* KY794938.1	100
Cladosporium sp.	Cladosporiaceae	*Cladosporium subcinereum* NR_148193.1	100
Fusarium sp.	Nectriaceae	*Fusarium armeniacum* KF944456.1	100
Sordaria sp.	Sordariaceae	*Sordaria fimicola* KX986578.1	100
Arthrinium sp.	Apiosporaceae	*Arthrinium sacchari* KY782634.1	100
unidentified species			

[a]Endophytes were isolated from leaves after surface sterilization (n = 10 tree genotypes). 12 out of 13 isolated endophytes were classified to the genus level via sequencing of the internal transcribed spacer (ITS) region of the nuclear ribosomal cistron (with primers ITS1F/ ITS4). The sequences obtained were compared to the NCBI sequence database (Supporting Information File 1, Table S1). Isolated fungi with multiple 99–100% identity hits on several species within the same genus were identified only to the genus level, but we still list the single best hit in the table.

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Table 2: Volatiles emitted from endophytic fungi growing in culture on potato dextrose agar (PDA).[a]

			Endophyte species											
Volatiles class	Volatile organic compound	Kovats' RI	*Alternaria infectoria*	*Alternaria* sp. 1	*Stemphylium* sp.	*Aureobasidium* sp. 1	*Aureobasidium* sp. 2	*Didymella glomerata*	*Didymella* sp. 1	*Didymella* sp. 2	*Cladosporium* sp.	*Fusarium* sp.	*Sordaria* sp.	*Arthrinium* sp.	unidentified
aliphatic alcohol	ethanol (**17**)[b]			X		X	X	X	X	X		X	X	X	X
aliphatic ketone	2-butanone	598						X		X					
aliphatic ester	ethyl acetate	611				X									
aliphatic alcohol	2-methyl-1-propanol (**7**)	623	X	X	X	X	X	X	X	X				X	
–	unknown 1	658													X
aliphatic alcohol	3-hydroxy-2-butanone	710				X	X	X	X	X			X		X
aliphatic alcohol	**3-methyl-1-butanol (6)**[b]	730			X	X	X	X	X	X			X	X	X
aliphatic alcohol	2-methyl-1-butanol	732	X	X											
–	unknown 2	776													X
aliphatic ester	3-methylbutyl acetate	881				X								X	
aromatic hydrocarbon	ethenylbenzene	891									X				
–	unknown 3	907				X									
–	unknown 4	1044												X	
–	unknown 5	1054	X												
aromatic alcohol	**2-phenylethanol (3)**[b]	1115				X	X							X	
sesquiterpene	unknown 6	1335	X	X											
sesquiterpene	unknown 7	1343	X	X											X
sesquiterpene	α-cubebene	1355													X
–	unknown 8	1356						X							
–	unknown 9	1361						X							
–	unknown 10	1369						X							
sesquiterpene	unknown 11	1372													X
sesquiterpene	α-copaene	1381													X
–	unknown 12	1391													X
sesquiterpene	unknown 13	1395													X
sesquiterpene	sativene (**16**)	1401													X
sesquiterpene	α-gurjunene	1415	X	X											
sesquiterpene	unknown 14	1416										X			
sesquiterpene	unknown 15	1419													X
sesquiterpene	unknown 16	1420										X			
sesquiterpene	unknown 17	1423													X
sesquiterpene	aristolene (**15**)	1424	X	X											
sesquiterpene	**(*E*)-β-caryophyllene (1)**[b]	1425									X				
sesquiterpene	unknown 18	1426													X
sesquiterpene	unknown 19	1433	X	X											
sesquiterpene	unknown 20	1433						X							X
sesquiterpene	bicyclosesquiphellandrene	1436										X			
sesquiterpene	β-gurjunene[c]	1437	X	X											
sesquiterpene	unknown 21	1438													X
sesquiterpene	unknown 22	1440										X			
sesquiterpene	unknown 23	1442	X	X											

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Table 2: Volatiles emitted from endophytic fungi growing in culture on potato dextrose agar (PDA).[a] (continued)

sesquiterpene	α-guaiene[c]	1447	X	X				
sesquiterpene	unknown 24	1448				X		
sesquiterpene	unknown 25	1453				X		
sesquiterpene	unknown 26	1454						X
sesquiterpene	(*E*)-β-farnesene (**2**)[c]	1456				X		
sesquiterpene	unknown 27	1462						X
sesquiterpene	unknown 28	1467						X
sesquiterpene	unknown 29	1469				X		
sesquiterpene	unknown 30	1472	X	X				
sesquiterpene	β-chamigrene[c]	1474				X		
sesquiterpene	unknown 31	1475	X					
–	unknown 32	1475						X
sesquiterpene	α-selinene[c]	1477	X	X				
sesquiterpene	γ-muurolene	1478						X
sesquiterpene	unknown 33	1483						X
sesquiterpene	unknown 34	1486	X	X				
sesquiterpene	β-selinene	1488	X	X				
sesquiterpene	unknown 35	1489						X
sesquiterpene	valencene[b]	1494	X	X				
sesquiterpene	unknown 36	1498	X	X				
sesquiterpene	**α-muurolene (8)**	1500						X
sesquiterpene	β-himachalene	1502				X		
sesquiterpene	β-bisabolene	1508				X		X
–	unknown 37	1525				X		
sesquiterpene	unknown 38[d]	1525						X
sesquiterpene	unknown 39	1533				X		
sesquiterpene	unknown 40	1544				X		
oxygenated ST	unknown 41	1549						X
–	unknown 42	1553					X	
sesquiterpene	unknown 43	1564						X
–	unknown 44	1584						X
oxygenated ST	unknown 45	1609	X	X				
–	unknown 46	1629						X
–	unknown 47	1650						X
–	unknown 48	1656						X
–	unknown 49	1702						X

[a]Volatiles were verified with authentic standards, or identified by comparing their mass spectra with reference spectra from databases (Wiley, NIST). Kovats' retention indices (RI) were calculated and compared to databases. Volatile organic compounds collected as background from fungal-free PDA plates were removed from the final dataset. Volatiles released from both the endophytic fungi and black poplar, as listed in previous reports [57,58], are depicted in bold. [b]Verified with authentic standards, otherwise verified with calculated Kovat's indices compared with Pubchem [60] or [c]NIST [61] library. [d]Kovat's indices and mass spectra suggest strongly resemblance to β-or γ-cadinene.

assembly as template revealed two genes with high similarity to other fungal *TPS* genes. The genes were designated *CxTPS1* and *CxTPS2*. For functional characterization, the complete open reading frames of *CxTPS1* and *CxTPS2* were amplified from cDNA, cloned, and heterologously expressed in *Escherichia coli*. To determine mono-, sesqui-, and diterpene-forming activity, the bacterial raw protein extracts were assayed with the substrates GPP, FPP, and GGPP, each in the presence of the co-substrate magnesium chloride.

Both protein extracts containing the respective enzymes accepted the substrate GPP and produced monoterpenes (Figure 2). CxTPS1 produced myrcene (**9**) and (*E*)-β-ocimene (**10**) in similar amounts. CxTPS2 produced (*E*)-β-ocimene (**10**) as the major product and minor amounts of myrcene (**9**), (*Z*)-β-ocimene (**11**), and linalool (**5**) (Figure 2). Only one sesquiterpene product was formed by each TPS: CxTPS1 produced (*E*,*E*)-α-farnesene (**12**) and CxTPS2 produced (*E*)-β-caryophyllene (**1**). With GGPP, no enzyme activity was recorded for

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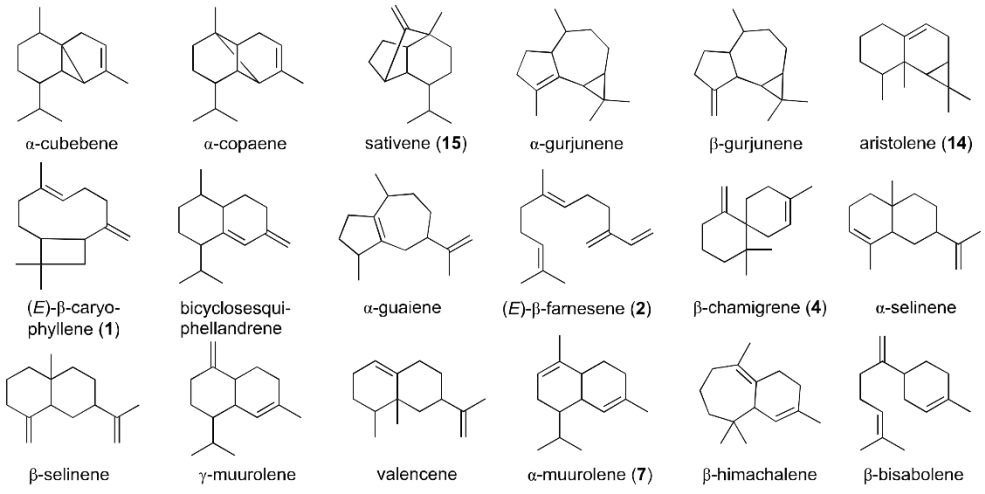

Figure 1: Chemical structures of sesquiterpenes emitted from endophytic fungi (Table 2) isolated from black poplar leaves.

CxTPS2, while CxTPS1 converted this substrate to (*E,E*)-β-springene (**13**) as the minor compound and major amounts of (*E,E,E*)-α-springene (**14**) (Figure 2).

Two terpene synthases from *Cladosporium* sp. are not closely related to each other

To investigate the phylogenetic relationships of CxTPS1 and CxTPS2 from *Cladosporium* sp. to other known terpene synthases from plant-associated Ascomycota that exhibit a pathogenic, endophytic or saprophytic lifestyle, we performed multiple sequence alignments and a subsequent dendrogram analysis.

According to the tree shown in Figure 3, CxTPS2 and CxTPS1 are not closely related to each other. While CxTPS2 forms a clade with sesquiterpene synthases of four pathogenic fungi and one endophyte, CxTPS1 is loosely related to a gene of the pathogenic fungus *Botrytis cinerea*. Further, CxTPS2, which produces (*E*)-β-caryophyllene (**1**), is more closely related to other sesquiterpene synthases from pathogens than to the caryophyllene synthases from the two endophytes *Hypoxylon* sp. CI4A and *Hypoxylon* sp. CO27.

Discussion

We were able to identify 12 different endophytic fungi from leaves of mature black poplar trees with a culture-dependent method and analyzed their volatile blends when growing on potato dextrose agar. Most of the tested fungi produced various aliphatic or aromatic alcohols, which are commonly produced by endophytic fungi and are known to act as antimicrobial agents (Table 2) [63]. Sesquiterpenes make up the largest proportion of fungus-produced terpenoids [64] and in our study we also detected several sesquiterpenes, e.g., (*E*)-β-caryophyllene (**1**), β-chamigrene (**4**), aristolene (**15**), sativene (**16**), and α-muurolene (**8**). However, monoterpenes were completely absent from the volatile bouquets of the endophytic species in our study. Weikl et al. who compared the volatiles released from *Alternaria alternata* and *Fusarium oxysporum* also did not detect any monoterpenes [41]. However, other studies on *Phomopsis* sp., *Cladosporium cladosporioides*, and *Hypoxylon anthochroum* showed that these endophytic fungi are able to produce monoterpenes like sabinene, α-pinene and 1,8-cineole, respectively [51,65,66]. In general, fungal volatile profiles are very species-specific [67], which also holds true for the species tested in our study (Table 2). However, the differences in the literature may arise from the use of different strains, volatile collection methods or variation in age, growth medium and environmental conditions, such as moisture, pH, temperature, and nutrient levels, or co-cultivation [27,41,67,68]. In our study, we measured the volatile profiles of endophytes cultivated on PDA medium at 28 °C in the dark. These profiles may differ from those released by endophytes growing under natural conditions in poplar leaves, in the possible presence of competing microbes.

While our knowledge about the volatile profiles of endophytic fungi has increased in recent years, only little is known about endophyte terpene synthases that may catalyze volatile terpene formation [44,45]. For the endophytic fungus *Cladosporium* sp., we identified and characterized two TPS, CxTPS1 and CxTPS2

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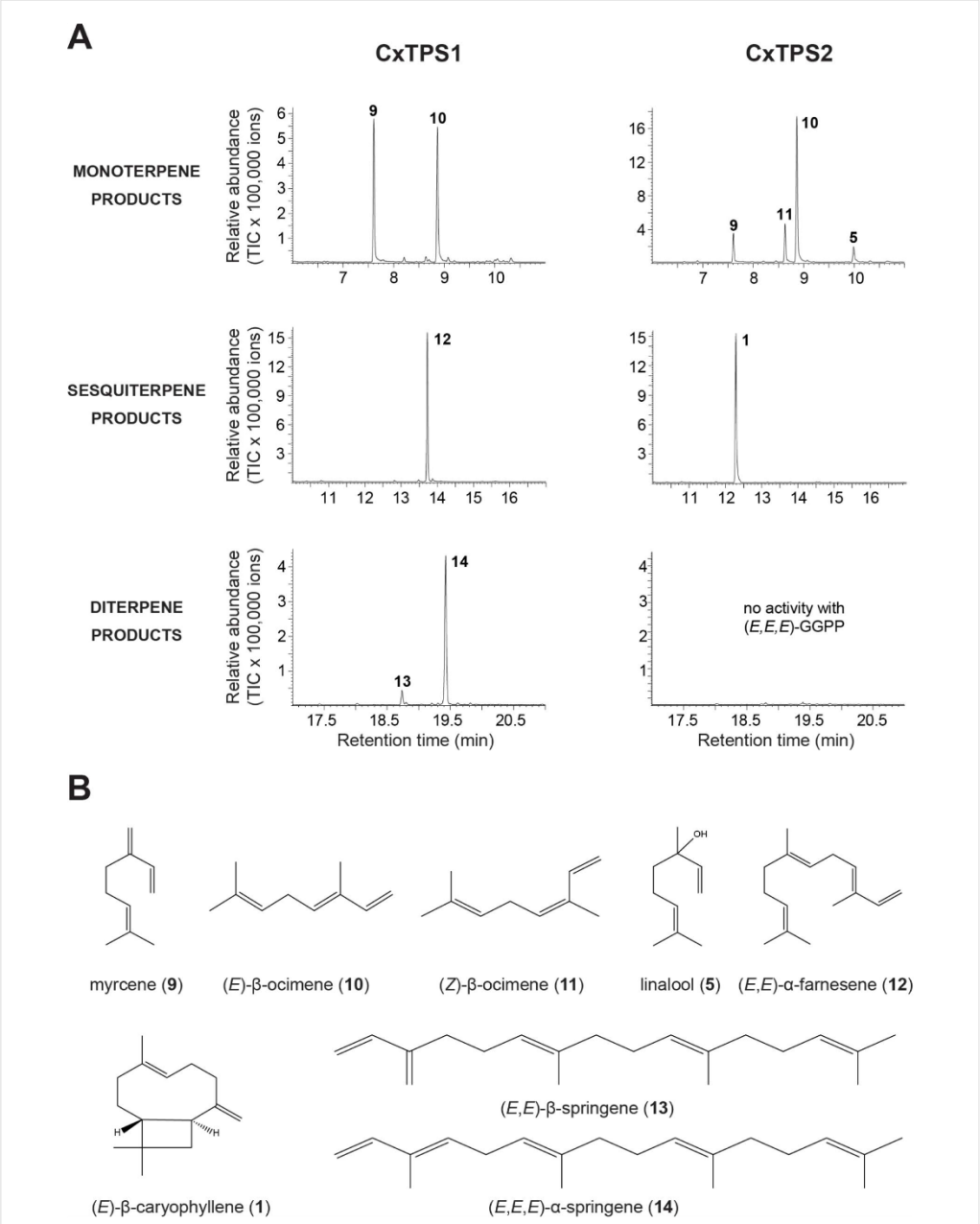

Figure 2: Terpene synthase activity of CxTPS1 and CxTPS2. A) Genes were heterologously expressed in *Escherichia coli* and partially purified proteins were assayed with GPP, (*E*,*E*)-FPP, or (*E*,*E*,*E*)-GGPP as substrates in the presence of 10 mM $MgCl_2$. Enzyme products were extracted from the assays with hexane and analyzed using gas chromatography–mass spectrometry. Myrcene (**9**); (*E*)-β-ocimene (**10**); (*Z*)-β-ocimene (**11**); linalool (**5**); (*E*,*E*)-α-farnesene (**12**); (*E*)-β-caryophyllene (**1**); (*E*,*E*)-β-springene (**13**); (*E*,*E*,*E*)-α-springene (**14**). B) Structures of the enzyme products of CxTPS1 and CxTPS2, including (*E*)-β-caryophyllene (**1**) which was the only terpene detected from *Cladosporium* sp. cultures.

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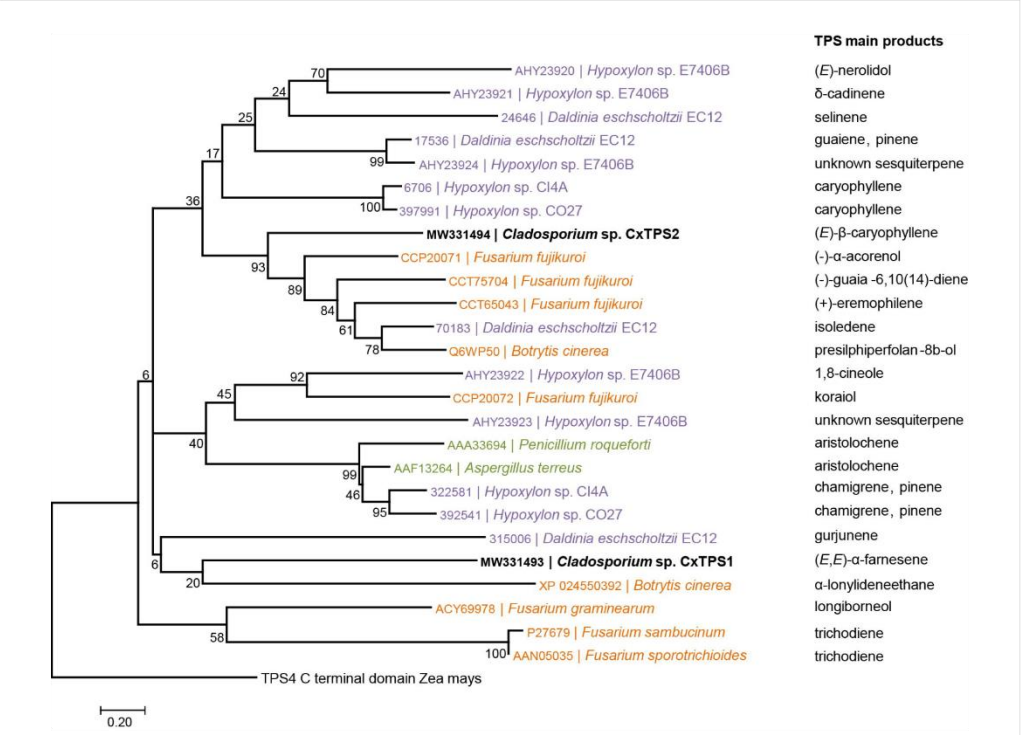

Figure 3: Dendrogram analysis (rooted tree) of CxTPS1 and CxTPS2 (bold) from *Cladosporium* sp. and characterized TPS proteins and their main products from other plant-associated Ascomycota. The tree was inferred using the Maximum Likelihood method based on the Poisson correction model and $n = 1000$ replicates for bootstrapping. Bootstrap values are shown next to each node. The tree is drawn to scale, with branch lengths measured in the number of amino acid substitutions per site. The alpha-domain of maize TPS4 [62] was chosen as an outgroup. TPS proteins from different Ascomycota are highlighted according to their different lifestyle: endophytic (purple), pathogenic (orange) and saprophytic (green).

(Figure 2). CxTPS1 was a multifunctional enzyme in vitro and produced the monoterpenes myrcene (**9**) and (*E*)-β-ocimene (**10**) from GPP, the sesquiterpene (*E*,*E*)-α-farnesene (**12**) from FPP, and the diterpenes (*E*,*E*)-β-springene (**13**) and (*E*,*E*,*E*)-α-springene (**14**) from GGPP. CxTPS2, in contrast, showed a narrower substrate specificity and converted GPP to myrcene (**9**), (*E*)-β-ocimene (**10**), (*Z*)-β-ocimene (**11**), and linalool (**5**) and FPP to (*E*)-β-caryophyllene (**1**). In a previous work on fungal terpene synthases, Hohn and Vanmiddlesworth found a narrow substrate specificity for the trichodiene synthase from *Fusarium sporotrichioides*, where only the sesquiterpene trichodiene was detected with FPP, while other substrates were not accepted [69]. In contrast, bi-functionality was also observed for the pinene and guaiene synthase from *Daldinia eschscholzii* EC12 and the pinene and guaiene synthase from *Hypoxylon* sp. EC28 (Figure 3) [45]. The multifunctionality of CxTPS1 and CxTPS2 was only observed when the fungal TPS was expressed heterologously in *E. coli* and assayed in vitro whereas the fungus itself only emitted (*E*)-β-caryophyllene (**1**) when growing on agar medium. Thus, we speculate that GPP, the substrate for monoterpene production, is not available in *Cladosporium* sp. In contrast, the emission of the monoterpene α-pinene has been reported for *Cladosporium cladosporioides* CL-1 [66]. Interestingly, we could not detect the emission of (*E*,*E*)-α-farnesene (**12**), a product of the in vitro assay of CxTPS1 in our fungal cultures, although the fungus must have the ability to produce the substrate FPP in sufficient quantity as it also produces the sesquiterpene (*E*)-β-caryophyllene (**1**). It might be that *CxTPS1* is not expressed in the fungus under our culture conditions or that (*E*,*E*)-α-farnesene (12) is further metabolized. To our knowledge, (*E*,*E*)-α-farnesene (**12**) has never been detected so far from any Cladosporium species.

To test whether there is a relationship between fungal lifestyle and their terpene synthases, we compared sequences of the terpene synthases CxTPS1 and CxTPS2 from *Cladosporium* sp.

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with the sequences of other known terpene synthases from plant-associated Ascomycota exhibiting a pathogenic, endophytic or saprophytic lifestyle. One clade was indeed evident that contained only terpene synthases from endophytes. However, a close relationship between fungal lifestyle and their terpene synthase sequences is not observable, since different terpene synthases from the same fungal species clustered together with terpene synthases from pathogens and/or endophytes (Figure 3). CxTPS2 forms a clade with sesquiterpene synthases from four pathogenic fungi and one endophyte, while CxTPS1 is loosely related to sequences of the pathogen *Botrytis cinerea* (Figure 3). We speculate that TPS from fungi that share the same lifestyle are not clustered together because some endophytes switch from being asymptomatic leaf inhabiting fungi to becoming either latent pathogens or saprophytes [21,24,70-73]. Furthermore, it is hypothesized that endophytes may have evolved directly from pathogens, since both must defeat plant protective barriers [38,74]. Nevertheless, the bootstrap values in the dendrogram are generally too low to make a clear statement about the relationship between terpene synthases and fungal lifestyle, and more work on this question is needed [63,75].

The volatiles found to be emitted from black poplar endophytic fungi in this study could have important biological activities. For instance, ethanol (**17**) and 2-phenylethanol (**3**) are known to have antifungal and phytotoxic activity and so could help the endophyte to defend its niche within the plant against other endophytic competitors [63]. The other endophyte VOCs could promote plant growth (e.g., 2-methyl-1-propanol (**7**) [76], (*E*)-β-caryophyllene (**1**) [77], and sativene (**16**) [66]), induce plant immunity (e.g., (*E*)-β-caryophyllene (**1**) [77]), and increase photosynthetic capacity (e.g., 2-methyl-1-propanol (**7**) [78]) (Table 2) [63]. Some of the analyzed compounds are also known to play a crucial role in plant–insect interactions, where they are involved in direct and indirect plant defenses or in attracting herbivorous insects. For example, (*E*)-β-caryophyllene (**1**) emitted by *Cladosporium* sp. (Table 2) is known to act as a signal cue for the planthopper *Sogatella furcifera* [15], while this compound also attracts nematodes that feed on attacking insect herbivores [79]. Nearly all of the endophytic fungi isolated in this study were able to produce at least some volatiles known from the literature to mediate plant–insect interactions.

Of the 13 endophytes studied, 11 of them release volatiles previously reported from black poplar foliage (Table 2) [57-59]. These compounds include the alcohols 3-methyl-1-butanol (**6**) and 2-phenylethanol (**3**) and the sesquiterpenes (*E*)-β-caryophyllene (**1**), and α-muurolene (**8**) (Table 2). This raises the question of whether endophytic fungi contribute to the overall plant volatile bouquet by producing the above-mentioned volatiles. If so, this would directly affect our interpretation of certain plant-fungus and plant–insect interactions [34,37,38]. Recently, it has been shown that the pathogenic rust fungus (*Melampsora larici-populina*) alters the volatile blend of black poplar trees by contributing 1-octen-3-ol and 3-octanone, which attract caterpillars of the generalist herbivore *Lymantria dispar* [57]. Jallow et al. showed that an endophytic fungus (*Acremonium strictum*) alters the volatile composition of the tomato plant *Solanum lycopersicum* and attracts *Helicoverpa armigera* moth for oviposition [80]. The endophytic fungus *Beauveria bassiana* also increased the emission of some terpenes from tomato plants resulting in a stronger defense response against the beet armyworm (*Spodoptera exigua*) [81]. In these cases, it is not known whether the increased terpene emission results from biosynthesis by the plant or the fungus. Future work should include measurements of plant and fungal TPS expression to determine the origin of these compounds. For this, identification of TPS genes in both plants and their fungal partners is crucial.

Conclusion

We showed that endophytic fungi isolated from mature black poplar trees emitted species-specific volatile blends. Almost all the endophytes here produced short-chain aliphatic alcohols that are known to have antifungal and phytotoxic effects and may be produced to compete with other microbial species. Several also produce diverse mixtures of sesquiterpenes. Interestingly, several VOCs emitted from the endophytes were earlier reported to be emitted by black poplar. We characterized two terpene synthases from one of the endophytic fungi to lay the groundwork for comparing the biosynthesis of plant vs fungal volatiles. More knowledge about the formation of these compounds could contribute to the greater understanding of their roles in plant–insect, plant–plant and plant–microbe interactions.

Experimental

Endophyte isolation from plant material

Endophytes were isolated from leaves of mature black poplar (*Populus nigra*) trees growing in a natural population in a floodplain forest in northeastern Germany (52°34'1''N, 14°38'3''E). The trees were around 25 m in height and approximately 70 years old. Five branches in the lower canopy (1–7 m) from each of the 10 tree genotypes were collected and from each branch, five leaves were randomly harvested. Those leaves did not show any symptoms of pathogen infection. A culture-dependent method was used to isolate endophytic fungi growing within the leaf blades. Under a clean bench, the leaves were surface sterilized (0.5% NaOCl for 2 min, followed by 70% of EtOH for 2 min) and rinsed three times by immersion in sterile distilled water. Then, four pieces (approximately 7 × 7 mm) of

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one leaf blade were placed equidistantly on potato dextrose agar (PDA; Sigma-Aldrich). Water from the last washing step was coated on PDA medium to test whether the surface of the leaves had been adequately sterilized. Petri dishes were sealed with Parafilm and incubated in the dark at 25 °C. Plates were inspected daily and morphologically distinct colonies were brought into pure culture on PDA medium using the same culturing conditions as above. Fresh mycelium was harvested from pure cultures for molecular identification of the morphospecies.

Molecular identification of endophytic fungi

DNA was extracted from fresh mycelium (approximately 5 cm in diameter) growing on PDA. The mycelium was flash frozen in liquid nitrogen and ground using plastic pestles in 1.5 mL Eppendorf tubes. After homogenization of the mycelium, 500 µL extraction buffer (100 mM Tris HCl, pH 8; 10 mM EDTA, pH 8; 2% w/v SDS) and 100 µL proteinase K (Sigma) were added and the mixture was incubated for 1 h at 60 °C. For separation of polysaccharides, 180 µL 5 M NaCl and 80 µL 10% CTAB were added and the mixture incubated further for 10 min at 65 °C.

To extract nucleic acids by phase separation, 860 µL chloroform/isoamyl alcohol (24:1) was added and incubated on ice for 30 min. The samples were centrifuged for 10 min (15,000 rpm), and the upper, aqueous phase was then transferred to a new tube and DNA was precipitated in 395 µL of 100% isopropanol (−20 °C). After centrifugation (4 °C, 20 min, 15,000 rpm) the pellet was washed with 750 µL 70% ethanol, centrifuged at 15,000 rpm (10 min), dried, and finally dissolved in 50 µL Milli-Q water (pH 6). DNA concentration and purity were determined with a NanoDrop 2000c spectrophotometer (Peqlab Biotechnology AG, Erlangen, Germany).

The primer pair ITS1F and ITS4 (Supporting Information File 1, Table S2) was used to amplify the highly conserved internal transcribed spacer region of the fungal rRNA cistron [82,83]. The reaction mix for DNA amplification (50 µL/tube) contained 2.5 µL of each primer (Sigma), 0.5 µL GoTaqX® Polymerase (Promega, Madison, WI, USA), 10 µL of GoTaqX® Reaction Buffer (Promega) and 1 µL 10 mM dNTPs (Thermo Fisher Scientific). The template volume was adjusted to a final DNA concentration of approximately 500 ng/mL. Ultrapure water (Milli-Q® Synthesis A10) was added up to a final volume of 50 µL. PCR was performed in a gradient thermal cycler (Whatman Biometra 96T) using the following program: initiation and activation of polymerase (95 °C/5 min); followed by 35 cycles of denaturation (95 °C/30 s), annealing (65 °C/30 s) and elongation (72 °C/90 s) and a single, final elongation step (72 °C/10 min).

For gel electrophoresis, 4 µL PCR product was mixed with one drop loading dye (0.3 mL 30% glycerol and 2.5 mg bromphenol blue/mL) and applied to an 1% agarose gel (1 g agarose/100 mL 0.5% TBE; 5 µL Midori Green). A 1 kb DNA ladder (Gene Ruler, Thermo Fisher Scientific) was applied to determine the fragment size of the products. Electrophoresis was performed in 0.5% TBE buffer (Thermo Fisher Scientific) for 30 min at 135 V (150 mA). The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Purified PCR products were sequenced using the Sanger method on a ABI Prism® Gen-Analysator 3130xl (Applied Biosystems, Weiterstadt, Germany). The obtained sequences were aligned using Geneious 6.0.5 [84] and compared to the NCBI sequence database [85] (Supporting Information File 1, Table S1). In case of isolates with multiple 99–100 % identity hits on several species within the same genus, we identified these only to the genus level, but still list the single best hit and its accession number (Table 1, Supporting Information File 1, Table S1).

Static headspace volatile collection from cultures and analysis

VOCs were collected from endophytes that had grown on PDA medium (25 mL) in an incubator (dark/28 °C) until the mycelium reached a diameter of 5 cm (± 0.5 cm). For each fungal species, seven replicates were used with fungus-free petri dishes with PDA medium used as blanks. Volatiles were trapped for 1 h by using four polydimethylsiloxane (PDMS) tubes. To prevent PDMS tubes from touching the mycelium, the tubes were placed with watchmaker forceps on loops of stainless steel wire that were kept at a distance of approximately 5 mm from the mycelium. PDMS tubes were prepared following the method described in Kallenbach et al. [86]. The experiment was performed under a clean bench at room temperature. After volatile collection PDMS tubes were immediately removed from the wire and stored in glass vials at −20 °C until further analysis.

Volatiles trapped on PDMS tubes were analyzed by GC–MS (GCMS-QP2010 Ultra, Shimadzu, Duisburg, Germany) coupled to a thermal desorption unit (TD-20, Shimadzu, Duisburg, Germany). A single PDMS tube from each replicate was placed in a glass tube (Supelco; Sigma-Aldrich). Desorption was achieved by a He flow (60 mL min^{-1}) at 200 °C for 8 min in the glass tube and the analytes were trapped on a Tenax® (Buchem BV, Apeldoorn, Netherlands) adsorbent trap at −17 °C. The trap was then heated to 230 °C, and the analytes injected onto the GC column (Rtx®-5MScolumn with 30 m × 0.25 mm × 0.25 µm (Restek GmbH, Bad Homburg, Germany)). The gas chromatograph was operated at a column flow rate of 1.5 mL/min (He), split injection (split ratio: 5). The oven was

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set to 45 °C, held for 3 min, increased to 250 °C with a gradient of 6 °C/min and subsequently increased to 300 °C at 100 °C/ min with a 3 min hold. Electron impact (EI) mass spectra were recorded at 70 eV in scan mode from 43 to 350 *m/z* at a scan speed of 1111 Da/s (interface temperature, 250 °C; source temperature, 230 °C). Fungal volatiles were identified by comparing their mass spectra with those of authentic standards or reference spectra from databases (Wiley, Version 8, National Institute of Standards and Technology (NIST, Version 11)) using GCMS SOLUTION v.4.20 (Shimadzu). In addition, non-isothermal Kovats retention indices were calculated, based on chromatographic retention times of a saturated alkane mixture (C_7–C_{40}; Sigma-Aldrich, Taufkirchen, Germany) [87]. The calculated Kovats retention indices were compared with indices published in Pubchem [60] or NIST [61] from the same or a similar type of GC column. Differences between calculated retention index and literature data were within ±5 points. Identified volatiles with a similarity hit above 90% and that were present in five out of seven replicates were included in this study, whereas VOCs which were also collected by blanks were removed from the final dataset. A representative total ion chromatogram for each fungus is shown in Supporting Information File 1, Figure S1. Mass spectra of unknown compounds are shown in Supporting Information File 1, Figure S2.

Fungal RNA extraction, reverse transcription, and sequencing

Total RNA was isolated from fresh mycelium (approximately 5 cm in diameter) growing on PDA using the RNeasy® Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA concentration was assessed using a spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific). RNA was treated with DNase I (Thermo Fisher Scientific) prior to cDNA synthesis. Single-stranded cDNA was prepared from 1 µg of DNase-treated RNA using SuperScriptTM III reverse transcriptase and oligo (dT12-18) primers (Invitrogen, Carlsbad, CA, USA).

For transcriptome sequencing, total RNA was extracted from fungal material as described above, a TruSeq RNA-compatible library was prepared, and PolyA enrichment was performed before sequencing on an IlluminaHiSeq 3000 sequencer (Max Planck Genome Centre, Cologne, Germany) with 25 Mio reads, 150 base pairs, paired end. Trimming of the obtained Illumina reads and de novo assembly were both performed with the program CLC Genomics Workbench (Qiagen Bioinformatics) using default parameters or parameters specified as follows: bubble size, 100; automatic word size; minimum contig length, 600. A BUSCO analysis (Supporting Information File 1, Figure S3) was performed to validate the completeness of the transcriptome.

Identification and heterologous expression of terpene synthase genes

To identify putative terpene synthases, a TBLASTN analysis with *Aspergillus terreus* aristolochene synthase (pdb 20A6) as query and the de novo transcriptome of *Cladosporium* sp. as a template was performed using the software BioEdit 7.0.9.0 [88]. Two terpene synthase-like sequences were found and designated as *CxTPS1* and *CxTPS2,* respectively. The complete open reading frames of *CxTPS1* and *CxTPS2* were amplified from cDNA using the primers shown in Supporting Information File 1 (Table S2) and cloned into pET100/D-TOPO vector (Thermo Fisher Scientific). The *E. coli* strain BL21 Star™ (DE3) (Thermo Fisher Scientific) was used for heterologous expression. The culture was grown at 37 °C, induced at an OD_{600} = 0.6 with 1 mM IPTG, and subsequently placed at 18 °C and grown for another 20 hours. The cells were collected by centrifugation and disrupted by a 4 × 20 s treatment with a sonicator (Bandelin UW2070, Berlin, Germany) in chilled extraction buffer (10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 10% (v/v) glycerol). Cell fragments were removed by centrifugation at 14,000*g* and the supernatant was further processed via an Illustra NAP-5 gravity flow desalting column (GE Healthcare, Chicago, IL, USA) and eluted in extraction buffer.

Enzyme assays were performed in a Teflon-sealed, screw-capped 1 mL GC glass vial containing 50 µL of the heterologously expressed protein and 50 µL assay buffer containing 50 µM substrate (GPP, (*E,E*)-FPP, or (*E,E,E*)-GGPP) and 20 mM $MgCl_2$. Assays were overlaid with 100 µL hexane and incubated for 60 minutes at 30 °C. One microliter of the hexane phase was injected into the GC–MS machine and the analysis was conducted using an Agilent 6890 Series gas chromatograph coupled to an Agilent 5973 quadrupole mass selective detector (interface temp, 270 °C; quadrupole temp, 150 °C; source temp, 230 °C; electron energy, 70 eV). Chromatographic separation was achieved with an initial oven temperature of 45 °C held for 2 min, which was then increased to 180 °C with a gradient of 6 °C min^{-1}, and then further increased to 300 °C with a gradient of 60 °C min^{-1} and a hold of 2 min. Compounds were identified by comparing their retention times and mass spectra to those of authentic standards, or by reference spectra in the Wiley and NIST libraries.

Sequence analysis and phylogenetic tree construction

For the estimation of a phylogenetic tree, we used the MUSCLE algorithm (gap open, −2.9; gap extend, 0; hydrophobicity multiplier, 1.2; max. iterations, 8; clustering method, upgmb) implemented in MEGA7 [89] to compute an amino acid alignment. Based on the MUSCLE alignment, the tree was constructed with MEGA7 using a Maximum Likelihood

algorithm (Poisson model). All positions with less than 80% site coverage were eliminated. A bootstrap resampling analysis with 1000 replicates was performed to evaluate the tree topology. For the phylogenetic tree, we included identified and characterized terpene synthases from plant-associated Ascomycota.

Accession numbers

Sequence data for *CxTPS1* (MW331493) and *CxTPS2* (MW331494) can be found in the NCBI GenBank [85] under the corresponding identifiers. Raw reads of the RNAseq experiment were deposited in the NCBI Sequence Read Archive under the BioProject accession PRJNA682522.

Supporting Information

Supporting Information File 1

Sequences of isolated endophytic fungi and identification according to NCBI database, primer used in this study, representative total ion chromatograms of single endophytic volatile blend, mass spectra of unknown volatile organic compounds, and BUSCO analysis of *Cladosporium* sp. de novo assembly.
[https://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-17-118-S1.pdf]

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ORCID® iDs

Peter H. W. Biedermann - https://orcid.org/0000-0003-4234-5659
Jonathan Gershenzon - https://orcid.org/0000-0002-1812-1551
Tobias G. Köllner - https://orcid.org/0000-0002-7037-904X
Sybille B. Unsicker - https://orcid.org/0000-0002-9738-0075

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5. MANUSCRIPT III

Angaben zum Eigenanteil

(gemäß der Durchführungsbestimmung zu § 8 Abs. 2 der Promotionsordnung vom 23.09.2019 der Fakultät für Biowissenschaften der FSU Jena, Anlage 1, Formular 1)

Manuskript Nr. 3

Titel des Manuskriptes: A fungal endophyte modifies leaf phytochemistry and shapes insect communities in poplar

Autoren: Christin Walther, Beate Rothe, Michael Reichelt, Pamela Medina van Berkum, Jonathan Gershenzon and Sybille B. Unsicker

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Der Kandidat / Die Kandidatin ist (bitte ankreuzen)

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Anteile (in %) der Autoren / der Autorinnen an der Publikation

Autor/-in	Konzeptionell	Datenanalyse	Experimentell	Verfassen des Manuskriptes	Bereitstellung von Material
Christin Walther	50	80	80	80	0
Beate Rothe	0	0	10	0	0
Michael Reichelt	0	10	5	5	0
Pamela Medina van Berkum	0	10	0	5	0
Jonathan Gershenzon	5	0	0	5	100
Sybille B. Unsicker	45	0	5	5	0

A fungal endophyte modifies leaf phytochemistry and shapes insect communities in poplar

Christin Walther[1], Beate Rothe[1], Michael Reichelt[1], Pamela Medina van Berkum[1], Jonathan Gershenzon[1] and Sybille B. Unsicker[1*]

Address: [1]Department of Biochemistry, Max Planck Institute for Chemical Ecology, Hans-Knöll Str. 8, 07745 Jena, Germany

* Corresponding author: Phone: +49(0)3641 571328; sunsicker@ice.mpg.de

Summary

Plants are exposed to a plethora of herbivores and microorganisms. Endophytic fungi isolated from grasses are a prominent example of a mutualistic plant-microbe interaction, however, the role of endophytic fungi in tree-insect interactions is hardly explored today. In this study, we investigated the influence of the endophytic fungus *Cladosporium* sp. on the constitutive and induced defense response of black poplar (*Populus nigra*) trees. Furthermore, we tested the consequences of fungus-mediated changes in poplar defense compounds for insect herbivore feeding preference in lab experiments and for insect community assembly on phytometer trees in the field. In an *in planta* choice assay, larvae of the generalist gypsy moth (*Lymantria dispar*) preferred uninoculated control leaves over endophyte-inoculated leaf tissue. Exposure of *P. nigra* to the endophyte *Cladosporium* sp. increased constitutive defenses as well as induced defense compounds. Additionally, we were able to identify and quantify the endophyte produced alkaloid stachydrine in leaves of inoculated plants, with this compound being shown to be attractive to the specialist beetle *Chrysomela tremulae*, while being a deterrent to the generalist caterpillar *Amata mogadorensis*. Further, we could demonstrate, that the endophyte shapes the insect community in a field experiment, by attracting species of the order Hemiptera and deterring coleopteran and hymenopteran species. The findings show that the role of endophytes as defense mutualists in woody plants is species dependent.

Keywords

Alkaloids, *Cladosporium*, endophyte, insect community, *Populus nigra* (black poplar), salicinoids, stachydrine, tripartite interaction

Introduction

Plants are in close association with a plethora of microbial species, colonizing every organ of a plant from the surface as well as inside of plant tissues. Fungal and bacterial microbes, which live within the plant tissues (e.g., leaves, flowers, roots) and undergo, for at least a part of their life cycle, a quiescent stage are called endophytes (Petrini 1991). With endophyte colonization being widespread in the plant kingdom, its influence on plant-insect interactions is highly discussed (Hyde & Soytong 2008). Fungal grass endophytes (clavicipitaceous endophytes) are a prominent example of mutualistic plant-microbe interactions (Hartley & Gange 2009, Saikkonen *et al.* 2010), while contrastingly the ecological significance of nonclavicipitaceous endophytes, which occur also in trees, seem to be more equivocal (Eberl *et al.* 2019, Meister *et al.* 2006, Rodriguez *et al.* 2009).

It has been shown, that endophytic metabolites are detectable in the plant matrix, which may impact plant-insect interactions. For instance, the rugulosin producing endophyte *Phialocephala scopiformis* isolated from white spruce (*Picea glauca*) is one example of a mutualistic relationship between an endophyte and a tree, where rugulosin negatively affects the growth of different herbivorous insects (Miller *et al.* 2002, Miller *et al.* 2008, Sumarah *et al.* 2008, Sumarah & Miller 2009, Sumarah *et al.* 2010). However, most of the literature concerning endophytic metabolites and how they influence plant-insect interactions is dominated by the alkaloid producing *Epichloë* and *Neotyphodium* species in fescue grasses (Clay 1988, Popay & Bonos 2005, Saikkonen *et al.* 2013). Alkaloids, being nitrogen-containing compounds typically derived from amino acids, are mainly found in herbaceous plants and fungi, and are known for their detrimental effects on herbivore performance (Dewick 2002, Kaur 2020). For example, the annual ryegrass *Lolium multiflorum* inoculated with the endophytic fungus *Epichloë occultans*, which is able to synthesize loline alkaloids, reduced the performance of the aphid *Rhopalosiphum padi* (Bastías *et al.* 2017b). Since alkaloids are widespread in fungi, woody plants harboring an alkaloid-producing endophyte may therefore use the alkaloid for their own benefit against herbivore insects as a common pattern in forest ecosystems.

It has been shown, that endophytes can manipulate plant defense against pathogens (Hartley *et al.* 2015, Mejía *et al.* 2014, Soliman *et al.* 2013). Qin *et al*. (2019) found increased total phenolic content and phenylalanine ammonia lyase activity in *Achnatherum sibiricum* when the plant was inoculated with an *Epichloë* endophyte and further treated with methyl jasmonate simultaneously, which led to an enhanced resistance to the locust *Locusta migratoria.* In another experiment, inoculation of *Theobroma cacao* with the endophyte *Colletotrichum tropicale* lead to changes in host specialized

chemistry (Christian *et al.* 2020). However, it remains unknown, which specialized compounds were regulated in response to the endophyte colonization and how this would affect herbivore insect behavior. More research is needed in order to observe endophytic mediated changes in phytochemistry, especially in response to herbivory.

Studies investigating endophytic mediated changes in the phytochemistry are especially rare in trees, even though trees host a plethora of different insect herbivores. It has been shown that trees benefit from endophytic infection by reduced insect herbivory (Albrectsen *et al.* 2010, González-Teuber 2016), and pathogen damage in the field, as shown for some endophyte species in *Populus trichocarpa* reducing poplar-rust infection (Busby *et al.* 2016). However, we know little about the role of endophytic fungi for poplar-insect interaction and their possible influence to shape a whole insect community. With specific reference to the field, plants are exposed to a wide range of different insects, where a single endophyte may negatively affect one herbivore insect and positively affect another (Eberl *et al.* 2019, Gange *et al.* 2012). Therefore, field studies with monitored arthropod communities, would help to understand the complex plant-herbivore-endophyte interaction.

As primary producers, plants developed numerous defense strategies and compounds against herbivorous insects with toxic, repellent, or anti-nutritive properties (Jander & Howe 2008), which can be produced constitutively or induced in response to wounding or herbivore attack (Wu & Baldwin 2010). Numerous plant species produce phenolic compounds as efficient defenses against herbivores and pathogens (Boeckler *et al.* 2011, Boeckler *et al.* 2013). In poplar trees condensed tannins and salicinoids are the major groups of phenolic compounds, and can make up to 30% of the leaf dry weight, with these salicinoids being shown to negatively affect the performance of generalist insect herbivores (Boeckler *et al.* 2016, Hemming & Lindroth 1995, Lindroth 1991). Furthermore, salicinoids can be induced upon herbivory, but literature reports regarding induction are inconsistent (Boeckler *et al.* 2013, Fields & Orians 2006, Osier & Lindroth 2001, Ruuhola *et al.* 2001, Stevens & Lindroth 2005, Young *et al.* 2010). In smaller amounts, Salicaceae also contain other flavan-3-ols (e.g., catechin, proanthocyanidin B1) and phenolic acids (e.g., caffeic acid, coumaric acid) (Boeckler *et al.* 2013). While phenolic acids are mainly known as herbivore defenses (Lindroth & Peterson 1988), catechin and proanthocyanidin B1 act as defenses against fungal pathogens (Ullah *et al.* 2019) rather than against insect herbivores (Boeckler *et al.* 2013).

The aim of the study was to test how a single endophytic fungus might manipulate constitutive and induced defense compounds of young black poplar trees (*Populus nigra*) under controlled conditions and how this might influence the feeding behavior of herbivore insects. Therefore, we inoculated black

poplar trees with the endophyte *Cladosporium* sp., and compared changes in constitutive and induced defense compounds upon herbivory. Via LC-MS analysis we searched for compounds in black poplar leaves, originating from the endophyte and tested their potential as possible herbivore feeding attractants or deterrents. To reveal the ecological significance of the endophyte in a more natural scenario, we performed a field experiment with inoculated trees and observed the visiting arthropod community.

Materials and Methods

Plants and insects

Young black poplar trees (*Populus nigra* L.) were reared under sterile conditions in a growth chamber. Briefly, 10 cm cuttings from the apex of saplings (50 cm tall) grown in a growth chamber were surface sterilized by immersing the cuttings in 100 mL water containing 20 mL 2% NaOCl and 1.2 mL Tween 20 for 3 min. The cuttings were rinsed five times with sterile distilled water. The ends of the cuttings were removed and 1 cm long cuttings were cut and placed in a petri dish with growth medium (McGowan's Woody Plant media WPM, Duchefa). After root development, cuttings were placed in Magenta™ boxes (Sigma-Aldrich) filled with the same medium as described before and placed in a growth chamber (day/night: 21/19 °C, photoperiod 16 h). After 8 weeks, the small plants were transferred into 1 L pots filled with soil (1:1 sand and soil; Klasmann potting substrate; Klasman-Deilmann, Geeste, Germany) and were further grown in a climate chamber (day/night: 21/19 °C, photoperiod 16 h). After another 4 weeks, the plants were again transferred to another climate chamber (day/night: 20/18 °C, photoperiod 16 h, humidity 60%). Two weeks later, 10 leaves per plant (with a height of at least 50 cm) were inoculated with endophyte fungal spore solution or with fungus free control solution as described below.

Gypsy moth (*Lymantria dispar*) caterpillars were hatched from eggs (kindly provided by Hannah Nadel, US Department of Agriculture, Buzzards Bay, MA, USA) and reared on artificial diet (Frontier Scientific Services Agriculture, Newark, USA) in a climate chamber (60% humidity, 20 °C, photoperiod 14 h) until the start of the experiment. Eggs from *Amata mogadorensis* were obtained from a private breeder (https://www.entomologenportal.de) and reared on black poplar saplings under laboratory conditions. Beetles and egg clutches from *Chrysomela tremulae* were collected from old-growth black poplar trees and reared on black poplar saplings under laboratory conditions.

Endophytic Fungus

Spores were collected from 4 weeks old *Cladosporium* sp. cultures grown on potato dextrose agar (PDA) in the dark at 25 °C. *Cladosporium* sp. was previously isolated from mature black poplar trees growing in a natural population in a floodplain forest in northeastern Germany (52°34'13.6''N, 14°37'57.1''E) as described in Walther *et al.* (2021). Spores from fungal cultures were scratched off in approximately 12 mL of solution containing 0.01% Triton X-100 (Sigma-Aldrich), 0.9% (w/v) NaCl (Roth), 0.3% (w/v) glucose (Roth) and 0.2% (w/v) peptone (Roth). The spore solution was filtered through sterile glass wool to separate the spores from the mycelium, and the filtrate diluted to 10^7 spores/mL by using a hemocytometer. To determine the spore germination rate, the spore solution was diluted to approximately 100 spores/mL. From this solution 100 mL were plated on PDA medium and germinating spores were counted (Tab. S1).

Plant treatments and harvesting

To examine the impact of herbivory on black poplar infected with the endophytic fungus *Cladosporium* sp., plants were split into four treatments (fungus, fungus + herbivory, uninfected control, uninfected control + herbivory). For each treatment, 9-10 fully developed leaves of four plants were sprayed (approximately 1.5 mL per leaf) with either endophyte spore solution (as described above) or a spore-free control solution (0.01% Triton X-100, 0.9% (w/v) NaCl, 0.3% (w/v) glucose and 0.2% (w/v) peptone) on both sides of the leaf. To avoid contamination with other microorganisms and contamination of the control treatment, plants were wrapped in polyethylene terephthalate (PET) bags (Bratschlauch, Toppits, Minden, Germany), that were opened 2 days post infection (dpi). To explore the effect of herbivory on endophyte-treated and uninfected control plants, 15 gypsy moth caterpillars (4^{th} to 5^{th} instar) were released per plant on the inoculated leaves 15 dpi. After 48 h caterpillars were removed. On the following day, herbivore-treated leaves were weighed, photographed, flash-frozen in liquid nitrogen, and lyophilized. Leaf damage was analyzed with Adobe Photoshop® 2020 as described in Boeckler *et al.* (2013).

Preference assays

To elucidate whether the pyrrolidine alkaloid stachydrine might affect herbivore preferences, we conducted choice assays on leaf discs with larvae of *L. dispar* (3^{rd} instar, n = 20), *Amata mogadorensis* ($4\text{-}5^{th}$ instar, n = 20) and beetles of *Chrysomela tremulae* (n = 20). Leaves (8^{th}-15^{th} leaf from apex) from 10 trees were coated with either a control solution containing 0.01% Silwet (Silwet®Gold, UPL Deutschland GmbH, Brühl, Germany) or a stachydrine solution containing 0.01% Silwet, and

1.5 µg/mL stachydrine solution per 40 cm² leaf surface (Tab. S3B; respective to 45 nmol/g dw stachydrine). After leaves dried, 16 mm leaf discs were cut and offered in a modified Petri dish arena (Boeckler *et al.* 2014) to the insects. All insect species were reared on black poplar leaves prior to the experiment. *L. dispar* caterpillars were allowed to feed for 48 h, while beetles of *C. tremulae* fed for 24 h and *A. mogadorensis* for 3 h. The remaining leaf tissue was photographed and analyzed by using Adobe Photoshop® 2020. To evaluate the stachydrine concentration in the leaf material, leaves were flash frozen and analyzed (see below) (Tab. S3D).

To study the effect of endophytic infection on herbivore preference *in planta*, plants were inoculated with either endophyte spore solution (n = 4) or a control solution (n = 4) as described above. Two leaves, one from an endophytic plant 15 dpi and one from an uninfected control plant, were wrapped together in a cellophane bag and one *L. dispar* caterpillar (2nd instar) was added inside the cellophane bag. A total of 21 replicates were set up, and the caterpillars were allowed to feed for 48 h. Afterwards leaf damage was analyzed with Adobe Photoshop® 2020 as described in Boeckler *et al.* (2013).

Field experiment

To examine the impact of the endophytic fungus *Cladosporium* sp. on the insect community of black poplar trees, fungus-infected and uninfected control trees were transferred to a field site in a floodplain forest with a natural population of old-growth black poplar located in Northeastern Germany (52°34’03.1’’N, 14°38’06.8’’E). Trees for this experiment were grown in climate chambers (as described above) for 2 weeks, before they were treated with either *Cladosporium* sp. spore solution (n = 10) or control solution (n = 10) as described above. At 15 dpi the trees were transferred in pots to the field and placed at fixed intervals of 1 m on a 4 x 5 grid (Fig. S1). Plants were watered twice per day with an automated drip irrigation system and pots were fixed with tent pegs. A fence (Wita Pro, Grube KG Forstgerätestelle, Bispingen, Germany) was installed at a 2 m distance from the experimental plants to protect them against herbivory and trampling by large mammals. To monitor visiting insects, trees were observed four times per day (9 a.m., 12 a.m., 3 p.m., 6 p.m.) for nine days by two experimenters. All arthropods observed on the trees were classified at least to order. Due to the time-consuming nature of counting wingless aphids on the trees, this was only done twice a day. At 20 days after the start of the experiment, leaves that had been inoculated either with endophyte spore solution or a control solution were harvested. Leaves showing leaf chewer damage and non-damaged leaves were harvested separately. All leaves were photographed, flash-frozen in liquid nitrogen and lyophilized for further analysis. Leaf damage was analyzed with Adobe Photoshop® 2020 as described in Boeckler *et al.* (2013).

Quantification of endophytic genomic DNA

To quantify *Cladosporium* sp. abundance in the leaves, genomic DNA was extracted using Invisorb Spin Plant Mini Kit (Stratec Biomedical AG, Birkenfeld, Germany) following the manufacturer's instructions. The genomic DNA was quantified with a NanoDrop 2000c Spectrophometer (Peqlab Biotechnology GmbH, Erlangen, Germany) and diluted to 100 ng/µL. To quantify fungal genomic DNA, primers specific to the internal transcribed spacer (ITS) region of *Cladosporium* sp. (Tab. S2) were used. The reaction mixture contained Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent), DNA (1 µL), and forward and reverse primers (10 µmol each). The quantitative PCR was performed in a CFX Connect Real-Time PCR Detection System (Bio-Rad) using the following program: initiation and activation of polymerase (95 °C/5 min), 35 cycles of denaturation (95 °C/30 s), annealing (65 °C/30 s), and elongation (72 °C/90 s), a single, final elongation step (72 °C/10 min). Data were analyzed using Bio-Rad CFX Manager3.1 (ΔΔcq). For normalization poplar *ACTIN2*-specific primers (Ramírez-Carvajal *et al.* 2008) were used. A non-template control was included in each run and primer efficiencies were tested, followed by a gel electrophoresis to verify the amplicon length. The sequence was further verified by cloned PCR amplicons.

Leaf chemical analysis

Extraction

A 10 mg quantity of lyophilized ground leaf material was extracted with 1 mL methanol containing internal standards for the later quantification of phytohormones (40 ng/mL D6-jasmonic acid (D6-JA), D6-abscisic acid (D6-ABA), D4-salicylic acid (D4-SA), and 8 ng/mL D6-jasmonic acid-isoleucine (D6-JA-Ile)), phenolics (0.8 mg/mL phenyl-β-glucopryranoside (Sigma Aldrich), and phenolic acids (10 ng/mL trifluoro-methyl-cinnamic acid (triF-methyl-CA, Alfa Aesar). The suspension was homogenized by 30 s shaking in a paint shaker (Skandex SO-10M, Fluid Management Europe, The Netherlands), followed by 30 min shaking at 240 rpm on a horizontal shaker (IKA® Labortechnik, Steifen im Breisgau, Germany) and centrifuged at 6363 g for 5 minutes. The supernatants were split for HPLC-UV and LC-MS/MS measurements.

Phenolic compound analysis

For analyzing phenolic compounds (salicinoids, flavonoids, and flavan-3-ols) the supernatant was diluted 1:2 with ultrapure water (Milli-Q® Synthesis A10). Phenolic compounds were measured by HPLC-UV as described in Boeckler *et al.* (2013) on a HPLC 1100 Series (Hewlett Packard, Berlin,

Germany) on a reversed phase column (Nucleodur Sphinx, RP 5 µm, 250 mm x f.6 mm, Machery-Nagel, Düren, Germany), with ultrapure water and acetonitrile as mobile phases A and B, respectively in the following gradient (min/% acetonitrile): 0/14; 22.0/58; 22.1/100; 25.0/100; 25.1/14; 30.0/14 with a constant flow rate of 1 mL/min. The column oven temperature was set to 25 °C. The analytes were quantified relative to the peak area of the internal standard, phenyl-β-glucopyranoside.

Phytohormone and phenolic acid analysis

The measurements were conducted on a HPLC-MS/MS system as described in Fernández-Milmanda *et al.* (2020) with some modifications on an HPLC 1260 Infinity II (Agilent Technologies, Santa Clara, CA, USA) coupled to a triple quadrupole mass spectrometer (QTrap® 6500+, AB Sciex, Waltham, MA, USA) in multiple reaction monitoring (MRM) mode. The analytes were separated on a reversed phase column (ZORBAX Eclipse XDB-C18, 1.8 µm, 4.6 mm x 50 mm, Agilent Technologies, Santa Clara, CA, USA), with 0.05% formic acid (diluted in water) and acetonitrile as mobile phases A and B, respectively in the following gradient (min/% acetonitrile): 0-0.5/5; 0.5-6/5-37.4; 6.02-7/100; 7-9.5/5; with a constant flow rate of 1.1 mL/min. Negative ionization mode was used at an ionization energy of -4500 eV for the electrospray ionization source with a declustering potential of -30 V. Other MS source parameters include a curtain gas of 40 psi, electrospray and drying gas of 60 psi, with the temperature of the drying gas being set to 650 °C. Details of the instrument parameters and response factors for quantification can be found in Tab. S7. Peak integration was carried out utilizing the software MultiQuant™ 3.0.3 (Sciex, Waltham, Massachusetts, USA). Phytohormones and phenolic acids were quantified using the respective internal standards, and the results for JA, JA-Ile, OH-JA, OH-JA-Ile, and COOH-JA-Ile were summed up to get concentration for “jasmonates”.

Phenylacetaldoxime analysis

Phenylacetaldoxime was analyzed in the same extracts as used for phytohormone and phenolic acid analysis as described in Irmisch *et al.* (2013) with some modifications. The measurements were conducted on a HPLC-MS/MS system with the same reversed phase column as described above. The column was eluted with 0.2 % formic acid (diluted in water) and acetonitrile as mobile phases A and B, respectively in the following gradient (min/ % acetonitrile): 0/10; 0-4.0/10-70; 4.0-4.1/70-100; 4.1-5.0/100; 5.0-5.1/100-10; 5.1-8.0/10; with a constant flow rate of 1.1 mL/min. Positive ionization mode was used at ionization energy of 5500 eV for the electrospray ionization source with a declustering potential of 20 V. Other MS source parameters include a curtain gas of 40 psi, electrospray and drying gas of 70 psi, with the temperature of the drying gas being set to 650 °C. The

MRM for phenylacetaldoxime was the following: *m/z* 136.1-119.1; collision energy (CE) 17 V). Analyte concentrations were deduced from the external standard curves made with authentic standards synthesized as described in Irmisch *et al.* (2013). Peak integration was carried out utilizing the software MultiQuant™ 3.0.3 (Sciex, Waltham, MA, USA).

Stachydrine analysis

Leaf extracts of black poplar infected with the endophyte *Cladosporium* sp. and control leaf extracts were compared by LC-MS using a Bruker Esquire 6000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in positive ion mode in the range *m/z* 60–1,200 (Skimmer voltage, 40 eV; capillary exit voltage, 113.5 eV; capillary voltage, 4000 V; nebulizer pressure, 35 psi; drying gas, 11 l/min; gas temperature, 330°C) coupled to an Agilent 1100 series HPLC (Agilent Technologies, Waldbronn, Germany). Elution was accomplished using a Nucleodur Sphinx RP column (250 × 4.6 mm, 5 µm; Macherey-Nagel, Düren, Germany). Mobile phases were 0.2% formic acid (v:v) (A) and acetonitrile (B) with the following gradient (min/% acetonitrile): 0/14; 22.00/58; 22.10/100; 25.00/100; 25.10/14; 30.00/14, flow rate of 1 mL/min. Comparison of the mass spectrometer total ion chromatogram of fungus inoculated and uninoculated control leaf extracts (using the software package Metabolite Detect 1.1, Bruker Daltonics, Bremen, Germany) identified a compound with an *m/z* of 144 ($[M+H]^+$) in the inoculated leaf extract. This was tentatively identified as stachydrine by its identical retention time with an authentic stachydrine standard (Roth, Karlsruhe, Germany).

For further structure confirmation of stachydrine, fungal mycelium was scratched from PDA plates and flash frozen in liquid nitrogen. A quantity of 10 mg of lyophilized ground fungal material were extracted with 1 mL methanol for analysis using a Dionex Ultimate 3000 series UHPLC (Thermo Scientific) and a Bruker timsToF mass spectrometer (Bruker Daltonik, Bremen, Germany). UHPLC was used applying a reversed-phase Zorbax Eclipse XDB-C18 column (100 mm × 2.1 mm, 1.8 µm, Agilent Technologies, Waldbronn, Germany) with a solvent system of 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.3 ml/min. The elution profile was the following (min/% acetonitrile): 0-0.5/5; 0.5-11.0/5-60; 11.0-11.1/60-100; 11.1-12.0/100; 12.0-12.1/100-5; 12.1-15.0/5. Electrospray ionization in positive ionization mode was used for the coupling of LC to MS. The mass spectrometer parameters were set as follows: capillary voltage 4.5 KV, end plate offset of 500 V, nebulizer pressure 2.8 bar, nitrogen at 280 °C at a flow rate of 8 L/min as drying gas. Acquisition was achieved at 12 Hz with a mass range from *m/z* 50-1500. At the beginning of each chromatographic analysis 10 µL of a sodium formate-isopropanol solution (10 mM solution of NaOH in 50/50 (v/v %) isopropanol water containing 0.2% formic acid) was injected into the dead volume of the sample injection for re-

calibration of the mass spectrometer using the expected cluster ion *m/z* values. The target compound showed a signal at *m/z* of 144.1018 for the $[M+H]^+$ ion ($C_7H_{14}NO_2$, theoretical value: 144.1019; Δ 0.73 ppm) with a fragment ion at *m/z* 84.0808 ($C_5H_{10}N$) after collision induced dissociation (Fig. S2) fully consistent with that of authentic stachydrine.

For quantification of stachydrine, the sample extract was diluted 1:10 with ultrapure water (Milli-Q® Synthesis A10) containing isotopically labeled amino acid mix including proline (^{13}C, ^{15}N labelled amino acid mix at a concentration of 10 µg of the mix per mL; from Isotec, Miamisburg, OH, USA). The measurements were conducted on the triple quadrupole HPLC-MS/MS system with the same column as described above. Elution was carried out with 0.05 % formic acid (diluted in water) and acetonitrile as mobile phases A and B, respectively with the following gradient (min/% acetonitrile): 0-1.0/3; 1.0-2.7/3-100; 2.7-3.0/100; 3.1-6.1/3. Positive ionization mode was used at ionization energy of 5500 eV for the electrospray ionization source with a declustering potential of 20. Other MS source parameters include a curtain gas of 40 psi, electrospray and drying gas of 70 psi, with the temperature of the drying gas being set to 650 °C. The MRMs were the following: stachydrine (*m/z* 144 - 84; CE 19 V), U-^{13}C,^{15}N-proline (*m/z* 122 - 75; CE 19 V). Peak integration was carried out utilizing the software MultiQuant™ 3.0.3 (Sciex, Waltham, Massachusetts, USA). Stachydrine was quantified relative to the peak area of the internal standard U-^{13}C,^{15}N-proline applying an experimentally determined response factor of 0.23.

Statistical analysis

To evaluate the influence of the endophyte fungus *Cladosporium* sp. and the gypsy moth caterpillar on black poplar phytochemistry, a two-way ANOVA (sum of squares type I) was applied. “Fungus”, “herbivory” (both as a factor with two levels) and their interactions were treated as fixed variables and concentrations of the compounds as response variables. To test differences in stachydrine concentration of black poplar trees in the field, a generalized mixed model was performed, with “fungus”, “herbivory” (both as a factor with two levels) and their interactions were treated as fixed variables, and “tree” as a random variable. Percentage leaf area loss was included as a covariate and entered before the explanatory variables in the model to account for variation attributable to the amount of damage. To test whether the alkaloid stachydrine influences the feeding preference of specialist and generalist herbivore insects (leaf discs preference assay), a paired t-test or Wilcoxon signed-rank test was applied with of leaf area loss (cm^2) used as a response variable and treatment as a dependent variable (as a factor with two levels). To evaluate the preference of young gypsy moth larvae between uninoculated control plants and plants inoculated with the endophyte *Cladosporium*

sp. (*in planta* preference assay), we constructed a mixed effect model with “percentage of leaf area loss” (arcsine transformed) as response variable, “fungus” as a fixed variable and “caterpillar” nested in “tree” as a random variable. We excluded all replicates (caterpillars) which fed on one endophytic tree and the respective control trees, due to three outliers within this group. Lastly, we evaluated the impact of the endophytic fungus *Cladosporium* sp. on the insect community of black poplar trees. A general linear model (Poisson error distribution and log link function) was performed with “fungus” and “observed time” as fixed variables and the “total number of each arthropod group observed on the plant” as a response variable. To visualize the impact of the fungus on the arthropod community of black poplar trees, a cumulative visitation bipartite network was constructed. For the field experiment the difference in percentage of leaf area loss (arcsine transformed) between plants treated either with spores (fungus) or control solution (control) was tested with Student’s t-test.

Statistical analyses were done using IBM SPSS statistics 25 (SPSS, Chicago, IL, USA), except for the construction of the bipartite network (*bipartite* package) (Dormann *et al.* 2008) and mixed model regressions (*lme4* package) (Bates *et al.* 2014), that were performed on R version 3.6 (R Core Team 2021). All data were checked for statistical assumptions like normal distribution and homogeneity of variances. Whenever statistical assumptions were not fulfilled, data were log transformed, added by 1 following log transformation, or log-log transformed. Percentage data were arcsine transformed.

Results

Endophyte deters a generalist insect herbivore in laboratory bioassay

To test whether the fungal endophyte *Cladosporium* sp. might protect the plant against a generalist insect herbivore, we conducted an *in planta* preference assays (with leaves still attached to the plant) on young endophyte-infected (fungus) and uninfected (control) black poplar trees (Fig. 1A). In the preference assay, young gypsy moth larvae significantly preferred uninoculated over endophyte-inoculated plants (Fig. 1B).

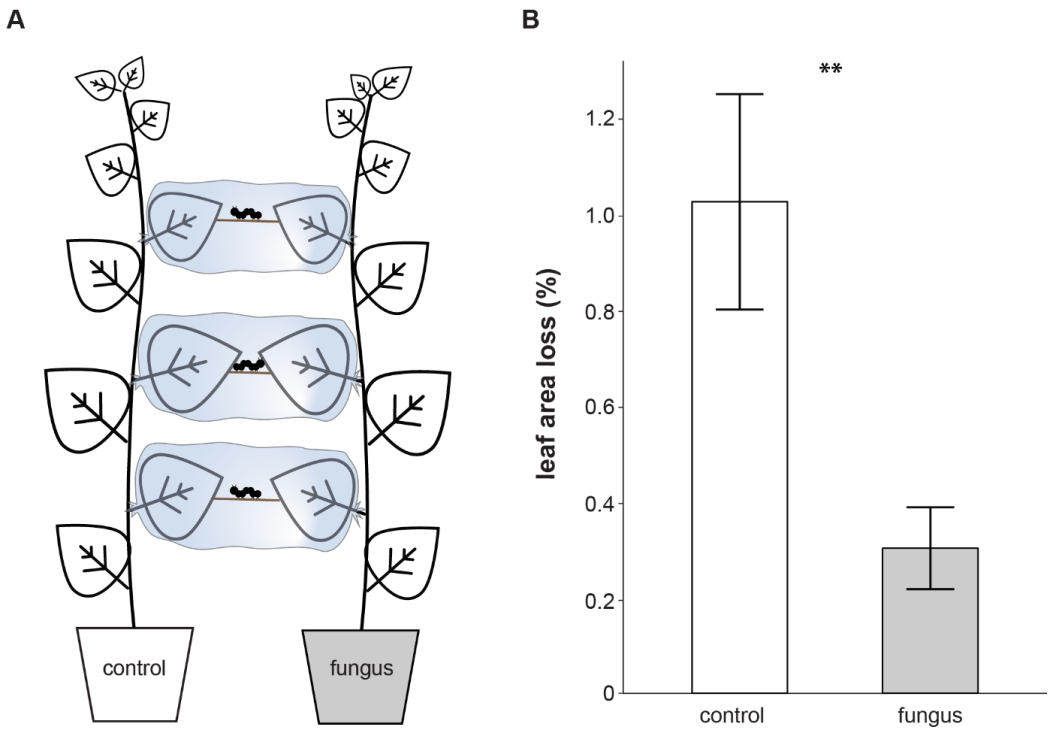

Figure 1: A Preference assay of young gypsy moth larvae between poplar plants inoculated with the endophyte *Cladosporium* sp. (fungus) and uninoculated (control) plants. Two leaves, one from each treatment, were wrapped in a cellophane bag (blue color) 15 dpi. Each bag contained one (2nd instar) gypsy moth larvae. Larval movement from one leaf to the other was facilitated by a wooden stick (brown color). After 48 h larvae were removed and leaf area loss was documented. **B** Preference was evaluated as leaf area loss (%) of total leaf area. Mean ± SE are shown (n = 21). Asterisks indicate significant difference of a linear mixed effect model with tree as a random factor (F = 10.47, p = 0.002; with outliers: F = 3.392, p = 0.071, n = 27).

The endophyte enhances the concentrations of some poplar defense compounds

To test if changes in known poplar defense compounds might be responsible for the caterpillar preference observed, we compared the levels of salicinoids, flavonoids, phenolic acids and phenylacetaldoxime in leaves of black poplar inoculated with the endophytic fungus *Cladosporium* sp. vs. uninoculated controls both with and without caterpillar herbivory.

Although non-significant, the quantitatively more abundant salicinoids, salicortin and homaloside D, showed a trend towards increased concentrations in the treatment with the fungus + herbivory compared to the other treatments (Fig. 2A). For the quantitatively less abundant salicinoids salicin and nigracin, their concentrations significantly increased in the presence of the endophytic fungus both with and without herbivory, while 6'-*O*-benzoylsalicortin was not affected (Fig. 2A). However, after herbivory there was a trend towards increased 6'-*O*-benzoylsalicortin concentration for the endophyte-inoculated plants compared to the uninoculated control (Fig. 2A).

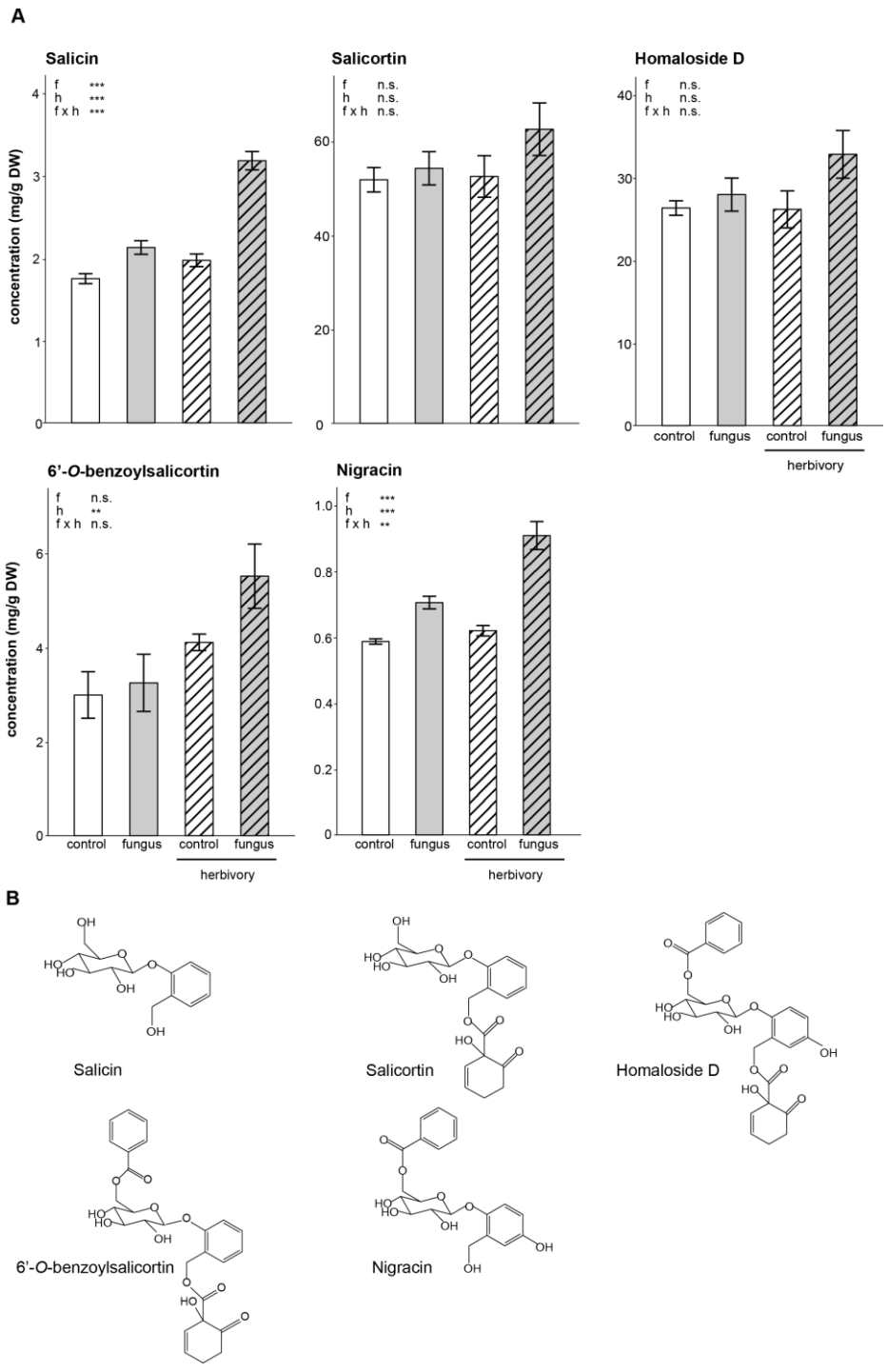

Figure 2: A Effect of fungal endophyte inoculation on salicinoid levels of black poplar leaves. Plants inoculated with the endophyte (fungus) were compared with uninoculated (control) plants either with or without herbivory by gypsy moth caterpillars. Trees were either inoculated with endophyte spore solution or a control solution 15 d before the onset of caterpillar feeding. Gypsy moth caterpillars were allowed to feed for 48 h. Results of two-way ANOVA (top left) to estimate the effect of fungus inoculation (f), caterpillar herbivory (h) and the interaction of both (f x h), (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. = not significant). Mean ± SE are shown (n = 4). On the x-axis, the herbivory treatment on control and endophyte inoculated plants is depicted by a vertical line. **B** Structures of major salicinoids of black poplar: salicin, salicortin, homaloside D, 6'-*O*-benzoylsalicortin and nigracin.

Turning to other types of defense compounds, there were no significant differences in catechin content across treatments (Fig. 3). In contrast, the concentration of proanthocyanidin B1 (PAB1) increased in response to the endophytic fungus and herbivory compared to the other treatments (Fig. 3). Among the phenolic acids, the concentration of caffeic acid increased significantly in response to the fungus, but only with herbivore damage, leading to a significant effect on the interaction of both treatments (Fig. 3). The phenolic acids, *p*-coumaric acid and ferulic acid, however, responded to the endophyte with a decrease in concentration, irrespective of herbivory (Fig. 3, Tab. S4-5). The fungus alone had no significant effect on phenylacetaldoxime concentration, but a trend is observable for increased concentrations in the combined treatment fungus + herbivory (Tab. S4-5).

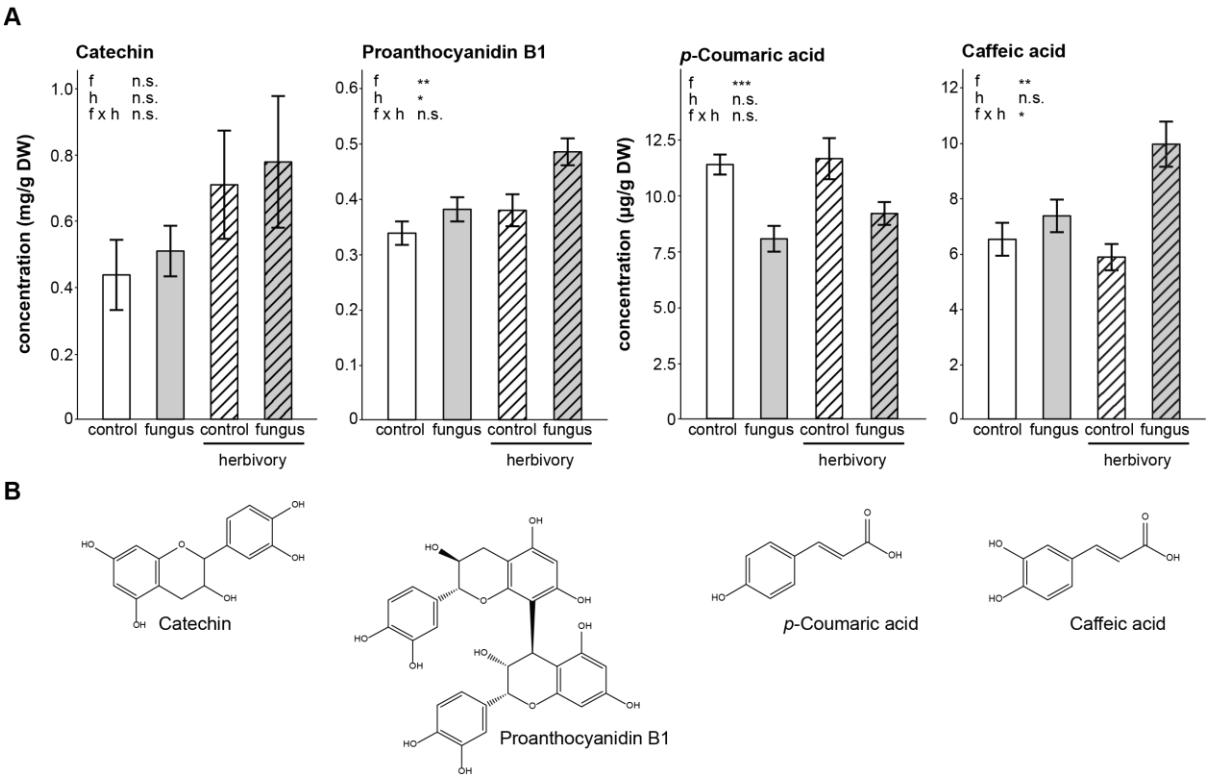

Figure 3: A Effect of a fungal endophyte inoculation on the levels of other defense compounds in black poplar leaves. Plants inoculated with the endophyte (fungus) were compared with uninoculated (control) plants either with or without herbivory by gypsy moth caterpillars. Trees were either inoculated with an endophyte spore solution or a control solution 15 d before the onset of caterpillar feeding. Gypsy moth caterpillars were allowed to feed for 48 h. Results of two-way ANOVA (top left) to estimate the effect of fungus inoculation (f), caterpillar herbivory (h) and the interaction of both (f x h), (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. = not significant). Mean ± SE are shown (n=4). On the x-axis, the herbivory treatment on control and endophyte inoculated plants is depicted by a vertical line. **B** Structures of the defense compounds: catechin, proanthocyanidin B1, *p*-coumaric acid, and caffeic acid.

Endophyte increases herbivore-induced jasmonate concentrations in poplar leaves

To explore what factors might cause the changes in defense compounds observed after endophyte infection, we measured the levels of various defense hormones in black poplar leaves after gypsy moth feeding on endophyte-inoculated and uninoculated control plants. The leaf area consumed by gypsy moth caterpillars did not differ between the endophyte and control plants (Fig. S3). The jasmonates significantly increased in response to herbivory (Fig. 4) and there was a trend towards increased concentration in the endophyte over control treatments after herbivory (Fig. 4). Salicylic acid (SA) concentrations were not affected by the presence of the endophyte or the herbivory treatment (Fig. 4). Caterpillar herbivory significantly increased abscisic acid (ABA) concentrations in black poplar leaves, but the endophytic fungus had no effect on the concentrations of ABA (Fig. 4).

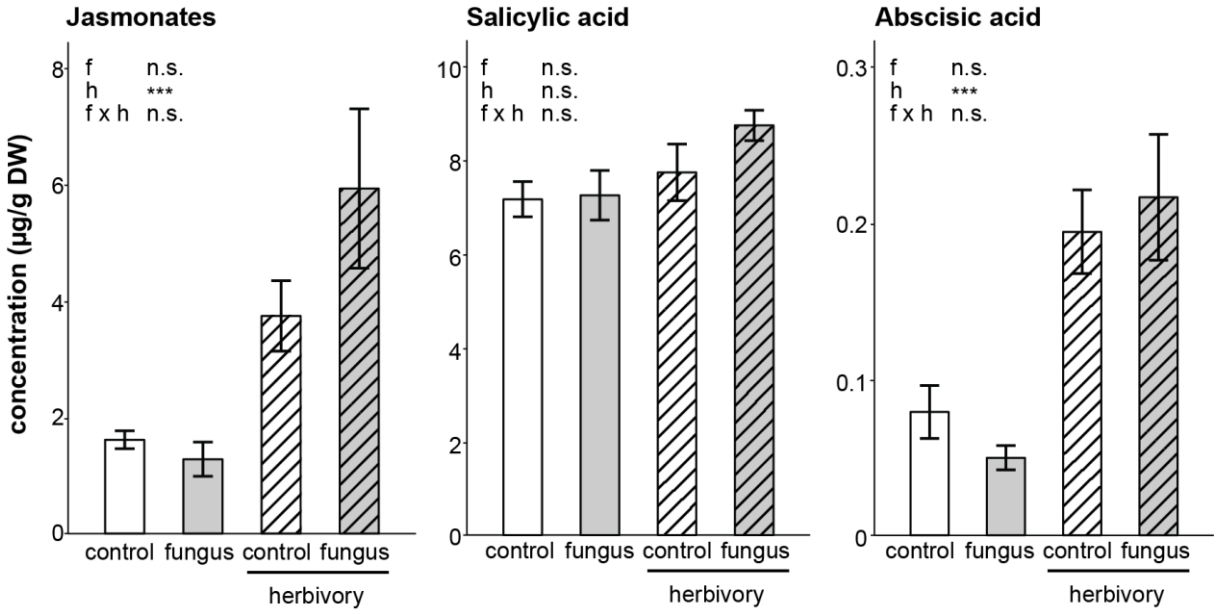

Figure 4: Effect of fungal endophyte inoculation on concentrations of phytohormones of black poplar leaves. Plants inoculated with the endophyte (fungus) were compared with uninoculated (control) plants either with or without herbivory by gypsy moth caterpillars. Trees were either inoculated with endophyte spore solution or a control solution 15 d before the onset of caterpillar feeding. Gypsy moth caterpillars were allowed to feed for 48 h. Results of two-way ANOVA (top left) to estimate the effect of fungus inoculation (f), caterpillar herbivory (h) and the interaction of both (f x h), (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. = not significant). Mean ± SE are shown (n = 4). On the x-axis, the herbivory treatment on control and endophyte inoculated plants is depicted by a vertical line.

Endophyte-specific alkaloid influences feeding preferences of various herbivore insects

By untargeted LC-MS analysis with a quadrupole-time-of-flight mass spectrometer, we detected a compound present in leaves of black poplar inoculated with the endophyte *Cladosporium* sp. but not in uninfected leaves. The compound was identified as the simple pyrrolidine alkaloid stachydrine, known from other fungi, algae and higher plants (Murata *et al.* 2011), by computation of its exact mass and sum formula, and by demonstrating an identical retention time and mass spectrum to those of an authentic standard (Fig. S2). In comparing endophyte and plant tissues, the fungal mycelium contained the highest amount of stachydrine with 6050 ± 460 nmol/g dw (Fig. 5A). However, readily detectable amounts (34.52 ± 8.53, and 45.80 ± 8.03 nmol/g dw) of stachydrine were also found in endophyte-treated plants. Trace quantities of stachydrine (1.03 ± 0.1 - 1.82 ± 0.22 nmol/g dw) were also measured in the uninoculated control treatments, likely due to contamination from the inoculated plants. Herbivory and the interaction of herbivory and endophyte inoculation did not have significant effects on stachydrine concentration (Fig. 5A).

To test whether stachydrine might influence the feeding decisions of specialist and generalist herbivore insects, we conducted a preference assay with leaf discs which were coated either with stachydrine or a control solution (Fig. 5B). Larvae of the generalist herbivore *A. mogadorensis* consumed significantly more leaf material from control leaves compared to stachydrine coated leaves (Fig. 5C). In contrast, beetles of the specialist herbivore *C. tremulae* significantly preferred stachydrine coated leaves (Fig. 5C). However, larvae of the generalist herbivore *L. dispar* did not show a significant preference for either treatments (Fig. 5C).

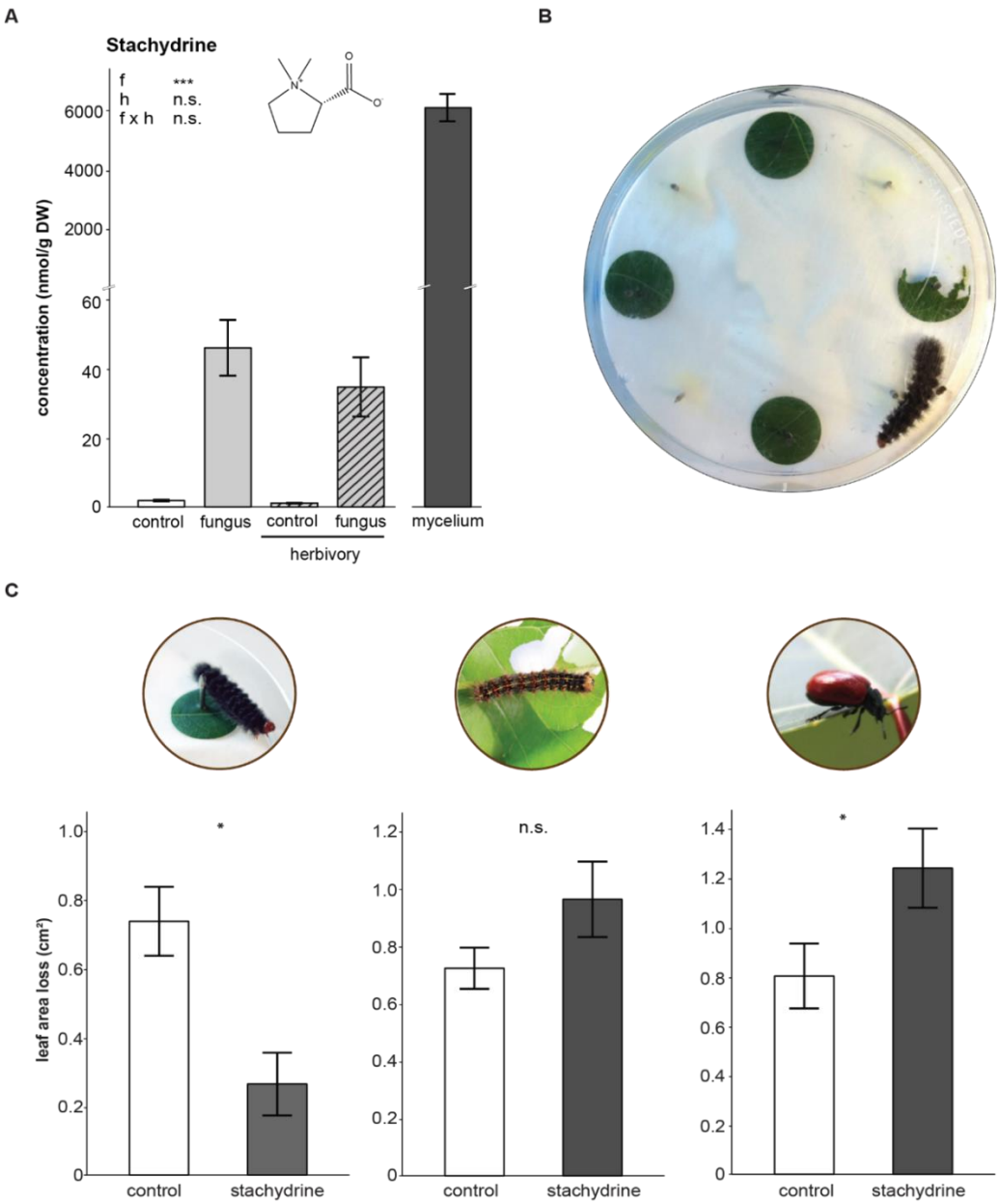

Figure 5: A Levels of the alkaloid stachydrine in leaves of black poplar trees inoculated with the endophyte *Cladosporium* sp. (fungus) compared to uninoculated (control) plants either with or without herbivory by gypsy moth caterpillars. We also measured the levels of stachydrine in cultured fungus (mycelium). Trees were either inoculated with endophyte spore solution or a control solution 15 d before the onset of caterpillar feeding. Gypsy moth caterpillars were allowed to feed for 48 h. Results of two-way ANOVA (top left) to estimate the effect of fungus inoculation (f), caterpillar herbivory (h) and the interaction of both (f x h). The concentration of stachydrine in the mycelium was not included in the statistical analysis. Mean ± SE are shown (n = 3-4). On the x-axis, the herbivory treatment on control and endophyte inoculated plants is depicted by a vertical line. **B** Preference arena containing leaf discs coated either with a stachydrine or control solution. *A. mogadorensis* (4-5th instar) were allowed to feed for 3 h, *L. dispar* (3rd instar) fed for 48 h and beetles of *C. tremulae* fed for 24 h. **C** Results of the preference assay for *A. mogadorensis*, *L. dispar* and *C. tremulae* (left to right). Asterisks indicate significant differences based on related samples Wilcoxon signed-rank test (*L. dispar, A. mogadorensis,*) or a paired t-test (*C. tremulae*) (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. = not significant). Preference was evaluated as leaf area loss (cm²). Mean ± SE are shown (n = 20).

Endophyte shapes insect communities in the field

To examine the impact of the endophytic fungus *Cladosporium* sp. on the arthropod community of black poplar trees, we transferred fungus-inoculated and uninoculated control saplings to a field site with mature black poplar trees (Fig. 6A). There was no difference in the amount of damage received by endophyte-inoculated vs. control trees (Fig. 6B).

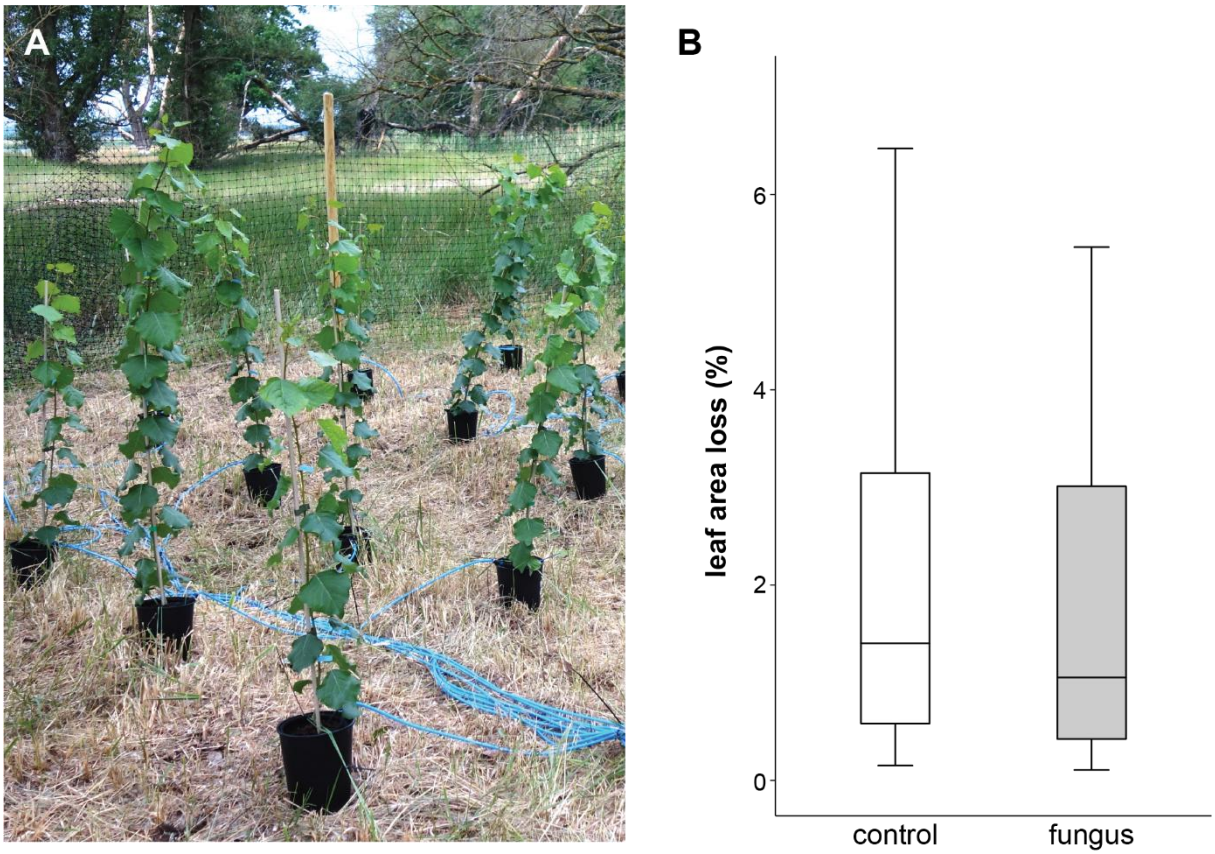

Figure 6: A View of experimental plot at field site in a floodplain forest in northeastern Germany containing mature black poplar trees. Trees in pots were placed in a grid of 4 x 5 plants in an alternate manner by treatment with a distance of 1 m between each plant and the others. Plants were watered twice per day. **B** Leaf area loss (%) of black poplar saplings treated with either a fungal endophyte spore solution (fungus) or a spore-free solution (control). Trees were brought to the field site 15 dpi. Leaves were harvested 35 dpi and leaf area loss was documented. Student's t-test (T = 0.090, p = 0.930, n = 10).

Differences were noted, however, in the composition of arthropod communities on the saplings after observations four times per day for nine days. Endophytic plants were visited significantly more often by species of the order Hemiptera, and the time and the interaction of time and treatment significantly influenced insect visitation. Aphids (Sternorrhyncha: Aphididae) made up the largest proportion of

hemipteran species, representing 93.75 % of the visitors (Tab. S8). In contrast, Hymenoptera were found significantly more often on control plants (Fig. 7). Formicidae made up the vast majority (94.16 %) of the individuals within the order Hymenoptera, whereas the rest consisted of species from the family Ichneumonidae (Tab. S8). Furthermore, coleopteran species were counted significantly more often on control plants, but time and the interaction of time and treatment did not have a significant impact (Fig. 7).

When the same chemical compounds analyzed in the laboratory experiment were also checked in the field plants, no similar patterns were observed except for stachydrine (Tab. S6). The stachydrine concentration was significantly higher in the presence of the endophyte fungus, but in contrast to the laboratory results herbivory also increased stachydrine significantly (Tab. S3B). We found higher amounts of stachydrine in our uninoculated control plants in the field experiment compared to the laboratory experiment (Tab. S3). However, the endophyte-inoculated plants had significantly higher amounts of stachydrine compared to the uninoculated control plants in both experiments (Tab. S3).

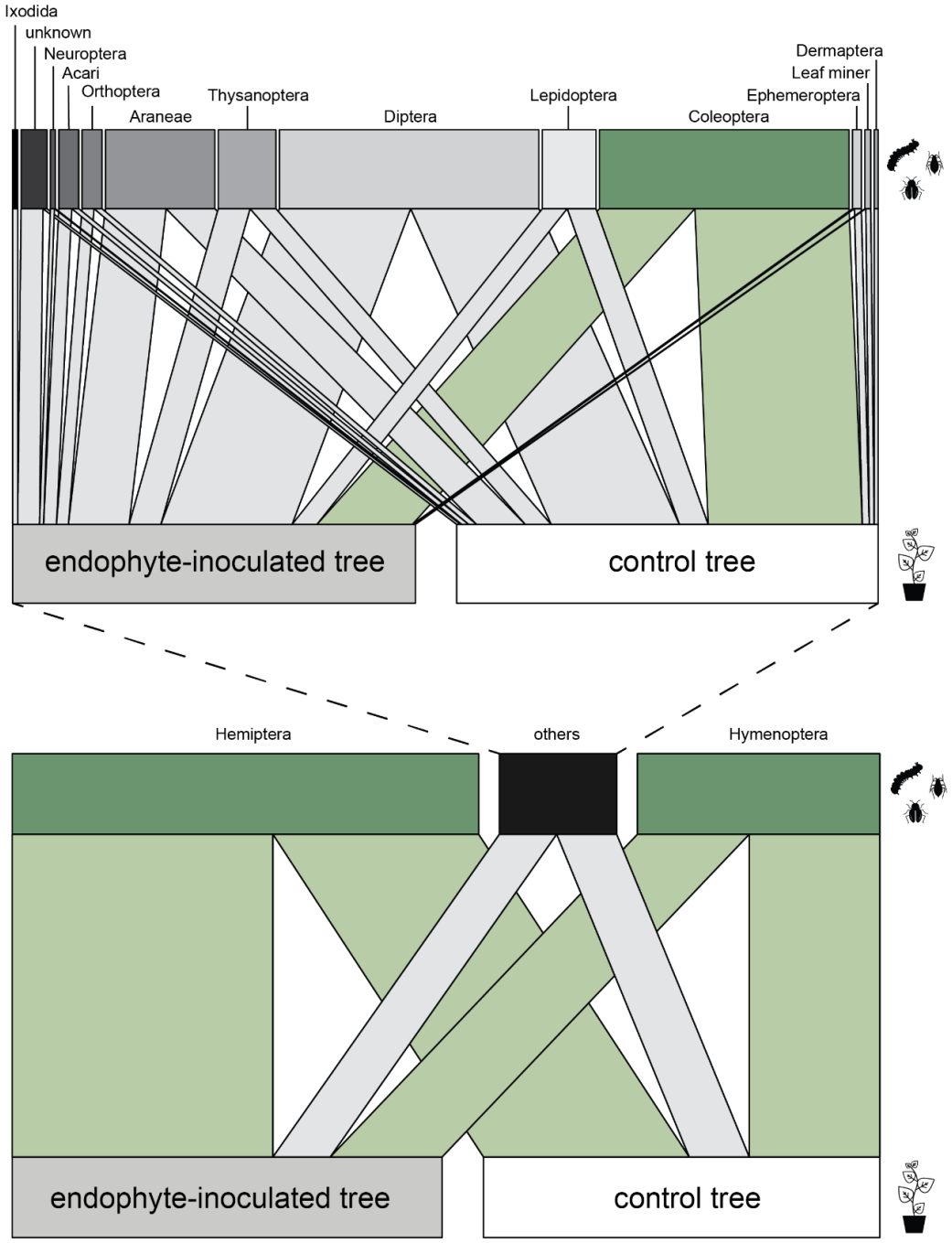

Figure 7: Bipartite network of plant-arthropod interactions for black poplar trees inoculated with either an endophyte spore solution (endophyte-inoculated tree) or a spore-free control solution (control tree) and placed in the field. Trees were inoculated 15 d before plants were brought to the field site. To monitor visiting arthropods, trees were observed four times per day (9 a.m., 12 a.m., 3 p.m., 6 p.m.) for nine days. The lower part of the figure shows a bipartite network for the whole arthropod community depicting the number of arthropods per order visiting endophyte-inoculated vs. control trees. The upper part of the figure shows a bipartite network for arthropods identified from "others" shown below. Thicknesses of lines are scaled to the abundance of individuals within an arthropod group on the treatment. Green colors highlight insect orders which significantly differ among the treatments (Poisson loglinear regression; **Coleoptera:** treatment $p < 0.001$, time $p < 0.001$, treatment x time $p = 0.159$; **Hemiptera:** treatment $p < 0.001$, time $p < 0.001$, treatment x time $p < 0.001$; **Hymenoptera:** treatment $p < 0.001$, time $p < 0.001$, treatment x time $p = 0.740$) ($n = 10$).

Discussion

Endophytic fungi may mediate plant-insect interactions either indirectly via induction and priming of plant-produced defenses or directly by producing toxic or deterrent allelochemicals on their own (Eberl *et al*. 2019 and references therein). Here we observed that the endophytic fungus *Cladosporium* sp., previously isolated from mature black poplar (*Populus nigra*) trees, deterred feeding by larvae of the gypsy moth (*Lymantria dispar*), a generalist herbivorous insect. The endophytic fungus was shown to act indirectly by increasing the levels of constitutive defenses in the plant and modifying the herbivore-induced response. The fungus also acted directly by producing an alkaloid, stachydrine, which was only detectable in endophyte-inoculated trees. Coating stachydrine on leaves deterred larvae of the generalist lepidopteran *Amata mogadorensis*, but attracted the specialist leaf beetle (*Chrysomela tremulae*). Further, a field experiment showed that the endophyte shapes the insect community of inoculated poplar.

Endophyte increases levels of poplar defense compounds

In this study it was shown that the fungal endophyte had a profound influence on the levels of the major poplar defense compounds known to be involved in protection against herbivores and pathogens. Salicinoids, which are exclusively produced by Salicaceae species, are known to be repellent or toxic to generalist insect herbivores, but not necessarily to specialists (Boeckler *et al.* 2016, Hemming & Lindroth 1995, Lindroth 1991) with their content variably increasing following herbivory (Boeckler *et al.* 2013, Fields & Orians 2006, Osier & Lindroth 2001, Ruuhola *et al.* 2001, Stevens & Lindroth 2005, Young *et al.* 2010). Nevertheless, here we could show that salicin and nigracin concentrations increase significantly in the presence of the fungal endophyte (Fig. 2A). Herbivory also led to higher levels of both compounds, and these increases were greater in the presence of the fungus (Fig. 2A). For the other major salicinoids in black poplar, homaloside D and salicortin, a trend towards higher concentrations was observed in fungus-treated plants after herbivory. These findings suggest that endophyte infection might represent an advantage for poplar in case of insect herbivore attack, as it increases the constitutive amounts of certain salicinoids as well as enhancing the herbivore-induced levels.

The flavan-3-ols catechin and proanthocyanidin B1 are known as anti-microbial defenses in poplar (Ullah *et al.* 2019), but their role in anti-herbivore defense is still unclear (Boeckler *et al.* 2014). While the levels of catechin did not respond to *Cladosporium* sp. infection, proanthocyanidin B1 significantly increased upon infection (Fig. 3), suggesting that this endophyte may also increase plant resistance to

pathogens. Other endophytic microorganisms have been shown to reduce pathogen damage, but the underlying mechanisms are poorly understood (e.g., Arnold *et al.* 2003, Hartley *et al.* 2015, Mejía *et al.* 2014, Rodriguez *et al.* 2009, Yue *et al.* 2000). Since the amount of proanthocyanidin B1 was highest after herbivory coupled with endophyte infection, we speculate that this compound might especially prevent pathogen infection at freshly wounded sites. The increased amounts of proanthocyanidin B1 might also negatively affect the endophyte itself, but this has not been tested.

Simple phenolic acids are also known to have detrimental effects on herbivorous insect performance. *p*-Coumaric acid has been shown to have a deterrent and toxic effect on the lepidopterans *Spodoptera litura* and *Amsacta albistriga* (Sambangi & Rani 2016), while caffeic acid inhibits the gut proteases of *Helicoverpa armigera* (Dixit *et al.* 2017). Furthermore, both compounds exhibit antimicrobial and antifungal activity (Aziz *et al.* 1998), and both may serve as precursors in lignin formation, which also defends plants against pathogen infection (Xie *et al.* 2018). Following endophyte infection, caffeic acid was found to increase while *p*-coumaric and ferulic acids decreased significantly upon fungal inoculation, irrespective of herbivory (Fig. 3, Tab. S4-5). The greater concentration of caffeic acid in endophytic plants may have resulted from conversion from *p*-coumaric acid (Zhong *et al.* 2000).

To date there has been little research on how endophyte infection affects the quantities of plant-produced anti-herbivore defense compounds. Christian *et al.* (2020) compared the metabolome from cacao (*Theobroma cacao*) inoculated with the endophytic fungus *Psychotria* sp. with endophytic free plants and found some alterations in the plant secondary chemical profile, but this was not connected with defensive potential. Thus, more research is needed to determine if the ability of endophytes to enhance plant defenses is a general trend.

Endophyte infection had only minor effects on known defense hormones

To test whether the increase in poplar defenses caused by the endophyte resulted from changes in any of the well-studied plant defense signaling pathways, we analyzed the levels of defense hormones after endophyte inoculation. There was no significant increase in the hormones jasmonates, ABA and salicylic acid (SA) (Fig. 4), although herbivory alone significantly increased the concentrations of jasmonates and ABA, agreeing with previous work in poplar (Boeckler *et al.* 2013) and a number of other plant species (Vos *et al.* 2013, Wasternack & Hause 2013). We observed a trend towards increased jasmonate concentration when endophyte-treated plants were subject to herbivory (compared to endophyte-free controls), which may partially explain the increased chemical defense profile of endophytic plants. A meta-analysis of the well-known *Epichloë*-grass endophyte system

reported that jasmonic acid was significantly elevated when grasses were infected with endophytes, thus boosting resistance against chewing insects (Bastías *et al.* 2017a). Additionally, the endophyte *Sphaeropsis* sp. B301 was reported to induce ABA levels in suspension cells of *Ginkgo biloba* leading to an increase in flavonoids. While jasmonic acid likely plays a role in the rise of defense in poplar after *Cladosporium* sp. infection, other jasmonate-independent signaling pathways may also be involved.

The symbiosis of a plant with an endophytic fungus often results in down-regulation of the SA pathway, as activation of the SA pathway is generally thought to have a negative impact on the growth and colonization of endophytic fungi (Bastías *et al.* 2018, Dupont *et al.* 2015, Johnson *et al.* 2003). For instance, Bastías *et al.* (2018) have shown that the presence of an *Epichloë*-grass endophyte suppressed the SA pathway, while the application of exogenous SA in turn suppressed endophyte growth and simultaneously reduced the production of endophytic alkaloids, which led to a higher susceptibility to aphids. In poplar leaves, no significant changes in levels of SA were observed after *Cladosporium* sp. inoculation (Fig. 4).

Endophyte produces a potential defense compound – the alkaloid stachydrine

The role of endophyte alkaloids in the anti-herbivore defense of their host plants is well documented for many grass endophytes (Bastías *et al.* 2017b, Faeth & Bultman 2002, Faeth & Hammon 1997), but there is little information about endophyte alkaloids in other types of endophyte-plant associations. Here, we were able to identify the pyrrolidine alkaloid stachydrine in the *Cladosporium* sp. fungal mycelium and in inoculated plants (Fig. 5A), which was initially detected in an untargeted screen for metabolites found in *Cladosporium*-inoculated, but not endophyte-free poplar leaves. Stachydrine has been isolated from *Citrus*, *Medicago*, *Chrysanthemum* and *Stachys* species, as well as from various algal and fungal taxa (Murata *et al.* 2011 and references therein), however this is the first report of an alkaloid in a plant of the Salicaceae (Desgagné-Penix 2017). Stachydrine shows a variety of activities against human diseases (Cheng *et al.* 2020), but in a more ecological context, stachydrine acts in a mixture as feeding stimulant for larvae of the citrus swallowtail butterfly (*Papilio xuthus*) and oviposition stimulant for their adults (Honda 1990, Murata *et al.* 2011). Other functions of this alkaloid in plant-insect interaction studies are not yet known.

To test the impact of stachydrine on poplar insect herbivores, we tested the preference of insects for leaf discs coated with stachydrine versus those coated with a control solution. While the specialist leaf beetle (*C. tremulae*) preferred the stachydrine coated leaf discs, the generalist *A. mogadorensis* favored the control, and the oligophagous *L. dispar* did show a significant preference (Fig. 5C). The

literature indicates that alkaloids are most effective against generalist insect herbivores since specialists can often detoxify them (Saunders *et al.* 1992). However, in order to determine a general pattern for herbivore response to stachydrine in poplar, studies with many more herbivores are necessary. Future experiments should therefore include varying concentrations of stachydrine to determine a threshold value for behavioral response, which may not have been reached in our experiments with *L. dispar*. The amounts of stachydrine in the coated leaves were somewhat lower than the amounts measured in leaf tissue inoculated with the *Cladosporium* sp. endophyte (Tab. S3D).

Alkaloids in general are toxic to herbivorous insects, although some species are able to detoxify or sequester them as protection against predators (Dobler *et al.* 2000, Haberer & Dobler 1999, Waller & Nowacki 2012). For example, the leaf beetle *Longitarsus anchusae* is able to detoxify pyrrolizidine alkaloids through *N*-oxidation and store them as protection against predators (Narberhaus *et al.* 2005). As *C. tremulae* and *L. anchusae* belongs to the same family, there is a possibility that the leaf beetle is able to sequester or detoxify the pyrrolidine alkaloid stachydrine. To examine the benefits of stachydrine for the specialist *C. tremulae* and the negative effects on *A. mogadorensis* requires further experiments to monitor the performance and physiology of these insects. The discovery of stachydrine in endophyte-infected poplar leaves hints at the unexplored diversity of endophyte-produced defenses in plants. Since most plant endophytes or endophyte-inoculated tissue have not been screened at all for defense compounds, many new discoveries are likely to be made in this area.

Endophyte deters a generalist insect herbivore and shapes insect communities

In order to integrate the effect of stachydrine with the effects of other endophyte changes in poplar defense compounds, we conducted a preference assay on whole poplar leaves with and without endophyte infection that were still attached to the plant (Fig. 1A). This experimental design better reflects a natural scenario, as it allows the exchange and transport of defense signaling, precursors, and products throughout the plant during feeding (Gange *et al.* 2019 and references therein). With whole attached leaves, *L. dispar* caterpillars significantly avoided endophyte-infected plants (Fig. 1B), consistent with the up-regulation of several poplar defense compounds upon endophyte infection or production of its own defenses (Fig. 2-5).

In the field, the patterns of herbivore visitation on endophyte-inoculated vs. uninoculated control poplar saplings were quite different from the preference tests in the laboratory. Lepidopterans did not distinguish among the two treatments, while coleopterans were counted more often on control plants (Fig. 7). The lack of congruence with laboratory results may stem from the absence of biotic

and abiotic factors that contribute to complex tripartite plant-herbivore-endophyte interactions (Hardoim *et al.* 2015). A lack of congruence with expectations was also seen in a field experiment with sleepy grass (*Achnatherum robustum*) containing the alkaloid producing endophyte (*Neotyphodium*), which revealed that endophyte infection caused increased herbivore abundance and species richness compared to plants without endophytes or plants infected with an endophyte lacking alkaloids.

In our field experiment, species of the family Hemiptera were observed significantly more on poplar plants harboring the endophyte than on those without endophyte infection, with the vast majority of Hemiptera present being aphids (Aphidoidea) (Tab. S8). Numerous studies on grasses with alkaloid-producing endophytes have shown the detrimental effects of endophytes on aphid performance (e.g., Bastías *et al.* 2017b, Shymanovich *et al.* 2015, Siegel *et al.* 1990). However, not every alkaloid produced by the fungi has a negative effect on the aphid population, as was shown for the alkaloid ergovaline (Siegel *et al.* 1990). Furthermore, Gange *et al*. (1996) could show in a field experiment that leaves of the *Acer pseudoplatanus* harboring the endophyte *Rhytisma acerinum* contained more aphids.

As ants are often involved in close mutualistic interactions with aphids, we expected that the increase in aphids on endophytic plants would result in higher numbers of ants as well. In contrast to this expectation, Hymenoptera (94.16 % of them belonging to the Formicidae) were counted more often on control plants. The mutualism between ants and aphids is based on collection of aphid sugary excrement by ants, which in turn provide aphids protection against predators and parasitoids (Banks 1962, Nielsen *et al.* 2010). Züst *et al*. (2017) argued that a successful ant-aphid mutualism is likely dependent on the honeydew quality, where they could show that aphids feeding on plants high in cardenolides, were visited less often by ants, since the aphids excreted cardenolides via their honeydew. If the aphids in our field experiment were able to excrete the alkaloid stachydrine via their honeydew, this may have led to the observed reduction in visitation by ants. Further analysis of honeydew is needed to test this hypothesis. The occurrence of more aphids on the endophytic plants suggests that the missing mutualistic interaction with ants has not reduced the population. Aphids might even benefit from the endophyte if the alkaloid stachydrine functions in protection against predators and parasitoids.

In general, the pattern of herbivore response to endophyte-inoculated vs. uninoculated plants depended on the herbivore species. The endophyte *Cladosporium* sp. we studied deterred the generalist herbivore *L. dispar* in the lab, and coleopteran and hymenopteran species in the field, while attracting hemipteran species in the field. Such differences in herbivore response have been reported

in previous endophyte-infected vs. uninfected plant comparisons (Gange *et al.* 2012) and illustrates that the role of endophytes as defense mutualists in woody plants is species dependent.

Conclusion

Here we could demonstrate that the endophyte *Cladosporium* sp. alters the concentrations of several poplar defense metabolites, produces the alkaloid stachydrine *in planta,* and modifies the behavior of herbivorous insects in the laboratory and in the field. Endophytes of herbaceous and woody plants include latent pathogens and dormant saprotrophs, and as a possible consequence are thought to be less mutualistic, leading to an ambiguity in their ecological role (Bahnweg *et al.* 2005, Davis & Shaw 2008, Herre *et al.* 1999, Suryanarayanan 2013, Van Bael *et al.* 2009). Therefore, as reflected in our own field experimental work, a more species-specific response of insect herbivores to woody plants harboring endophytic fungi is expected. Although more experimentation is needed to understand the complex nature of endophyte-tree system interactions, this work represents important steps towards.

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Supplemental Information

Table S1: Germination test of *Cladosporium* sp. spores for the lab experiment, the preference assay and the field experiment.

Table S2: Primers used in this study

Table S3: Relative endophyte abundance and stachydrine concentration from laboratory, coating and field experiment

Table S4: Leaf chemical analysis from laboratory experiment

Table S5: Statistical analysis from phytochemistry of the laboratory experiment

Table S6: Leaf chemical analysis from the field experiment

Table S7. Parameters used in LC-MS/MS analysis of phytohormones and phenolic acids

Table S8: Arthropods attracted during the field experiment

Figure S1: Planting scheme of the field experiment

Figure S2: Chromatogram and mass spectra of stachydrine

Figure S3: Herbivore damage from control and inoculated trees from the laboratory experiment

6. DISCUSSION

In recent decades, researchers have gained a good understanding of the chemical and molecular patterns underlying plant-insect interactions. However, plants provide a large number of different niches that harbor a wide diversity of microorganisms. Therefore, a more holistic perception is needed in plant-insect interaction studies to include the "plant microbiome" (Vandenkoornhuyse *et al.* 2015). Some plant-associated microorganisms, such as mycorrhizal fungi, are well-known to promote plant productivity and health under natural conditions (Trivedi *et al.* 2020). But knowledge about endophytes is still in its infancy, especially of horizontally transmitted endophytes in woody plants. Trees typically have long generation times and therefore evolve more slowly than pathogens and herbivore insects. For this reason, it has been suggested that a beneficial microbiome may add a layer of phenotypic plasticity that could help the tree to defend against potential attackers (Albrectsen & Witzell 2012, Albrectsen *et al.* 2018, Christian *et al.* 2017b). However, the role of horizontally transmitted endophytes as defense mutualists is ambiguous compared to the well-established vertically transmitted *Epichloë* endophytes. New insights about the interactions between endophytic fungi and their host trees would therefore make important contributions to basic and applied knowledge on forest protection.

6.1 Endophytes produce VOCs overlapping with the volatile bouquet of their host plant

Compared to the intense research on the biosynthesis and bioactivity of plant-derived volatile organic compounds (VOCs), we only know little about volatiles produced by endophytic fungi isolated from trees. In **manuscript II** we investigated the volatile profile of 13 different endophytic species isolated from old growth black poplar trees. Overall, the volatile profile of the endophytic fungi was species-specific, meaning that none of them shared the same volatile profile, as shown in other studies (Hung *et al.* 2015). Most of the endophytic fungi produced aliphatic or aromatic compounds (**manuscript II**, Farh & Jeon 2020), with some of them known to have bioactive properties. For instance, endophytic fungi are able to release volatiles with antifungal and phytotoxic activity to defend their niche within the plant matrix, like ethanol or 2-phenylethanol (Farh & Jeon 2020). From 77 detected volatiles, 50 different sesquiterpenes were emitted by seven endophytic fungi (**manuscript II**). In general, sesquiterpenes make up the largest proportion of fungus-produced compounds (Souza *et al.* 2011) and it has been shown that they are able to mediate plant-plant, plant-microbe, and microbe-microbe interactions (Ditengou *et al.* 2015, Schmidt *et al.* 2015). For

instance, sativene and (*E*)-β-caryophyllene are known to promote plant growth and plant immunity against attackers (Paul & Park 2013, Yamagiwa *et al.* 2011).

Almost all endophytic fungi isolated from black poplar were able to produce volatiles that are known from plant-insect interaction, highlighting the importance of endophytic fungi as potential driver for the ecology of plant-insect interactions. Some of the volatiles produced by the endophytic fungi are also produced by the host plant black poplar itself (**manuscript II**), including the alcohols 3-methyl-1-butanol and 2-phenylethanol, and the sesquiterpenes (*E*)-β-caryophyllene, and α-muurolene. Especially (*E*)-β-caryophyllene is known for its bioactive potential, as it is was shown to attract insect herbivores or mediate tri-trophic interactions (Rasmann *et al.* 2005, Wang *et al.* 2015b). Other studies have shown that endophytes are able to manipulate the volatile profile of their host plants. For example, the tomato plant *Solanum lycopersicum* emits a different volatile profile when inoculated with the endophyte *Acremonium strictum*, which leads to attraction and oviposition of the moth *Helicoverpa armigera* (Jallow *et al.* 2008). The emission of several terpenes was also increased upon endophytic infection with the fungus *Beauveria bassiana* in tomato plants, affecting the defense response against *Spodoptera exigua* (Shrivastava *et al.* 2015). In these cases, it is not clear whether the endophyte triggers the production of terpenes by the host plant or if the fungus directly contributes to the overall volatile bouquet of the plant as a holobiont via its own production of terpenes. To solve this issue, the isolation and characterization of volatile biosynthetic enzymes, such as terpene synthases (TPSs), from the endophyte is crucial, but information in the literature are scarce. So far, only twelve TPSs have been characterized for endophytic fungi isolated from leaf tissues (**manuscript II**). As *Cladosporium* sp. is a cosmopolitan fungus and is also frequently isolated as an endophyte (Bensch *et al.* 2012, Brown 1998, Riesen 1985), we screened this isolate for potential TPSs, as it produced (*E*)-β-caryophyllene on medium (**manuscript II**). We identified and characterized two TPSs, CxTPS1 and CxTPS2. The heterologous expression of the TPS genes showed, that CxTPS1 produced a variety of different terpenes, while CxTPS2 showed a narrower product profile. Both TPSs produced several monoterpenes that were not detected when the fungus grew on medium. Therefore, we speculate that geranyl diphosphate (GPP), the substrate for monoterpene production, is not available in the fungus when growing on artificial medium. However, among sesquiterpenes, we found that CxTPS1 produced (*E,E*)-α-farnesene, while CxTPS2 produced (*E*)-β-caryophyllene. The fungus itself only emitted (*E*)-β-caryophyllene when growing on medium (**manuscript II**). Thus, we speculate, that *CxTPS1* might not be expressed in the fungus under the culture conditions used, or that (*E,E*)-α-farnesene is produced and further metabolized. The identification and characterization of endophytic TPSs allows us to elucidate the impact of endophytic fungi on the plant volatile blend,

which could directly affect our interpretation of certain plant-fungus and plant-insect interactions (Albrectsen *et al.* 2010, Albrectsen *et al.* 2018, Jia *et al.* 2020).

6.2 Endophytic fungus mediates plant defenses and deters a generalist caterpillar

Tripartite interactions between endophytes, host plants and herbivorous insects in woody species are understudied and have focused mostly on the correlation between endophyte infection and herbivore load (**manuscript I**, Albrectsen *et al.* 2010, Bittleston *et al.* 2011) or between the concentrations of certain plant specialized compounds, plant genotype and endophyte infection (Albrectsen *et al.* 2018, Bailey *et al.* 2005). Little is known about how a single endophyte influences plant specialized compounds alone and in response to insect herbivory. In **manuscript III,** we investigated the impact of the endophyte *Cladosporium* sp. on the phytochemistry of black poplar trees and the consequences for herbivorous insects. We focused on differences in defense hormones and several defense compounds due to endophyte infection, herbivory and a combination of both treatments.

Colonization with the endophytic fungus *Cladosporium* sp. did not lead to an increase of either of the defense hormones, salicylic acid (SA) or jasmonates in black poplar (**manuscript III**). However, a trend towards increased jasmonate concentration was shown when endophyte infection was combined with herbivory by the generalist caterpillar *L. dispar*. Jasmonates are important signaling hormones in the anti-herbivore defense response of plants. Therefore, plants harboring *Cladosporium* sp. might be better protected from chewing insects. Studies investigating levels of defense hormones in response to endophyte infection and herbivory are best described for the grass-endophyte system, soil-borne bacteria, and soil-borne fungi, while literature on horizontally transmitted endophytes in woody plants is rare. It is argued that an endophyte infection causes a plant immune response similar to pathogen infection. Yet, endophytes may manipulate the effector-triggered susceptibility state and overcome plant defense responses, as was shown for beneficial soil-borne microorganisms (Zamioudis & Pieterse 2012 and references therein). For instance, the root endophytic fungus *Piriformospora indica* is able to induce the jasmonic acid (JA) signaling pathway in *Arabidopsis thaliana* to suppress SA signaling (Millet *et al.* 2010, Zamioudis & Pieterse 2012). Thereby, the activation of the SA signaling pathway, as it is the case for pathogens, would negatively affect successful endophyte colonization (Bastías *et al.* 2018, Bastías *et al.* 2017a, Dupont *et al.* 2015, Johnson *et al.* 2003). Other beneficial microorganisms are able to produce phytohormone-like compounds, like auxins and gibberellins that may negatively affect the SA signaling pathway, leading to the speculation that for successful

colonization beneficial microbes produce phytohormones to suppress the SA pathway (Zamioudis & Pieterse 2012). This is true, for example for the endophyte *Epichloë occultans* that is able to suppress the SA pathway in its host plant *Lolium multiflorum* (Bastías *et al.* 2018).

In this thesis, abscisic acid (ABA) was only induced upon herbivory, irrespective of the presence of an endophyte (**manuscript III)**. ABA is best known for enhancing plant tolerance against drought stress (Cutler *et al.* 2010, Jia *et al.* 2016), but has also gained attention as a defense against plant pathogens, as it regulates stomata closing, and thus prevents pathogens from entering the leaf tissue (Melotto *et al.* 2006, Sun *et al.* 2014, Ullah *et al.* 2019). Increases in jasmonates and ABA are well-known plant defense strategies against chewing insects (Boeckler *et al.* 2013, Vos *et al.* 2013, Wasternack & Hause 2013) that is also supported by the results obtained. As ABA and SA are not induced upon endophyte infection, it is possible that the endophyte *Cladosporium* sp. is not recognized as a pathogen by black poplar. In contrast, there was a trend towards increased levels of jasmonates upon endophyte infection accompanied by herbivory, indicating that black poplar might be better defended against herbivorous insects.

Literature on foliar endophytic fungi in poplars has mainly focused on the question which factors influence endophyte communities in poplar trees (Albrectsen *et al.* 2010, Albrectsen *et al.* 2018, Bailey *et al.* 2005). For instance, levels of condensed tannins, which are known to have antimicrobial activity, were negatively correlated to endophyte infection, while levels of salicinoids, a major group of defense compounds of Salicaceae, were not affected (Albrectsen *et al.* 2018, Bailey *et al.* 2005). However, there has been no information about the combined effect of herbivory and endophytic infection on leaf chemistry in black poplar. Here, we could show that the salicinoids, salicin, 6'-*O*-benzoylsalicortin and nigracin are induced upon caterpillar feeding, but also the endophyte alone and in combination with herbivory lead to an induction of salicin and nigracin (**manuscript III**). Fabisch *et al.* (2019) showed that salicin is induced upon herbivory, but findings from the literature are contradictory (Boeckler *et al.* 2013, Lackner *et al.* 2019). It has been often shown that salicinoids have detrimental effects on a broad spectrum of different herbivores, like generalist chewing insects, (e.g., *L. dispar*, *Malacosoma disstria*), leaf miner (e.g., *Phyllocnistis populiella*), and mammalian herbivores (Boeckler *et al.* 2011 and references therein). Thus, we speculate, that the endophyte *Cladosporium* sp. might be beneficial for the tree against generalist insect herbivores, as it increases the constitutive amounts of salicin and nigracin, as well as the herbivore-induced defense response (**manuscript III**).

In **manuscript III**, the patterns of phenolic acids in response to endophyte infection and herbivory were inconsistent, as caffeic acid was increased after endophyte infection as well as when combined

with herbivory, while *p*-coumaric and ferulic acid decreased. The phenolic acids caffeic acid and *p*-coumaric acid are specialized compounds with known anti-herbivore effects (Dixit *et al.* 2017, Sambangi & Rani 2016). Thus it needs to be tested how the responses observed would affect the preference and performance of insect herbivores. Furthermore, phenolic acids are known as signaling molecules in plant-microbe symbioses, especially for the initation of legume-rhizobia and arbuscular symbioses (Mandal *et al.* 2010 and references therein). Coumaric acid, for example, promotes the growth and colonization of white clover (*Trifolium repens*) and sorghum (*Sorghum bicolor*) roots by the arbusculuar mycorrhizal fungus *Glomus* in a dose dependent manner (Fries *et al.* 1997). It is known, that coumaric and gallic acids act as antimicrobial agents directly and indirectly as part of lignin biosynthesis (Aziz *et al.* 1998, Xie *et al.* 2018). As many different functions are described for phenolic acids, more studies are needed to elucidate the role of these compounds in the interactions among the endophyte *Cladosporium* sp., its host black poplar and insect herbivores.

The poplar flavan-3-ols catechin and proanthocyanidin B1 (PAB 1) are described as antifungal defense compounds effective against rust infection (Ullah *et al.* 2017). While levels of catechin did not respond to endophyte infection, PAB 1 significantly increased upon endophyte infection as well as herbivory (**manuscript III**). The pathogenic rust fungus *Melampsora larici-populina* is a common and serious threat of poplar trees that can lead to a dramatic economic loss in poplar plantations world-wide (Pinon *et al.* 1987). It has been shown that presence of the foliar symbiont *Cladosporium* sp., among other symbionts, reduced the disease severity of the rust fungus *M. x columbiana* in *Populus trichocarpa* (Busby *et al.* 2016). However, the underlying mechanisms were not considered in this paper. It is possible that the endophyte primed the plant defense response against rust infection, or that the increase of PAB 1 is a direct antimicrobial reaction to the endophyte.

To summarize, endophyte infection alone increased the concentrations of the black poplar defense compounds salicin, nigracin as well as the phenolics PAB 1, and caffeic acid, suggesting that an increase in defensive potential is a consequence of endophyte infection. The effect was even stronger after herbivory for some compounds. A trend towards increased jasmonate concentrations after endophyte infection and herbivory is also consistent with this view. Therefore, we tested, whether the endophyte plays a role as a defense mutualist against a generalist insect herbivore. We conducted an *in planta* choice assay with *L. dispar* with leaves still attached to the plant. Plants harboring the endophytic fungus suffered less leaf damage showing that the endophyte helped the plant to defend against a generalist herbivorous insect (**manuscript III**). Our data on differences in plant defense compounds between endophyte-infected and uninfected poplar foliage provide a chemical rationale for this defensive phenotype.

6.3 Endophytic fungus produces an alkaloid with bioactive properties

The protective role of endophytic fungi is well described for grass-associated endophytic fungi, with a strong focus on the fungal production of alkaloids. In **manuscript III**, we were able to identify the pyrrolidine alkaloid stachydrine in fungal mycelium of the endophyte *Cladosporium* sp., which is also detectable in inoculated black poplar plants. So far, no alkaloids were previously detected in Salicaceae (Desgagné-Penix 2017). In medicine, stachydrine has been used for the treatment of fibrosis, cardiovascular diseases, cancer, and inflammation (Cheng *et al.* 2020 and references therein). In general, alkaloids are effective defense compounds against generalist insects, while specialists are able to detoxify alkaloids (Narberhaus *et al.* 2005). However, literature about the role of stachydrine in plant-insect interactions is scarce. In a mixture, stachydrine was shown to act as feeding attractant for larvae of the citrus swallowtail butterfly (*Papilio xuthus*) and as oviposition stimulant for their adults (Honda 1990, Murata *et al.* 2011). Endophyte researchers have often looked for new bioactive compounds, but only a few studies focusing on woody plants that host endophytes have pursued this goal (**manuscript I**). For example, the rugulosin producing endophyte *Phialocephala scopiformis* isolated from white spruce (*Picea glauca*) was shown to reduce the performance of several insect herbivores (Miller *et al.* 2002, Miller *et al.* 2008, Sumarah *et al.* 2008, Sumarah & Miller 2009, Sumarah *et al.* 2010). Other examples are the taxol producing endophytes isolated from the western yew (*Taxus brevifolia*), which is used as an anti-cancer drug (Zhou *et al.* 2010 and references therein).

In order to test the impact of stachydrine on the preference of insect herbivores known to feed on poplar plants, leaf discs coated with stachydrine were offered to two generalist insects, *L. dispar* and *Amata mogadorensis*, and the specialist leaf beetle *Chrysomela tremulae.* Caterpillars of *A. mogadorensis* avoided feeding on leaf discs coated with stachydrine, while the specialist leaf beetle *C. tremulae* consumed significantly more leaf material containing the alkaloid (**manuscript III**). Wink & Schneider (1990) could show that *A. mogadorensis* avoided plant species with high amounts of allelochemicals, including alkaloids, when given the choice. The results support the abovementioned hypothesis, that alkaloids are more effective against generalist herbivorous insects, but a larger sample size of insect species is needed to validate this conclusion. However, *L. dispar* did not show any preference for either control or stachydrine coated leaf discs. The concentration of stachydrine in the coating experiment was lower than that of leaves infected with the endophytes (**supplemental of manuscript III**). Either the concentration of stachydrine was too low for an insect to respond to or the gypsy moth has no receptor for tasting stachydrine. Still, we could show with the previously discussed bioassay data that the endophyte defends its host plant against the generalist

gypsy moth, irrespective of this alkaloid. Based on a meta-analysis of alkaloid producing endophytes associated with grasses, it was proposed, that endophytes that produce alkaloids ineffective to a certain attacker, would still have a protective effect against a chewing insect attacker (Bastías *et al.* 2017a). This is explained by the promotion of the JA signaling pathway as a consequence of the endophyte colonization process and the associated suppression of the SA pathway, as it was shown for beneficial soil-borne microbes (Bastías *et al.* 2017a, Zamioudis & Pieterse 2012 and references therein). However, whether this proposal can be adopted for endophyte-tree-insect interactions is questionable as several cases are described where endophytes had a neutral or positive effect on insect herbivores (**manuscript I**). As the specialist leaf beetle *C. tremulae* preferred stachydrine coated leaves in a leaf disc assay, it needs to be tested whether this preference persists in an *in planta* choice assay. Since we detected increased amounts of salicin after endophyte infection, we might expect a stronger preference of the leaf beetles towards endophyte-infected poplar plants.

Our finding that a plant endophyte produces a nitrogen-containing defense compound is relevant for the assessment of the nutritional value of the plant. It can no longer be assumed from measurements of total nitrogen that all nitrogen-containing compounds are beneficial to herbivorous insects (Bastías *et al.* 2017b). Nitrogen incorporated in toxic defense compounds, like alkaloids, glucosinolates, or cyanogenic glucosides (Bastías *et al.* 2017b, Karban & Agrawal 2002, Schoonhoven *et al.* 2005, Walters 2011) may be harmful and so full chemical profiles of endophytes in woody plant species should be considered in future studies focusing on plant-insect interactions.

In the field, we were able to detect stachydrine along with the endophyte *Cladosporium* sp. in natural old growth black poplar trees (unpublished) showing the importance of this compound under natural conditions. Whether stachydrine is exclusively produced by *Cladosporium* sp., or also by other endophytes previously isolated from black poplar trees (**manuscript II**) needs to be tested. Teimoori-Boghsani *et al*. (2019) were able to detect stachydrine in the endophytic fungus *Fusarium dlaminii* isolated from *Salvia abrotanoides* roots. As we also found *Fusarium* sp. in old growth black poplar trees (**manuscript II**), further analysis needs to be done to find out, whether this endophyte is also able to produce stachydrine.

6.4 Endophytic fungus shapes arthropod community composition in the field

Having shown in **manuscript III** that the endophytic fungus *Cladosporium* sp. caused induction of several poplar-derived defense compounds and, in addition, contributed to the plant's defense chemistry by its own production of the alkaloid stachydrine, we next explored how this endophyte-plant interaction would affect the arthropod community under field conditions.

Endophyte infection caused significant changes in the number of visiting insects for Hemiptera, Hymenoptera and Coleoptera (**manuscript III**). Species of the order Hemiptera were counted more often on plants harboring the endophyte, with aphids of the suborder Sternorrhyncha making up the largest proportion with 93.75 % (**manuscript III**). Alkaloids are commonly known for their negative effects on various insect species, including aphids (Bastías *et al.* 2017a, Bastías *et al.* 2017b, Shymanovich *et al.* 2015). In contrast, Siegel *et al*. (1990) could not show a detrimental effect of alkaloids on aphid populations, concluding that not all alkaloids are effective against aphids. It is argued that plants with ineffective alkaloids would be even more susceptible to sap-sucking insects, taking into account that endophytes suppress the SA pathway (a well-known defense strategy against sucking insects) for their successful colonization (Bastías *et al.* 2017a). In our field data, we could not detect any changes of SA among the treatments (**supplemental of manuscript III**). Further, we could show that the endophyte is able to inhabit the mutualistic interaction between aphids and ants. In contrast to the findings for Hemiptera species preferring endophytic plants, we more frequently counted Hymenoptera species (94.16 % Formicidae) on non-endophytic control plants (**manuscript III**). It has been shown that the mutualistic relationship between aphids and ants likely depends on the quality of the honeydew. For example, aphids feeding on plants containing high levels of cardenolides, were visited less often by ants due to the aphids excreting cardenolides via the honeydew (Züst & Agrawal 2017). Whether aphids exposed to plants harboring *Cladosporium* sp. excrete stachydrine in their honeydew or are able to detoxify stachydrine, needs to be tested. Like Hymenoptera, Coleoptera species were also found more frequently on control plants, with species of the family Chrysomelidae occurring exclusively on control plants (**manuscript III**). These results are in contrast to our expectations derived from the increase of salicin after endophyte infection, a chemical defense tolerated by specialist chrysomelid beetles and the observed preference of a chrysomelid beetle for stachydrine (**manuscript III**). Such seeming contradictions underscore the complexity of endophyte-plant interactions under field conditions.

Furthermore, we could not detect a significant difference of the leaf area loss between plants harboring the endophyte or not (**manuscript III**). Other studies have found a negative correlation

between the density of endophytes and herbivory damage in trees (Albrectsen *et al.* 2010, González-Teuber 2016). However, studies focusing on a single endophyte and its impact on plant-insect interactions under natural conditions are scarce, especially those employing horizontally transmitted endophytes in herbaceous and woody plants. It is proposed that horizontally transmitted endophytes are less mutualistic compared to vertically transmitted endophytes (**manuscript I**) (Herre *et al.* 1999 and references therein, Van Bael *et al.* 2009) and act in a species-specific manner on herbivores or only on those with a certain level of feeding specialization (Gange *et al.* 2012). For example, this was shown with several herbivorous insects feeding on creeping thistle (*Cirsium arvense*) inoculated with different endophytes (Gange *et al.* 2012).

As endophytes affect the biochemistry of host plants and can equip their hosts with new allelochemicals, they may have a cascading effect on arthropod communities (Clay & Schardl 2002). Comparing chemical results obtained from the lab experiments with field data, however, revealed few similar patterns except for the presence of stachydrine (**manuscript III**). The differences between laboratory and field results might be explained by the longer time span the plants were exposed to endophytes in the field, the different dynamics for the induction and depletion of defense compounds under the two settings, or variability in the insect species present and the time span between the last herbivory event and the sampling time (Fabisch *et al.* 2019, Karban 2011, Wang *et al.* 2015a). Stachydrine, however, stands out as a stable marker for the endophyte, and as an allelochemical with the potential to shape insect communities.

To summarize, a single endophytic fungus influenced the preference of different insect orders and species in different ways: The generalist *L. dispar* in the lab, and coleopteran and hymenopteran species in the field were deterred by the endophyte, while hemipteran species were attracted. Therefore a general role of the endophyte *Cladosporium* sp. as defense mutualist can be rejected, but we instead showed a more species-specific response to insect herbivores, which is consistent with the study of Gange *et al.* (2012). These results highlight the significant impact of endophytic fungi on plant-insect interactions, as a single endophyte is able to shape a whole arthropod community.

6.5 Conclusion and future perspectives

Horizontally transmitted endophytic fungi can be found in almost all plants. However, the potential of endophytes to alter plant defense compounds or to produce compounds of their own, and its resulting effects on plant-insect interactions are poorly understood, particularly in woody plant species. The results obtained here should change our perspective on plant-fungus and plant-insect interactions as

the isolated endophytes were found to emit VOCs that were previously reported to be released from their host plant black poplar and are known to mediate plant-insect interactions. To confirm the endophyte's release of volatiles, we investigated the volatile biosynthetic machinery of the endophyte *Cladosporium* sp. by characterizing its terpene synthases. Furthermore, the endophyte *Cladosporium* sp. directly and indirectly influenced host tree-insect interactions via the production of a bioactive alkaloid and the modulation of typical poplar defense compounds. Taken together, these processes affected poplar herbivorous insects in a species-specific way, and shaped the arthropod community in the field. These findings emphasize the importance of endophytic fungi for tree-herbivore interactions and suggest that these relationships are more than just simple two-way interactions between an insect and a plant.

This work also provides a firm basis for further research on topics ranging from the identification of molecular mechanisms to a broader knowledge about the ecological consequences of endophyte infection on arthropod community compositions. Until now, we have only analyzed a small fraction of cultivable endophytes from black poplar trees. New molecular approaches like metabarcoding will help to gain a deeper understanding of the whole endophyte community and to investigate which factors might determine the diversity and distribution of endophytes. Further, the combination of metabolomics and arthropod community data would provide a more comprehensive survey of tree-endophyte-arthropod interactions. Since literature has shown that endophyte infection affects interactions among plants, as was shown for grasses (Clay & Schardl 2002 and references therein), infestations with endophytic microorganisms might influence the species composition of the plant community and its successional progress. Since black poplar is described as a key species in the initial phase of succession of riparian forests, further studies on its endophytes may reveal much about plant community dynamics.

Screening for new compounds produced by endophytic fungi using modern metabolomic approaches will shed light on tree-insect, as well as tree-pathogen interactions. As we already identified endophytic VOCs with antimicrobial activity and an endophytic alkaloid with bioactive potential against herbivorous insects, further knowledge of the chemistry of endophytic fungi might have useful application in forestry or commercial wood production. Future studies, should investigate the role of the alkaloid stachydrine in the endophyte itself. At present, only 1-2 % of plant species have been investigated for endophytic associations (Khare *et al.* 2018, Strobel & Daisy 2003). Thus more research in this area is urgently needed to increase our understanding of these microorganisms and the roles they play in interactions with the plant, its herbivores and other microbes.

7. SUMMARY

Each plant species is exposed to a plethora of different microorganisms that colonize all organs and tissue types, including the surface and internal tissues. Microorganisms that colonize the inside of a plant tissue but do not act as pathogens, for at least a part of their life cycle, are called endophytes. Endophyte colonization is widespread in plant species, and it has been shown that endophytes are able to affect plant-insect interactions, either directly via production of metabolites that deter or attract insects, or indirectly via altering plant defense response against attackers. The impact of endophytes on plant-insect interactions is best described for vertically transmitted grass-associated endophytes. On the contrary, only a few studies have focused on the interaction between endophytes and woody plants, even though forests cover 33 % of our planet's landmass. Little is known about phytochemical changes upon endophyte colonization in trees and their consequences for single insect species as well as arthropod communities.

In my thesis, I studied the role of endophytic fungi in tree-insect interactions. Volatile organic compounds (VOCs) play a prominent role in the direct and indirect defenses of plants against insects. Since fungi are also known to emit volatiles, I investigated the volatile profile emitted by endophytic fungi isolated from mature black poplar (*Populus nigra*) trees. Among the isolated fungi, I investigated the biosynthesis of terpenes in the cosmopolitan fungus *Cladosporium* sp. and its effect on the quantities of defense compounds produced by poplar trees alone and in response to herbivory. In addition, the fungus itself was examined for its production of bioactive compounds and their effects on plant-insect interactions.

The endophytic fungi isolated from mature black poplar trees showed species-specific volatile emission patterns. Most of these fungi produced a blend of short-chained aliphatic alcohols and a diverse mixture of sesquiterpenes. These mixtures included VOCs with known bioactive properties against microbes, herbivores, or other plants. Remarkably, most endophytic fungi emitted VOCs that were also known to be produced by their host plant black poplar (**Figure 1, II Endophytic VOC emission**). To understand what controls the formation of VOCs in endophytes, we investigated two terpene synthases, biosynthetic enzymes involved in the formation of volatile terpenes.

The inoculation of black poplar saplings with the endophyte *Cladosporium* sp. resulted in an increase of constitutive as well as herbivore-induced poplar defense compounds (**Figure 1, IIIa Phytochemistry**). The generalist insect herbivore *Lymantria dispar* avoided feeding on plants harboring the endophyte, which might be explained by the increased plant defense response in

endophyte-inoculated plants. Furthermore, leaves of inoculated plants contained the alkaloid stachydrine, which is produced by the endophyte *Cladosporium* sp.. In a preference assay with stachydrine-coated leaf discs, herbivore insects responded in a species-specific manner to the alkaloid, with the generalist caterpillar *Amata mogadorensis* avoiding the stachydrine-coated leaves, while the specialist beetle *Chrysomela tremulae* consumed more of the stachydrine-coated leaves (**Figure 1, IIIb Preference assays**). In a field experiment, the presence of the endophytic fungus *Cladosporium* sp. changed the composition of plant-visiting arthropods with plants containing the endophyte being visited more often by hemipteran species (mainly aphids), while control plants were visited more frequently by coleopteran and hymenopteran species (mainly ants) (**Figure 1, IIIc Field experiment**). These findings support the hypothesis that the role of horizontally transmitted endophytes as defense mutualists is species-specific.

This thesis highlights the importance of including endophytic microorganisms in future plant-insect interaction studies since these microbes were demonstrated to (1) produce known bioactive VOCs (2) influence the concentrations of plant defense compounds, (3) produce their own defense compounds, and (4) modify the behavior of herbivorous insects. Consideration of endophytes allows a more holistic approach to studying plant-insect interactions, which have historically been treated as strict two-way relationships. Adding endophytes to the mix will undoubtedly make experiments more complex, but will surely increase our understanding of both the mechanisms and outcomes of plant-insect interactions.

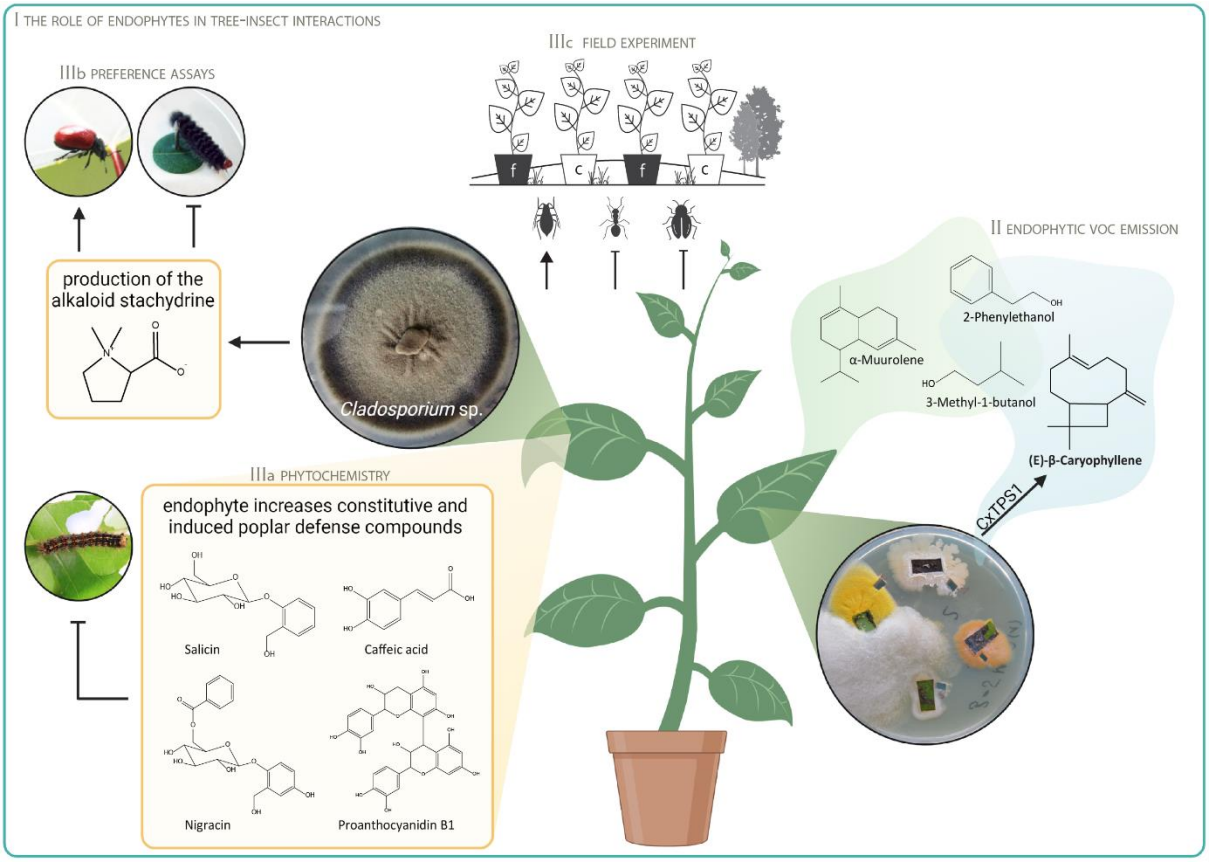

Figure 1. Graphical summary of the direct and indirect effects of endophytes on tree-insect interactions, focusing on black poplar (*Populus nigra*) -associated endophytic fungi and their influence on herbivorous insects. Even though endophytes can be found in all plants, their impact on the phytochemistry and the resulting consequences for plant-insect interactions is less studied, especially in woody plants (**I The role of endophytes in tree-insect interactions, manuscript I**). Endophytic fungi isolated from mature black poplar trees produce volatile organic compounds (VOCs), which are also known to be emitted by the host plant itself, e.g., 2-phenylethanol, 3-methyl-1-butanol, α-muurolene, and (*E*)-β-caryophyllene (**II Endophytic VOC emission, manuscript II**). In the endophytic fungus *Cladosporium* sp. (*E*)-β-caryophyllene is biosynthesized by the fungal terpene synthase CxTPS1. Further, the inoculation of the endophyte *Cladosporium* sp. in young poplar trees induces several anti-herbivore defense compounds that deter the generalist caterpillar *Lymantria dispar* (**IIIa Phytochemistry, manuscript III**). The endophytic fungus *Cladosporium* sp. is also able to produce the alkaloid stachydrine, which is detectable in inoculated plants. This compound shows a species-specific response, as it deters the larvae of the generalist caterpillar *Amata mogadorensis* and attracts the specialist beetle *Chrysomela tremulae* (**IIIb Preference assays, manuscript III**). Furthermore, the above-mentioned endophytic fungus can shape a whole arthropod community in the field, as it attracts hemipteran species (mainly aphids), and deters coleopteran and hymenopteran species (mainly ants) (**IIIc Field experiment, manuscript III**). These findings highlight the significance of endophytic fungi as mediators in tree-insect interactions. Figure created with BioRender.com.

8. ZUSAMMENFASSUNG

Jede Pflanzenart ist einer Vielzahl verschiedener Mikroorganismen ausgesetzt, die alle Organe und Gewebearten, einschließlich der Oberfläche und des inneren Gewebes, besiedeln. Mikroorganismen, die im Inneren eines Pflanzengewebes leben und zumindest einen Teil ihres Lebenszyklus keine Krankheiten in ihrer Wirtspflanze auslösen, werden als Endophyten bezeichnet. Die Besiedlung mit Endophyten ist im Pflanzenreich weit verbreitet, und es hat sich gezeigt, dass Endophyten in der Lage sind, die Wechselwirkungen zwischen Pflanzen und Insekten zu beeinflussen. Dies geschieht entweder direkt durch die Produktion von spezialisierten Verbindungen, die Insekten abschrecken oder anlocken, oder indirekt durch die Veränderung der pflanzlichen Abwehrreaktion gegen Angreifer. Die Auswirkungen von Endophyten auf Interaktionen zwischen Pflanzen und Insekten sind am besten für vertikal übertragene grasassoziierte Endophyten beschrieben. Im Gegensatz dazu haben sich nur wenige Studien auf die Interaktion zwischen Endophyten und Gehölzpflanzen konzentriert, obwohl Wälder 33 % der Landmasse unseres Planeten bedecken. Über die phytochemischen Veränderungen bei der Besiedlung von Bäumen mit Endophyten und deren Auswirkungen auf einzelne Insektenarten sowie auf Arthropodengemeinschaften ist hingegen wenig bekannt.

In meiner Dissertation untersuchte ich die Rolle endophytischer Pilze in Baum-Insekten-Interaktionen. Flüchtige organische Verbindungen (volatile organic compounds, VOCs) spielen eine wichtige Rolle bei der direkten und indirekten Abwehr von Pflanzen gegen Insekten. Da auch Pilze bekanntermaßen VOCs abgeben, untersuchte ich das Profil der flüchtigen Stoffe, die von endophytischen Pilzen abgegeben werden, welche zuvor aus ausgewachsenen Schwarzpappeln (*Populus nigra*) isoliert wurden. Unter den isolierten Pilzen untersuchte ich die Biosynthese von Terpenen in dem kosmopolitischen Pilz *Cladosporium* sp., sowie auch dessen Wirkung auf die pflanzlichen Abwehrstoffe, die von Pappelbäumen allein und als Reaktion auf Herbivorie gebildet werden. Darüber hinaus wurde der Pilz selbst auf die Produktion bioaktiver Verbindungen und deren Auswirkungen auf die Interaktionen zwischen Pflanzen und Insekten untersucht.

Die aus Schwarzpappeln isolierten endophytischen Pilze wiesen artspezifische Emissionsmuster flüchtiger Stoffe auf. Die meisten dieser Pilze produzierten eine Mischung aus kurzkettigen aliphatischen Alkoholen und verschiedenen Sesquiterpenen. Diese Mischungen enthielten VOCs mit bekannten bioaktiven Eigenschaften gegen Mikroben, Pflanzenfresser oder andere Pflanzen. Bemerkenswerterweise emittierten die meisten endophytischen Pilze Duftstoffe, von denen bekannt ist, dass sie auch von ihrer Wirtspflanze, der Schwarzpappel, produziert werden (**Abbildung 1, II**

Endophytic VOC emission). Um zu verstehen, was die Bildung von VOCs in Endophyten steuert, untersuchten wir zwei Terpensynthasen, biosynthetische Enzyme, die an der Bildung flüchtiger Terpene beteiligt sind.

Die Inokulation von Schwarzpappel-Setzlingen mit dem Endophyten *Cladosporium* sp. führte zu einem Anstieg der konstitutiven sowie der durch Herbivoren induzierten Abwehrstoffe der Pappel (**Abbildung 1, IIIa Phytochemistry**). Die generalistische Raupe *Lymantria dispar* vermied es, sich von Pflanzen zu ernähren, die den Endophyten enthielten, was durch die verstärkte Abwehrreaktion in Endophyten geimpften Pflanzen erklärt werden kann. Außerdem enthielten die Blätter der beimpften Pflanzen das Alkaloid Stachydrin, das vom Endophyten *Cladosporium* sp. produziert wird. In einem Präferenztest mit Stachydrin-beschichteten Blattscheiben reagierten pflanzenfressende Insekten artspezifisch auf das Alkaloid: während die generalistische Raupe *Amata mogadorensis* die Stachydrin-beschichteten Blätter mied, bevorzugte der spezialisierte Käfer *Chrysomela tremulae* die mit Stachydrin-beschichteten Blätter (**Abbildung 1, IIIb Preference assays**). In einem Feldexperiment veränderte das Vorhandensein des endophytischen Pilzes *Cladosporium* sp. die Zusammensetzung der pflanzenbesuchenden Arthropoden: Pflanzen, die den Endophyten enthielten, wurden häufiger von Hemipteren-Arten (hauptsächlich Blattläusen) besucht, während die Kontrollpflanzen häufiger von Coleopteren- und Hymenopteren-Arten (hauptsächlich Ameisen) aufgesucht wurden (**Abbildung 1, IIIc Field experiment**). Diese Ergebnisse stützen die Hypothese, dass die Rolle von horizontal übertragenen Endophyten als Verteidigungs-Mutualisten eher artspezifisch ist.

Diese Arbeit unterstreicht, wie wichtig es ist, endophytische Mikroorganismen in künftige Studien zur Interaktion zwischen Pflanzen und Insekten einzubeziehen, da diese Mikroben nachweislich (1) bekannte bioaktive flüchtige Stoffe produzieren, (2) die Konzentrationen pflanzlicher Abwehrstoffe beeinflussen, (3) ihre eigenen Abwehrstoffe produzieren und (4) das Verhalten pflanzenfressender Insekten verändern. Die Berücksichtigung von Endophyten ermöglicht einen ganzheitlicheren Ansatz bei der Untersuchung der Wechselwirkungen zwischen Pflanzen und Insekten, die in der Vergangenheit als streng zweiseitige Beziehung behandelt wurden. Durch die Einbeziehung von Endophyten werden die Experimente zweifellos komplexer, dafür tragen die Erkenntnisse zu einem besseren Verständnis über die Mechanismen und Ergebnisse der Interaktionen zwischen Pflanzen und Insekten bei.

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11. EIGENSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass mir die geltende Promotionsordnung der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena bekannt ist. Entsprechend § 5 Abs. 4 der Promotionsordnung bestätige ich, dass ich diese Dissertation selbst angefertigt habe und keine Textabschnitte eines Dritten oder eigener Prüfungsarbeiten ohne Kennzeichnung übernommen habe. Weiterhin habe ich alle benutzten Hilfsmittel und Quellen angegeben. Personen, die mich bei der Erhebung und Auswahl des Materials sowie bei der Erstellung der Manuskripte unterstützt haben, sind am Anfang der jeweiligen Kapitel der Manuskripte sowie im Anhang genannt oder werden, im Falle von Beiträgen geringeren Ausmaßes, in der Danksagung genannt. Ich habe keine Hilfe eines Promotionsberaters in Anspruch genommen und es wurden im Zusammenhang mit dem Inhalt der Dissertation keine Geldwerte oder Leistungen unmittelbar oder mittelbar an Dritte weitergegeben. Die Dissertation wurde nicht bereits zuvor als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Weiterhin wurde keine gleiche, in wesentlichen Teilen ähnliche oder andere Abhandlung als Dissertation bei einer anderen Hochschule eingereicht.

Christin Walther Jena, den 28.10.2021

12. CURRICULUM VITAE

Christin Walther, née Uhe

Born: 28.01.1989 in Beckendorf-Neindorf now Oschersleben (Bode), Germany

Address: Jansonstraße 17, 07745 Jena

Contact: cwalther@ice.mpg.de

Theses

2017 - present PhD student, Max Planck Institute for Chemical Ecology, Jena, *"Fungal endophytes mediate tree-insect interactions*." Supervision: Prof. Dr. Jonathan Gershenzon, Prof. Dr. Holger Schielzeth

2013 - 2016 M. Sc. Evolution Ecology and Systematics
"Analysis of common vole (Microtus arvalis) movement within an heter-geneous matrix". Supervision: Prof. Dr. Stefan Halle, Dr. Hans-Ulrich Peter

2010 - 2013 B. Sc. Biology
"*Comparison of food preference in bank vole (Myodes glareolus) depending on the reproductive state*". Supervision: Prof. Dr. Stefan Halle, Dr. Hans-Ulrich Peter

Education

2017 - present PhD student, Max Planck Institute for Chemical Ecology, Jena.

2013 - 2016 M. Sc. Evolution Ecology and Systematics (1.3), Friedrich Schiller University, Jena.

2010 - 2013 B. Sc. Biology (2.0), Friedrich Schiller University, Jena.

2005 - 2009 Abitur (1.8), Tagore Schule, Berlin.

Work Experience

2013 - 2016 Student assistant, Department Biochemistry, Max Planck Institute for Chemical Ecology, Jena.

Nov – Dec 2015 Internship, Department Biochemistry, Max Planck Institute for Chemical Ecology, Jena.

2014 - 2015 Tutor, Department Ecology, Friedrich Schiller University, Jena.

List of Publications

Walther C., Bauman P., Luck K., Rothe B., Biedermann P.H.W., Gershenzon J., Köllner T.G., and S.B. Unsicker (2021). Volatile emission and biosynthesis in endophytic fungi colonizing black poplar leaves. Beilstein Journal of organic chemistry. DOI: 10.3762/bjoc.17.118.

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List of Conference contribution

Oral presentation

2020 Volatile emission from endophytic fungi colonizing black poplar leaves. Talk presented at *19. IMPRS Symposium, IMPRS*, Jena, Germany

2018 Volatiles released from endophytic fungi of black poplar leaves. Talk presented at *Gordon Research Conference-Plant volatiles: The role of plant volatiles in communication*, Barga, Italy

2016 Analysis of common vole movement within an heterogeneous matrix. Talk presented at *19. Christmas Symposium*, Department Ecology, Friedrich Schiller University, Jena, Germany

2015 Sozialverhalten bei heimischen Ambrosiakäfern. Talk presented at *24. Internationale Naturschutztagung „Zoologischer und botanischer Artenschutz in Mitteleuropa“*, Bad Blankenburg, Germany

2015 Sozialverhalten bei Ambrosiakäfern. Talk presented at *106. Tagung Thüringer Enthomologen “Insektenmonitoring”*, Erfurt, Germany

Poster presentation

2021 Walther C., Eberl F., Medina van Berkum P., Reichelt M., Mindt E., Gershenzon J., Unsicker S.B. The chemical ecology of complex plant-insect-microbe interactions. Poster presented at *Scientific Advisory Board Meeting,* Max Planck Institute for Chemical Ecology, Jena, Germany.

2019 Uhe C., Gershenzon J., Unsicker S. Beetle feeding in poplar changes the preference and performance of gypsy moth caterpillars. Poster presented at *Institute Symposium*, Max Planck Institute for Chemical Ecology, Jena, DE

2019 Uhe C., Gershenzon J., Unsicker S. Beetle-induced plant response leads to a shift in feeding preference of *Lymantria dispar* caterpillar. Poster presented at *18th IMPRS Symposium*, Max Planck Institute for Chemical Ecology, IMPRS, Dornburg, Germany

2019 Uhe C., Gershenzon J., Unsicker S. Beetle feeding in poplar changes the feeding preference of gypsy moth caterpillars. Poster presented at *Gordon Research Conference - Plant-Herbivore Interaction*, Ventura, CA, US

2018 Uhe C., Baumann P., Biedermann P., Unsicker S. (2018). Volatiles released from black poplar endophytes. Poster presented at *17th IMPRS Symposium*, International Max Planck Research School, Dornburg, Germany

Awards

March 2019 IMPRS travel award for best poster at the 18th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany

Dec 2016 Travel award for best talk at 19. Christmas Symposium, Department Ecology, Friedrich Schiller University, Jena, Germany

Scientific Activities

Aug 2021 presented at Girls' Science Camp: Versteckte Mikroorganismen beeinflussen die Pflanzen-Insekten Interaktion

Apr 2020 engaged in Medienarbeit Filmbericht (Terra X): Rätselhafte Phänomene-Diamentenfieber und sprechende Pflanzen

Apr 2019 presented at EES Colloquium: Beetle feeding in poplar changes the feeding preference of gypsy moth caterpillars

Dec 2017 presented at EES Colloquium: Behavioral and nutritional ecology of insects in poplar

Nov 2017 presented at 6. Lange Nacht der Wissenschaften in Jena: Können Pflanzen sprechen? Ja, aber man kann es nicht hören, sondern riechen!

Language Skills

German (mother tongue), English (fluent), Spanish (basic)

13. SUPPLEMENTARY MATERIAL

13.1 Manuscript I

Table S1: Taxonomic classification of tree, fungal and insect species presented in Table 1 of the main manuscript. Information was withdrawn from USDA (https://plants.usda.gov) for tree species, Mycobank (http://www.mycobank.org) for fungal species and Bug Guide (https://bugguide.net) for insect species. Species within trees, pathogens, endophytes and insects are listed in alphabetic order.

	Species	**Family**	**Order**	**Phylum**
Tree species	*Acacia dealbata*	Fabaceae	Fabales	Magnoliophyta
	Arbutus unedo	Ericaceae	Ericales	Magnoliophyta
	Betula pendula	Betulaceae	Fagales	Magnoliophyta
	Betula pubescens	Betulaceae	Fagales	Magnoliophyta
	Cinnamomum yabunikkei	Lauraceae	Laurales	Magnoliophyta
	Cordia alliodora	Boraginaceae	Lamiales	Magnoliophyta
	Embothrium coccineum	Proteaceae	Proteales	Magnoliophyta
	Picea glauca	Pinaceae	Pinales	Coniferophyta
	Picea rubens	Pinaceae	Pinales	Coniferophyta
	Pinus nigra	Pinaceae	Pinales	Coniferophyta
	Populus nigra	Salicaceae	Malpighiales	Magnoliophyta
	Populus spp.	Salicaceae	Malpighiales	Magnoliophyta
	Populus tremula	Salicaceae	Malpighiales	Magnoliophyta
	Pseudotsuga menziesii	Pinaceae	Pinales	Coniferophyta
	Quercus emoryi	Fagaceae	Fagales	Magnoliophyta
	Quercus garrayana	Fagaceae	Fagales	Magnoliophyta
	Quercus robur	Fagaceae	Fagales	Magnoliophyta
	Quercus rubra	Fagaceae	Fagales	Magnoliophyta
	Quercus spp.	Fagaceae	Fagales	Magnoliophyta
	Salix viminalis	Salicaceae	Malpighiales	Magnoliophyta
	Salix x cuspidata	Salicaceae	Malpighiales	Magnoliophyta
Fungal species (pathogens)	*Drepanopeziza populi*	Dermateaceae	Helotiales	Ascomycota
	Erysiphe alphitoides	Erysiphaceae	Erysiphales	Ascomycota
	Marssonia betulae	Dermateaceae	Helotiales	Ascomycota
	Melampsora allii-fragilis	Melampsoraceae	Pucciniales	Basidiomycota
	Melampsora epitea	Melampsoraceae	Pucciniales	Basidiomycota
	Melampsora larici-populina	Melampsoraceae	Pucciniales	Basidiomycota
	Melampsoridium betulinum	Pucciniastraceae	Pucciniales	Basidiomycota
	Melanopsichium onumae	Ustilaginaceae	Ustilaginales	Basidiomycota
	Phytophtora plurivora	Pythiaceae	Peronosporales	Oomycota
	Sphaeropsis sapinea	Botryosphaeriaceae	Botryosphaeriales	Ascomycota
	Uromycladium spp.	Pileolariaceae	Pucciniales	Basidiomycota
Fungal species	*Asteromella sp.*[1]	Mycosphaerellaceae	Capnoidales	Ascomycota

	Species	Family	Order	Phylum
(endophytes)	*Aureobasidium sp.*	Dothioraceae	Dothideales	Ascomycota
	Diplodia pinea	Botryosphaeriaceae	Botryosphaeriales	Ascomycota
	Discula quercina	Gnomoniaceae	Gnomoniaceae	Ascomycota
	Fusicladium sp.	Venturiaceae	Pleosporales	Ascomycota
	Melanconium sp.	Melanconidaceae	Diaporthales	Ascomycota
	Phialocephala sp.	Vibrisseaceae	Helotiales	Ascomycota
	Plectophomella sp.[2]	Botryosphaeriaceae	Botryosphaeriales	Ascomycota
	Rhabdocline parkeri	Hemiphacidiaceae	Helotiales	Ascomycota
	Talaromyces pinophilus	Trichocomaceae	Eurotiales	Ascomycota
	Phialocephala scopiformis	Vibrisseaceae	Helotiales	Ascomycota
Insect species	*Acronicta psi*	Noctuidae	Lepidoptera	Arthropoda
	Acyrthosiphon pisum	Aphididae	Hemiptera	Arthropoda
	Arge sp.	Argidae	Hymenoptera	Arthropoda
	Atta colombica	Formicidae	Hymenoptera	Arthropoda
	Bassettia ligni	Cynipidae	Hymenoptera	Arthropoda
	Besbicus mirabilis	Cynipidae	Hymenoptera	Arthropoda
	Cameraria sp.	Gracillariidae	Lepidoptera	Arthropoda
	Choristoneura fumiferana	Tortricidae	Lepidoptera	Arthropoda
	Contarinia spp.	Cecidomyiidae	Diptera	Arthropoda
	Cynipidae	Cynipidae	Hymenoptera	Arthropoda
	Deporaus betulae	Rhynchitinae	Coleoptera	Arthropoda
	Dineura pullior	Tenthredinidae	Hymenoptera	Arthropoda
	Epirrita autumnata	Geometridae	Lepidoptera	Arthropoda
	Eriophyes rudis	Eriophyidae	Trombidiformes	Arthropoda
	Euceraphis betulae	Aphididae	Hemiptera	Arthropoda
	Lambdina fiscellaria	Geometridae	Lepidoptera	Arthropoda
	Lymantria dispar	Erebidae	Lepidoptera	Arthropoda
	Neodiprion sertifer	Diprionidae	Hymenoptera	Arthropoda
	Phratora vitellinae	Chrysomelidae	Coleoptera	Arthropoda
	Phratora vulgatissima	Chrysomelidae	Coleoptera	Arthropoda
	Plagiodera versicolor	Chrysomelidae	Coleoptera	Arthropoda
	Priophorus pallipes	Tenthredinidae	Hymenoptera	Arthropoda
	Tischeria ekebladella	Tischeriidae	Lepidoptera	Arthropoda
	Zeiraphera canadensis	Tortricidae	Lepidoptera	Arthropoda

[1] Synonyme for *Mycosphaerella sp.*
[2] Synonyme for *Dothiorella sp.*

13.2 Manuscript II

Table S1: Endophytes were identified to genus level via sequencing of ribosomal DNA (ITS1F/ ITS4). The obtained sequences were compared to the NCBI sequence database and the identity (%) of best hits with their accession number is given in the main document.

Species	Sequence
Alternaria infectoria	TGTCTTTTGCGTACTTCTTGTTTCCTGGGTGGGCTCGCCCGCCCTCAGGACCAACCACAAAC CTTTTGCAATAGCAATCAGCGTCAGTAACAACGTAATTAATTACAACTTTCAACAACGGATC TCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAA TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCC TGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTTTGTCTCCAGTTC GCTGGAGACTCGCCTTAAAGTCATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCACAAG TCGCGCTCTTCGCCAGCCAAGGTCAGCGTCCAGCAAGCCTTTTTTTCAACCTTTGACCTCGG ATCAGGTAGGGATACCCG
Alternaria sp. 1	TTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAAT CAGTGTCAGTAACAAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCG ATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGA ATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTT GTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTA AAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTCTATCAGC AAAGGTCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGACCTCGGATCAGGTAGGGATACC CGCTGAACTTAAGCATATCAATAAGCGGAGGA
Stemphylium sp.	AAAAATGTGGTCTTGATGGATGCTCAACCAAGGCCGATTCAAAGTGCAAGAATTGTGCTGC GCTCCGAAACCAGTAGGTCGGCTGCCAATCATTTTAAGGCGAGTCTCGTGAGAGACAAAGA CGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAGGCATGCCCTTTGGA ATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACAC TACGTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAG TTGTAATAATTACATTGTTTACTGACGCTGATTGCAATTACAAAAAGGTTTATGGTTTGGTCC TGGTGGCGGGCGAACCCGCCCAGGAAACAAGAAGTGCGCAAAAGACATGGGTGAATAATT CAGACAAGCTGGAGCCCTCACCGAGGTGAGGTCCCAACCCGCTTTCATATTGTGTAATGAT CCCTCCGCAGGTTCACC
Aureobasidium sp. 1	GTCCCAGGCGAGCGCCCGCCAGAGTTAAACCAAACTCTTGTTATTTAACCGGTCGTCTGAGT TAAAATTTTGAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGA ACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA ACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTCGAGCGTCATTACACCACTC AAGCTATGCTTGGTATTGGGCGTCGTCCTTAGTTGGGCGCGCCTTAAAGACCTCGGCGAGG CCACTCCGGCTTTAGGCGTAGTAGAATTTATTCGAACGTCTGTCAAAGGAGAGGAACTCTG CCGACTGAAACCTTTATTTTTCTAGGTTGACCTCGGATCAGGTAGGGATACCC

Species	Sequence
Aureobasidium sp. 2	ATAAAGGTTTCAGTCGGCAGAGTTCCTCTCCTTTGACAGACGTTCGAATAAATTCTACTACG CCTAAAGCCGGAGTGGCCTCGCCGAGGTCTTTAAGGCGCGCCCAACTAAGGACGACGCCC AATACCAAGCATAGCTTGAGTGGTGTAATGACGCTCGAACAGGCATGCCCCTCGGAATACC AAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTT ATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTTGAAAGTTTTG ATTTATTCAAAATTTTAACTCAGACGACCGGTTAAATAACAAGAGTTTGGTTTAACTCTGGC GGGCGCTCGCCTGGGACGAATCCCCAGCGGCTCGAGACCGAGCGGTCCCGCCAAAGCAAC AAGGTAGTTTTAACAACAAAGGGTTGGAGGTCGGGCGCTGAGCACCCTTACTCTTTAATGA TCCTTCCGCAGGTTCACCTACGGAAGNGGATNATTAAAGAGTAAGGGTGCTCAGCGCCCG ACCTCCAACCCTTTGTTGTTAAAACTACCTTGTTGCTTTGGCGGGACCGCTCGGTCTCGAGC CGCTGGGATTCGTCCCAGGCGAGCGCCCGCCAGAGTTAAACCAAACTCTTGTTATTTAACCG GTCGTCTGAGTTAAAATTTNGAATAAATNAAAACTTTNACAACGGANCTCTTGGTTCTCGCA TCGA
Didymella glomerata	CCGCCGATTGGNCAATTTAAACNATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGT TACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGA TAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCT TGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTT GGGTGTTTGTCTCGCCTCTGCGTGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTG ATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGTACATTT TTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCG G
Didymella sp. 1	CCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGT TACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGA TAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCT TGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTT GGGTGTTTGTCTCGCCTCTGCGTGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTG ATTTCGGAGCGCAGTACATCTCG
Didymella sp. 2	CTTTTAAGTACCTTACGTTTCCTCGGCGGGTCCGCCCGCCGATTGGACAATTTAAACCATTTG CAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGT TCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGT GAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCG AGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCCTCTGCGTGTAG ACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTT TGCACTCATAACGACGACGTCCAAAAGTACATT
Cladosporium sp.	TCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAAC TTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC AGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC ACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGC CTCGCTTGGTATTGGGCAACGCG
Fusarium sp.	GGGACGGCCCGCCGCAGGAAACCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTATAAAAA ACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAG CAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC ATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCC AGCTTGGTGTTGGGATCTGTGTGCAAACACAGTCCCCAAATTGATTGGCGGTCACGTCGAG CTTCCATAGCGTAGTA

Species	Sequence
Sordaria sp.	CGGGCCCCCGGATCCTCGGGTCTCCCGCTCGCGGGAGGCTGCCCGCCGGAGTGCCGAAAC CAAACTCTTGATATTTTATGTCTCTCTGAGTAAACTTTTAAATAAGTCAAAACTTTCAACAAC GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG CAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGTATTCTGGCGAGC ATGCCTGTTCGAGCGTCATTTCAACCATCAAGCTCTGCTTGCGTTGGGGATCCGCGTCTGAC GCGGTCCCTCAAAAACAGTGGCGGGCTCGCTAGTCACACCGAGCGTAGTAACTCTACATCG CTATGGTCGTGCGGCGGGTTCTTGCCGTAAAACCCCCAATTTCTAAGGTTGACCTCGGATCA GGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
Arthrinium sp.	AAAAATGTGGTCTTGATGGATGCTCAACCAAGGCCGATTCAAAGTGCAAGAATTGTGCTGC GCTCCGAAACCAGTAGGTCGGCTGCCAATCATTTTAAGGCGAGTCTCGTGAGAGACAAAGA CGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAGGCATGCCCTTTGGA ATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACAC TACGTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAG TTGTAATAATTACATTGTTTACTGACGCTGATTGCAATTACAAAAAGGTTTATGGTTTGGTCC TGGTGGCGGGCGAACCCGCCCAGGAAACAAGAAGTGCGCAAAAGACATGGGTGAATAATT CAGACAAGCTGGAGCCCTCACCGAGGTGAGGTCCCAACCCGCTTTCATATTGTGTAATGAT CCCTCCGCAGGTTCACC

Table S2: Primers used in this study.

Name	Sequence	Purpose
CxTPS1_Fwd	CACCATGAGCTCTAGCACGGGTC	cloning
CxTPS1_Rev	TCACGACGCCCCTCG	cloning
CxTPS2_Fwd	CACCATGTCAGACCCTACTCGCC	cloning
CxTPS2_Rev	TCAGCAACACTCCAGATAGCTAGG	cloning
ITS1F	CTTGGTCATTTAGAGGAAGTAA	amplify fungal rRNA ITS
ITS4	TCCTCCGCTTATTGATATGC	amplify fungal rRNA ITS

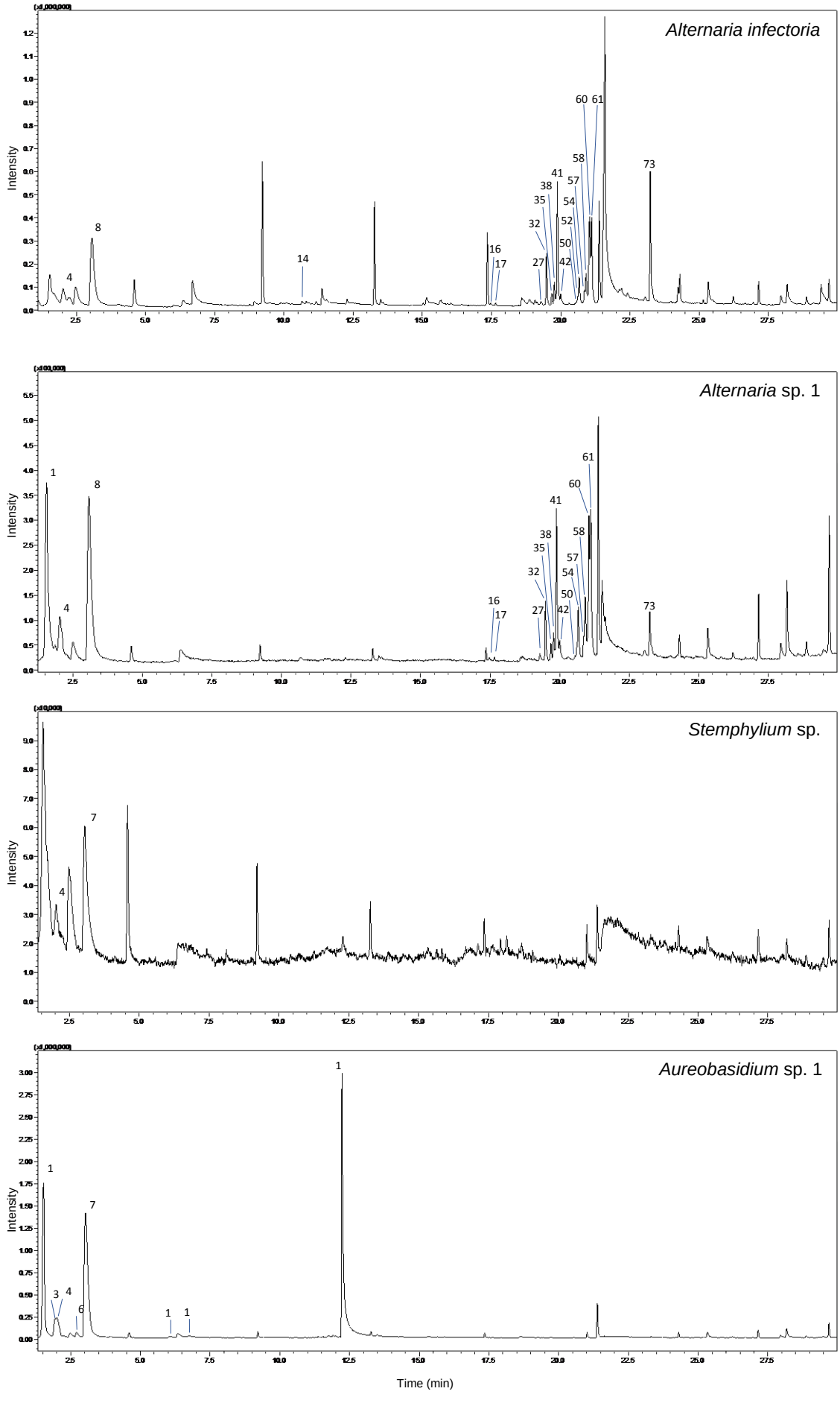
Table S3: Numbers for volatile organic compounds, which are shown in the total ion chromatograms of the volatile bouquet for each endophytic fungus in Figure S1.

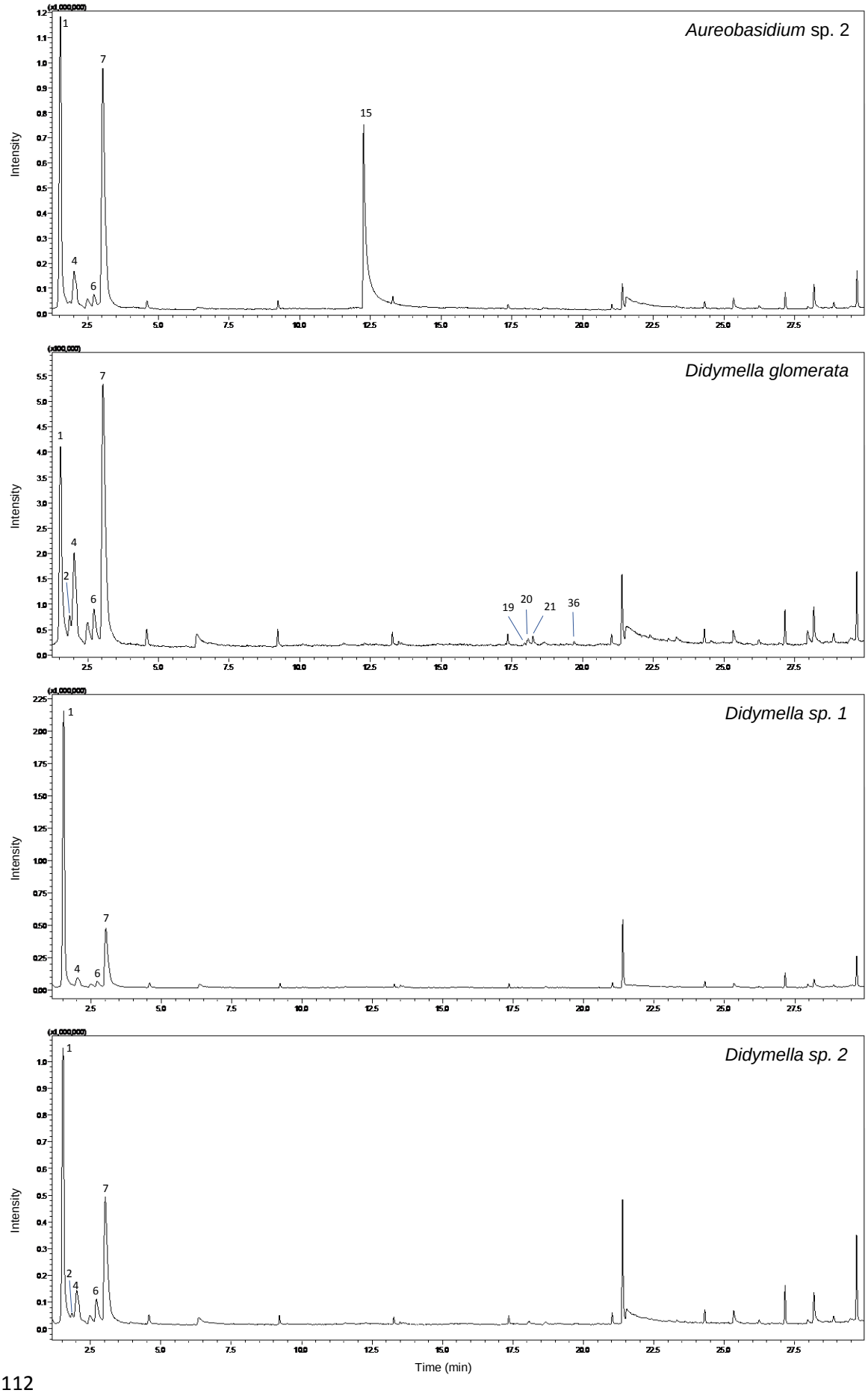
#	Volatile organic compound	R.T. (min)
1	Ethanol	1.525
2	2-Butanone	1.855
3	Ethyl Acetate	1.940
4	2-Methyl-1-propanol	2.020
5	unknown 1	2.265
6	3-Hydroxy-2-Butanone	2.720

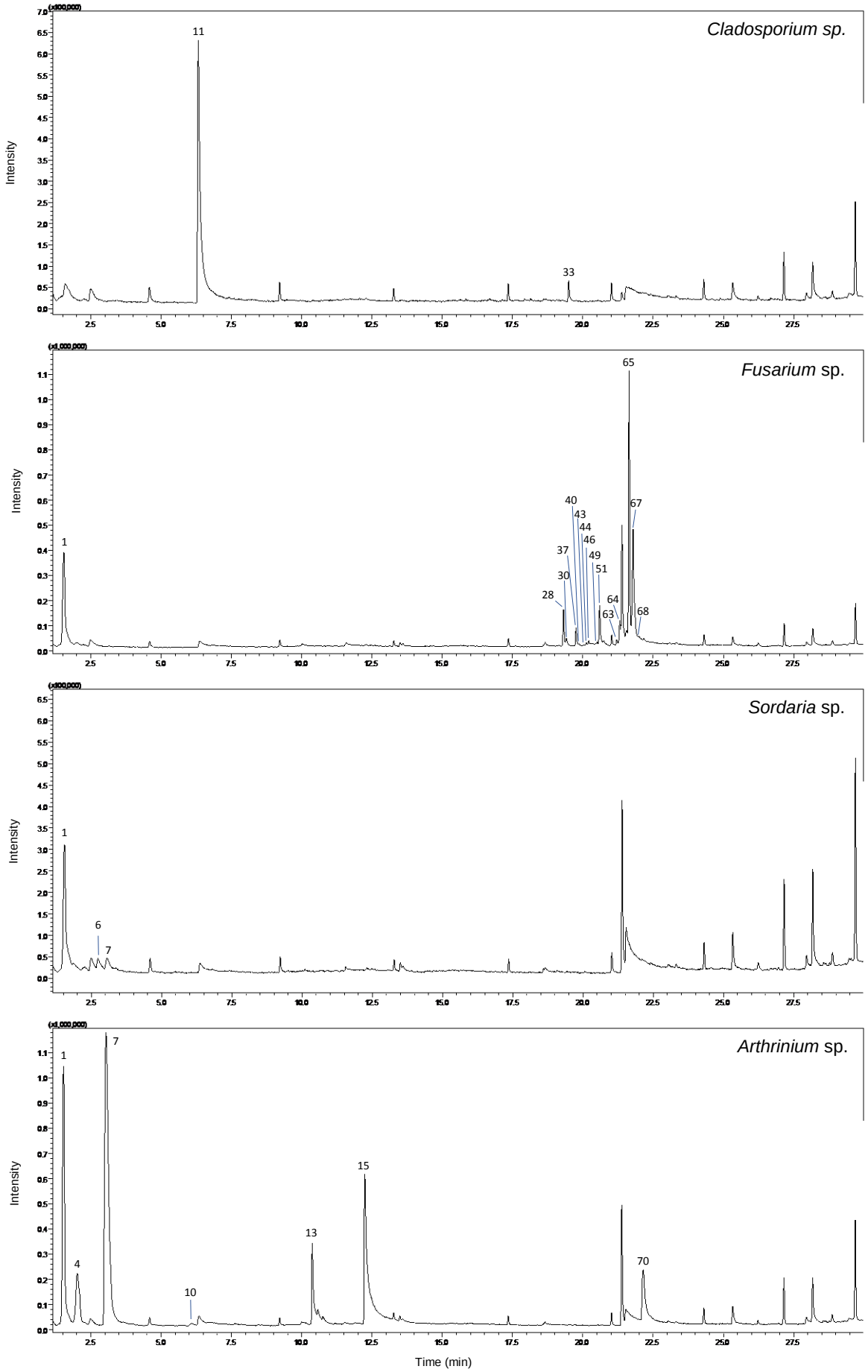
#	**Volatile organic compound**	**R.T. (min)**
7	3-Methyl-1-butanol	3.035
8	2-Methyl-1-butanol	3.070
9	unknown 2	3.780
10	3-Methylbutyl acetate	6.100
11	Ethenylbenzene	6.325
12	unknown 3	6.730
13	unknown 4	10.380
14	unknown 5	10.655
15	2-Phenylethanol	12.240
16	unknown 6	17.465
17	unknown 7	17.650
18	α-Cubebene	17.920
19	unknown 8	17.950
20	unknown 9	18.075
21	unknown 10	18.245
22	unknown 11	18.330
23	α-Copaene	18.525
24	unknown 12	18.765
25	unknown 13	18.850
26	Sativene	18.985
27	α-Gurjunene	19.280
28	unknown 14	19.300
29	unknown 15	19.380
30	unknown 16	19.400
31	unknown 17	19.460
32	Aristolene	19.485
33	(*E*)-β-Caryophyllene	19.500
34	unknown 18	19.535
35	unknown 19	19.675
36	unknown 20	19.695

#	**Volatile organic compound**	**R.T. (min)**
37	Bicyclosesquiphellandrene	19.745
38	β-Gurjunene	19.775
39	unknown 21	19.790
40	unknown 22	19.845
41	unknown 23	19.880
42	α-Guaiene	20.000
43	unknown 24	20.015
44	unknown 25	20.120
45	unknown 26	20.140
46	(*E*)-β-Farnesene	20.195
47	unknown 27	20.325
48	unknown 28	20.440
49	unknown 29	20.490
50	unknown 30	20.555
51	β-Chamigrene	20.585
52	unknown 31	20.610
53	unknown 32	20.610
54	α-Selinene	20.665
55	γ-Muurolene	20.685
56	unknown 33	20.800
57	unknown 34	20.860
58	β-Selinene	20.910
59	unknown 35	20.930
60	(+)-Valencene	21.040
61	unknown 36	21.115
62	α-Muurolene	21.170
63	β-Himachalene	21.195
64	β-Bisabolene	21.305
65	unknown 37	21.630
66	unknown 38	21.635

#	Volatile organic compound	R.T. (min)
67	unknown 39	21.775
68	unknown 40	21.985
69	unknown 41	22.075
70	unknown 42	22.150
71	unknown 43	22.355
72	unknown 44	22.720
73	unknown 45	23.235
74	unknown 46	23.875
75	unknown 47	24.485
76	unknown 48	24.675
77	unknown 49	25.980


Alternaria infectoria
Alternaria sp. 1
Stemphylium sp.
Aureobasidium sp. 1
Intensity
Time (min)


Aureobasidium sp. 2
Didymella glomerata
Didymella sp. 1
Didymella sp. 2
Intensity
Time (min)


Cladosporium sp.
11
33
Fusarium sp.
65
40
43
44
46
37
49
51
30
28
64
63
67
68
1
Sordaria sp.
1
6
7
Arthrinium sp.
1
7
15
13
4
10
70
Intensity
Time (min)

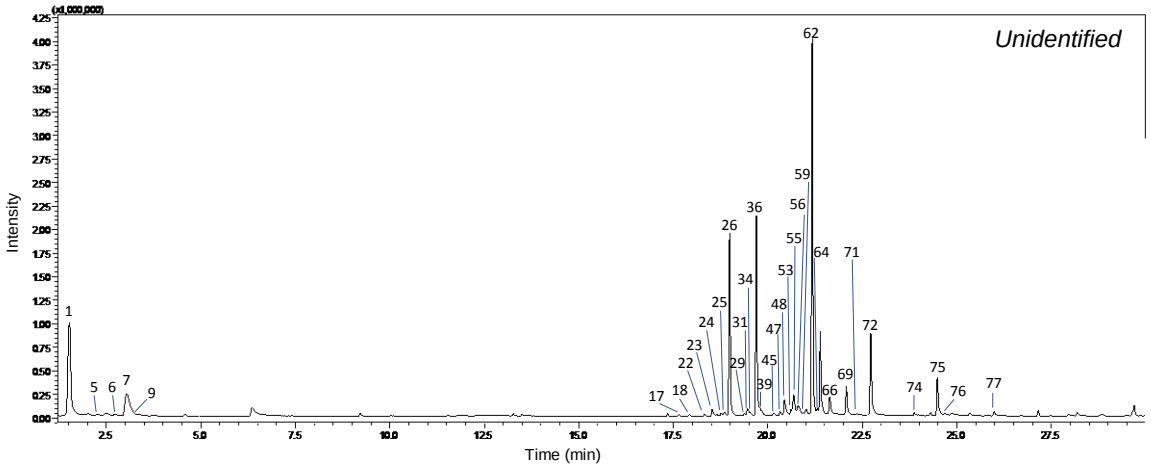
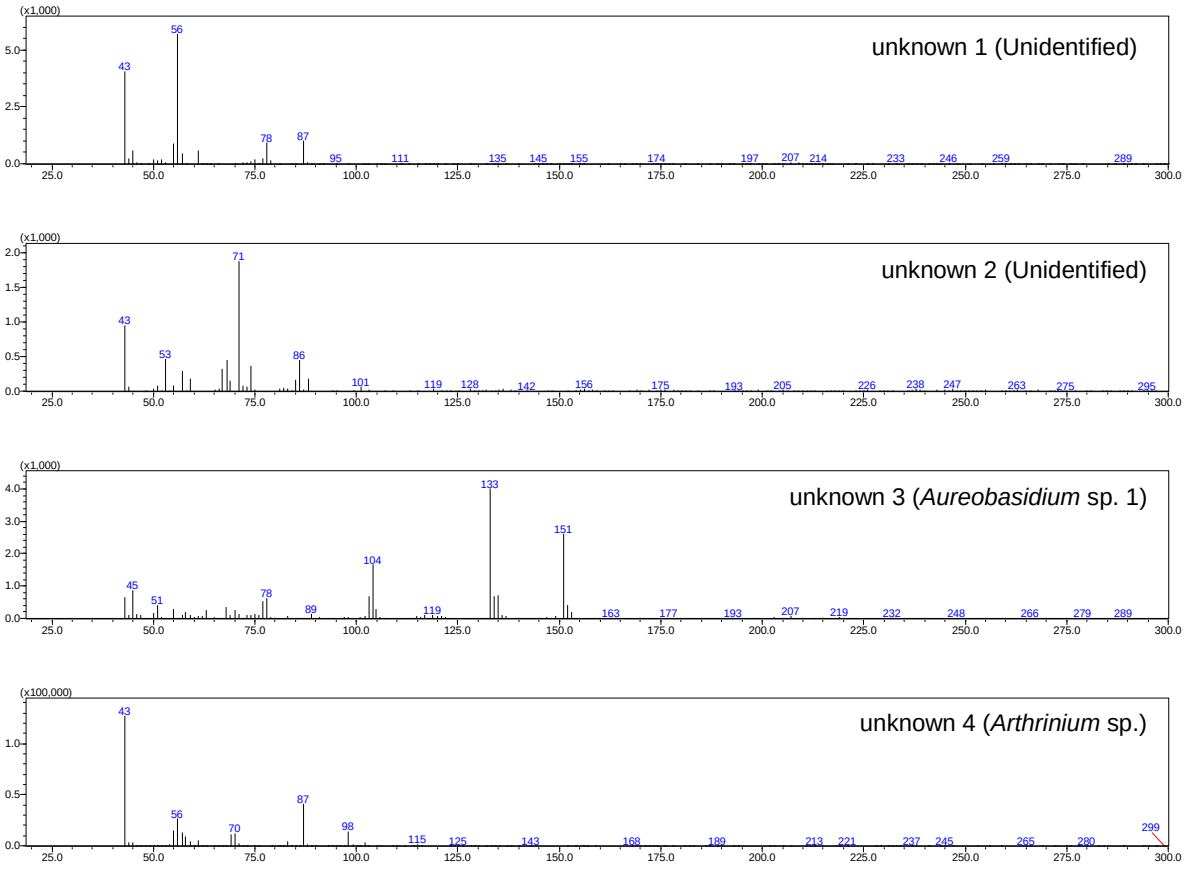
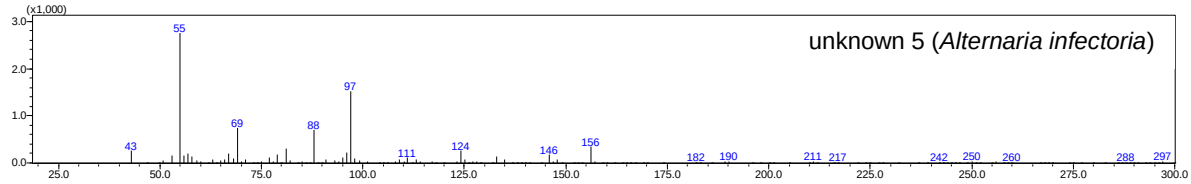
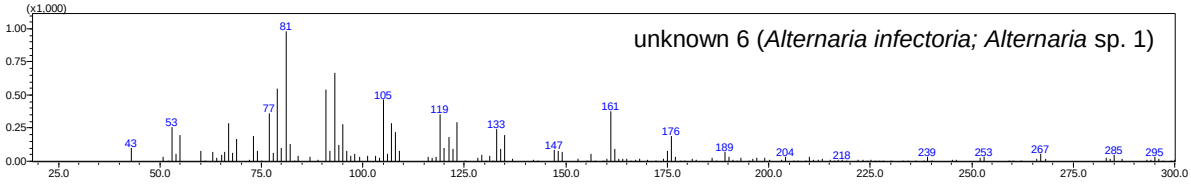
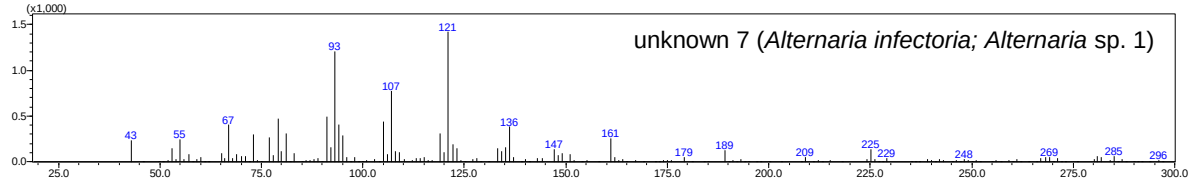


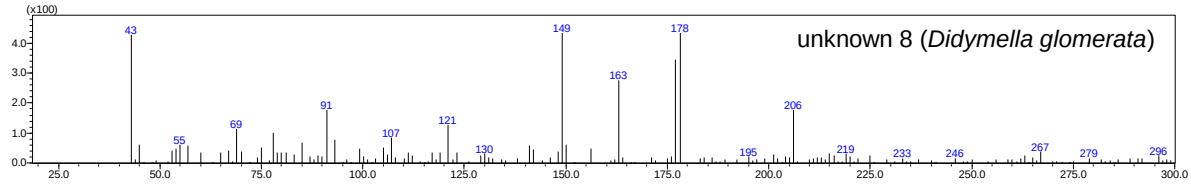
Figure S1: Representative total ion chromatogram of volatiles measured from different endophytes used in this study. Numbers indicate different volatile organic compounds, listed in Table S3. Peaks without numbers are either contamination from the PDMS tube or originate from the culture media itself.

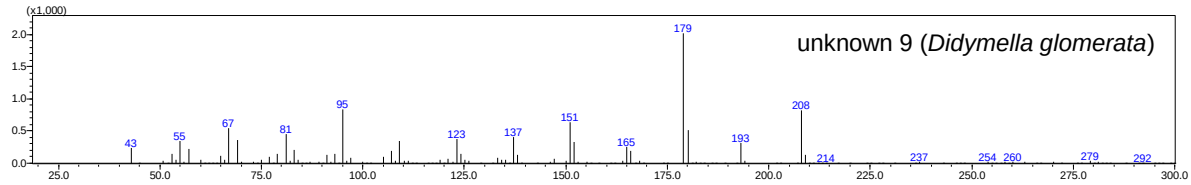



(x1,000)
unknown 5 (*Alternaria infectoria*)


(x1,000)
unknown 6 (*Alternaria infectoria; Alternaria* sp. 1)


(x1,000)
unknown 7 (*Alternaria infectoria; Alternaria* sp. 1)


(x100)
unknown 8 (*Didymella glomerata*)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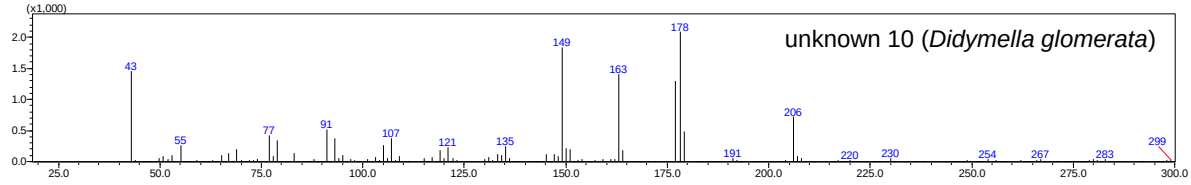
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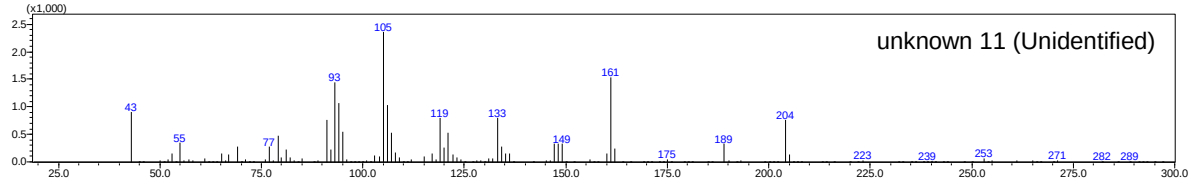
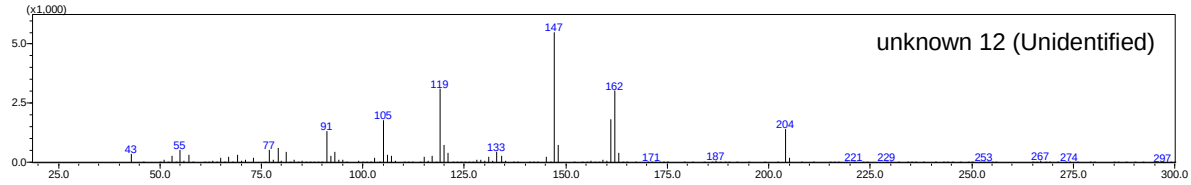

(x1,000)
unknown 11 (Unidentified)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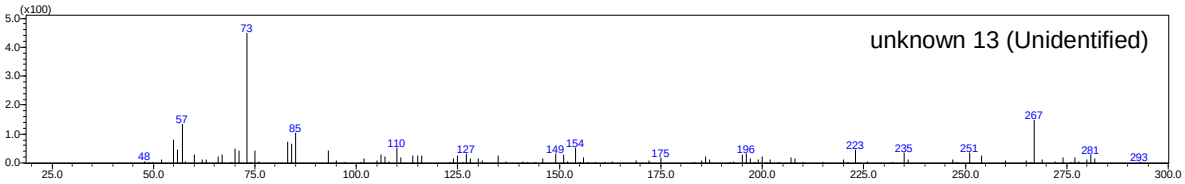
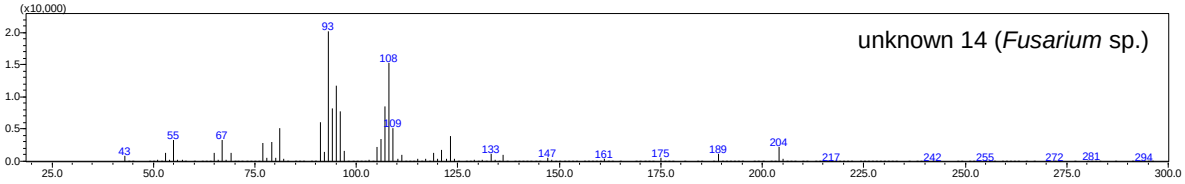

(x100)
unknown 13 (Unidentified)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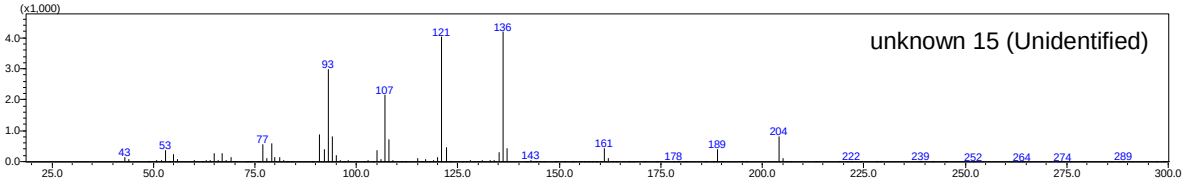
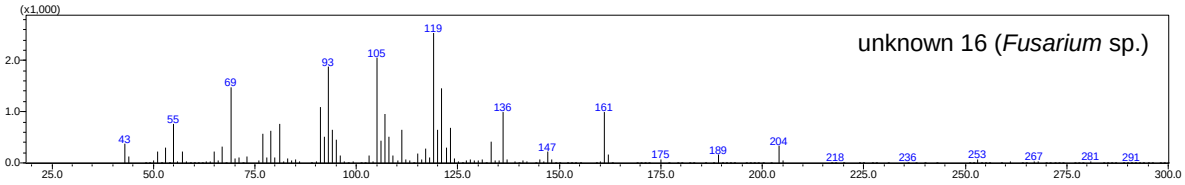

(x1,000)
unknown 15 (Unidentified)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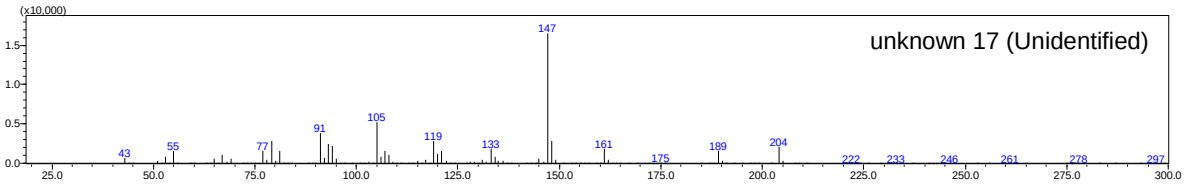
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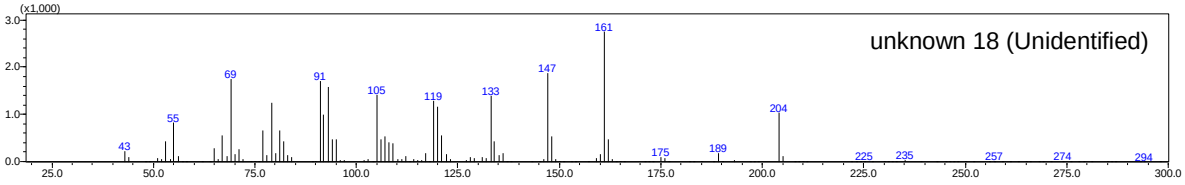
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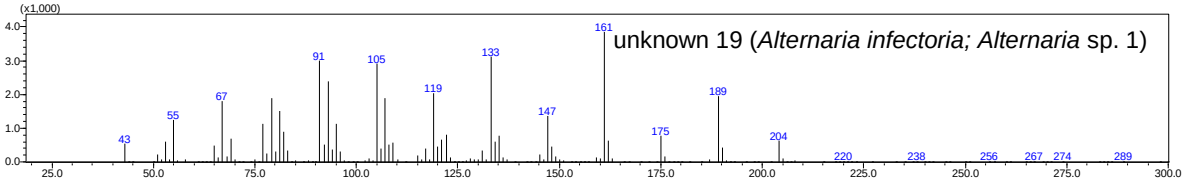
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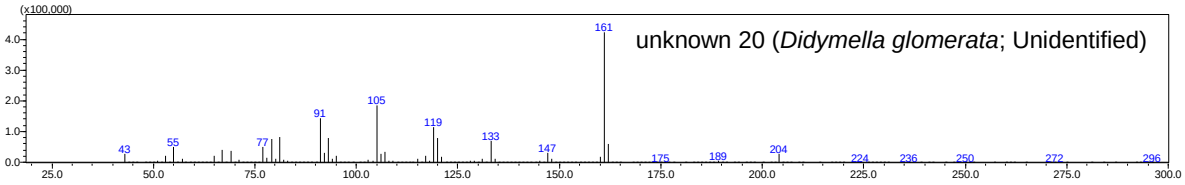
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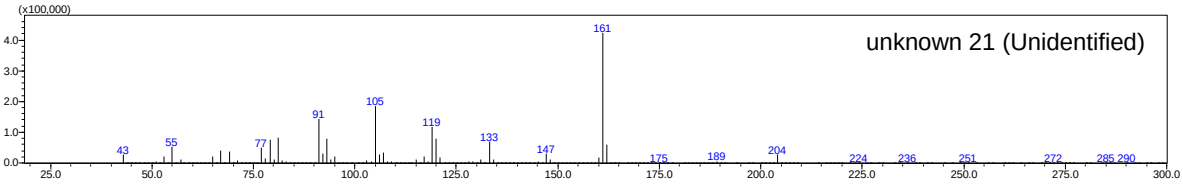
unknown 20 (*Didymella glomerata*; Unidentified)


(x100,000)
unknown 21 (Unidentified)
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91
119
133
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189
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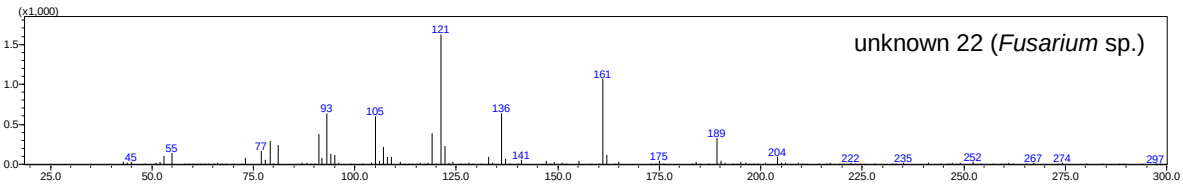
251
272
285
290


(x1,000)
unknown 22 (*Fusarium* sp.)
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105
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77
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204
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252
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274
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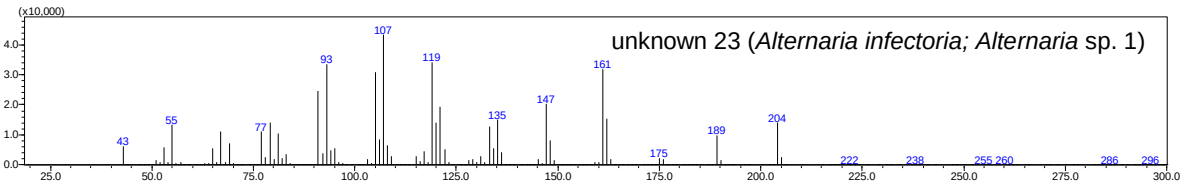
(x10,000)
unknown 23 (*Alternaria infectoria; Alternaria* sp. 1)
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119
161
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77
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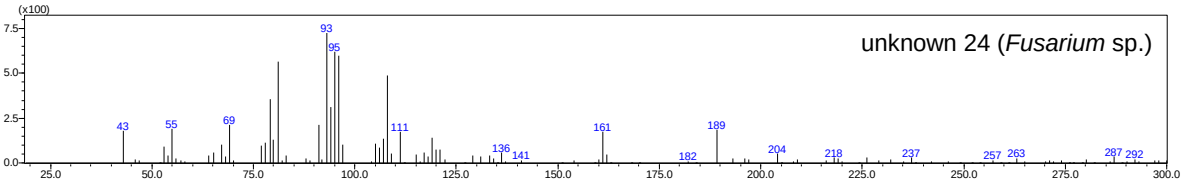
286
296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unknown 24 (*Fusarium* sp.)
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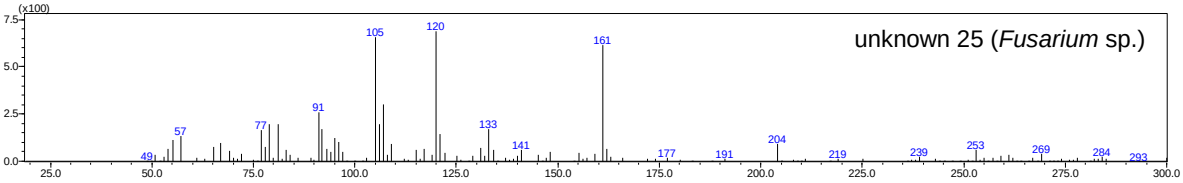
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unknown 25 (*Fusarium* sp.)
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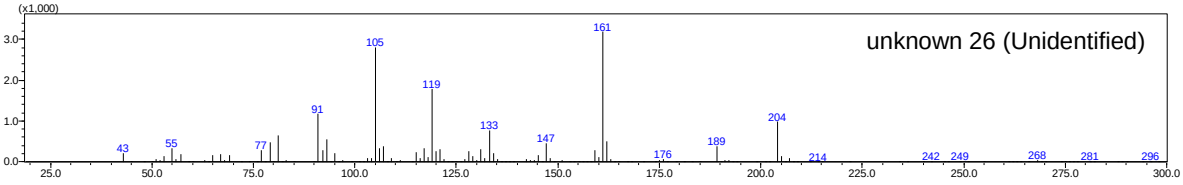
(x1,000)
unknown 26 (Unidentified)
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176
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214
242
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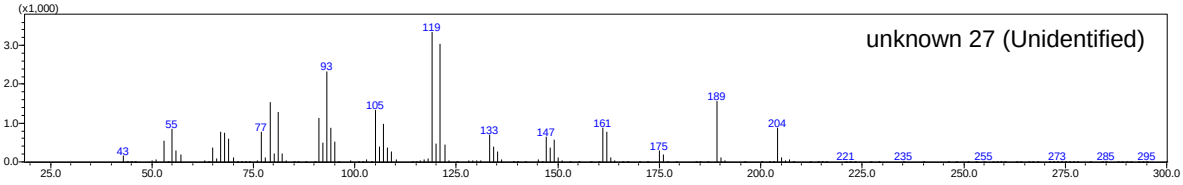
(x1,000)
unknown 27 (Unidentified)
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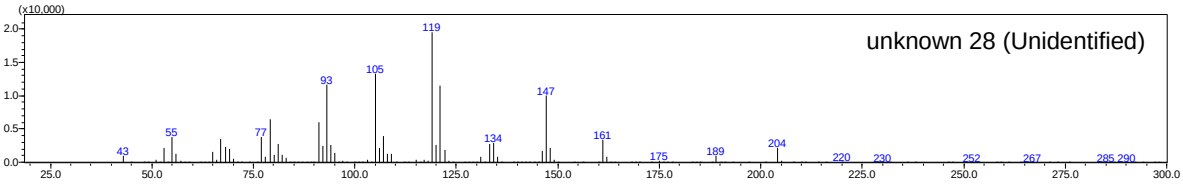
(x10,000)
unknown 28 (Unidentified)
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147
55
77
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161
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43
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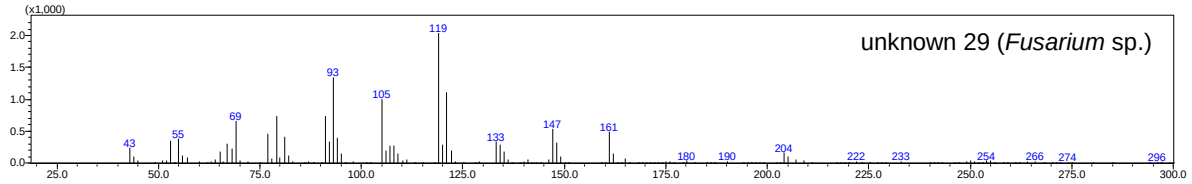
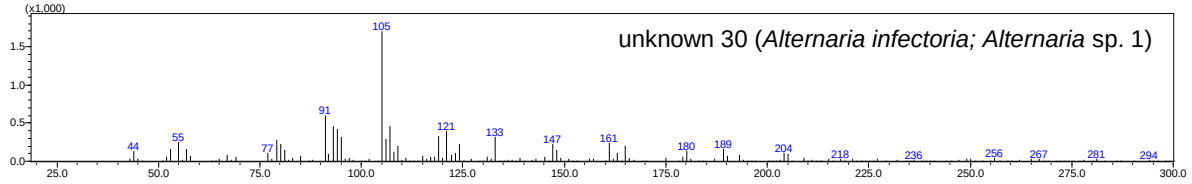
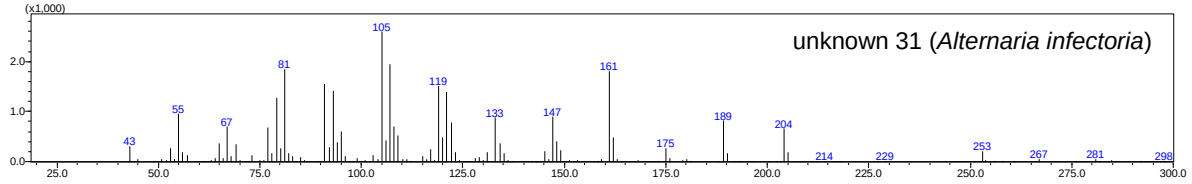
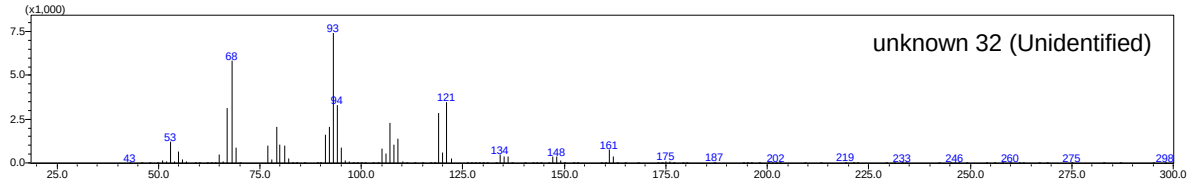
252
267
285
290


(x1,000)
unknown 29 (*Fusarium* sp.)


(x1,000)
unknown 30 (*Alternaria infectoria; Alternaria* sp. 1)


(x1,000)
unknown 31 (*Alternaria infectoria*)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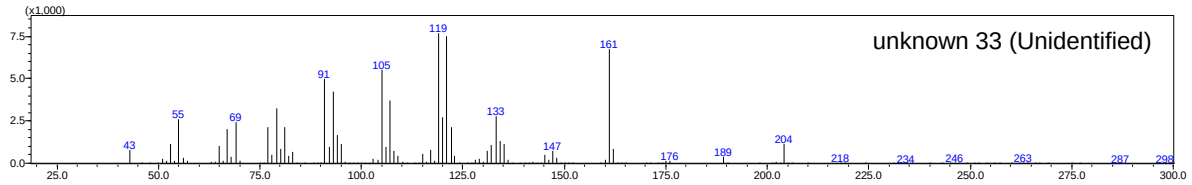
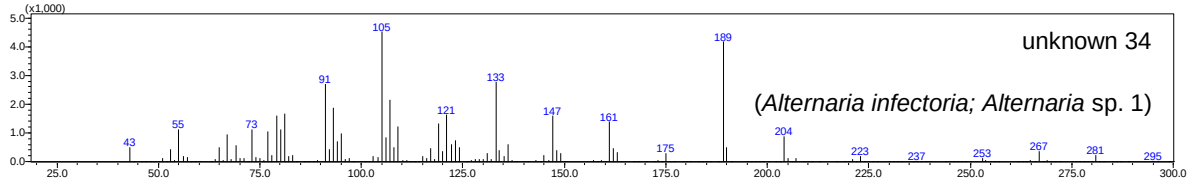
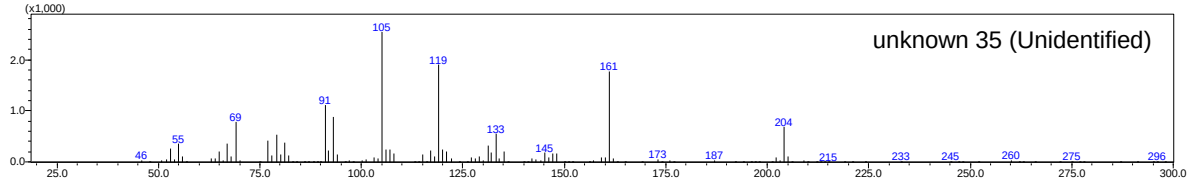
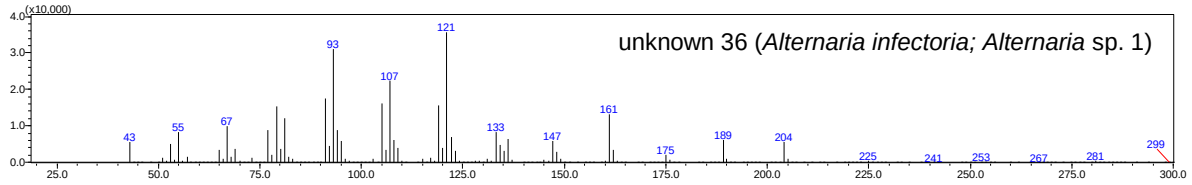
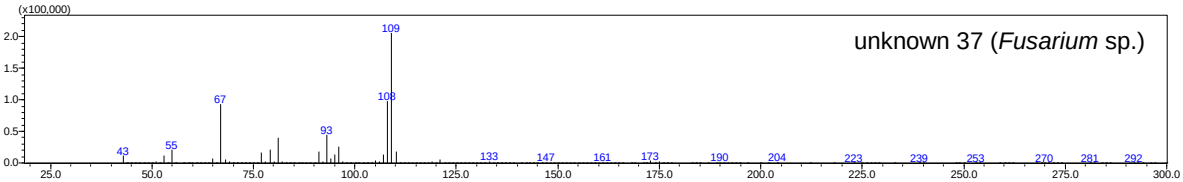
unknown 32 (Unidentified)

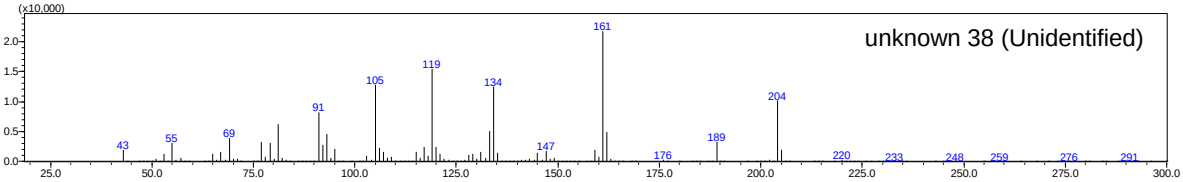

(x1,000)
unknown 33 (Unidentified)


(x1,000)
unknown 34
(*Alternaria infectoria; Alternaria* sp. 1)


(x1,000)
unknown 35 (Unidentified)


(x10,000)
unknown 36 (*Alternaria infectoria; Alternaria* sp. 1)


(x100,000)
unknown 37 (*Fusarium* sp.)
109
108
67
93
43
55
133
147
161
173
190
204
223
239
253
270
281
292


(x10,000)
unknown 38 (Unidentified)
161
119
105
134
91
204
43
55
69
147
176
189
220
233
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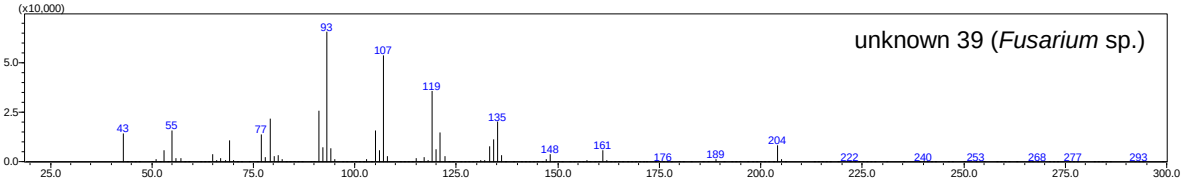
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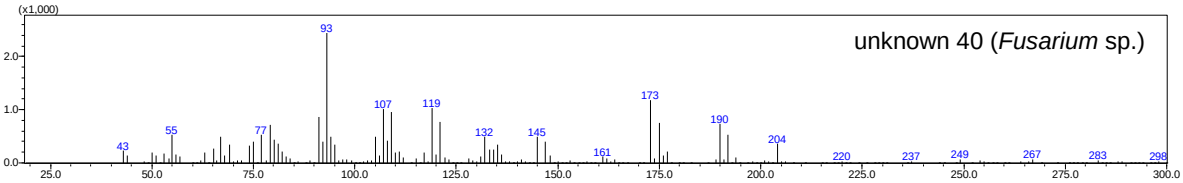
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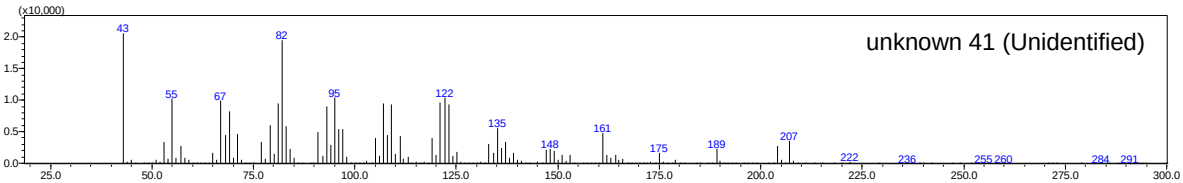
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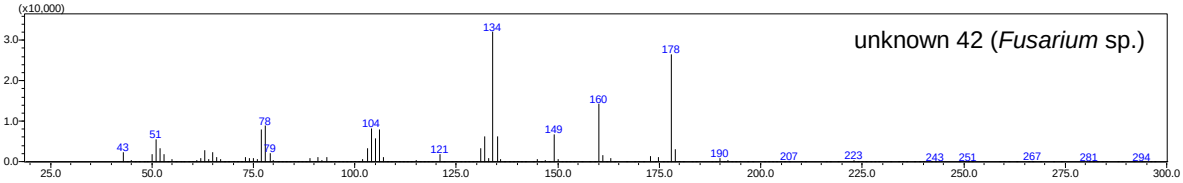
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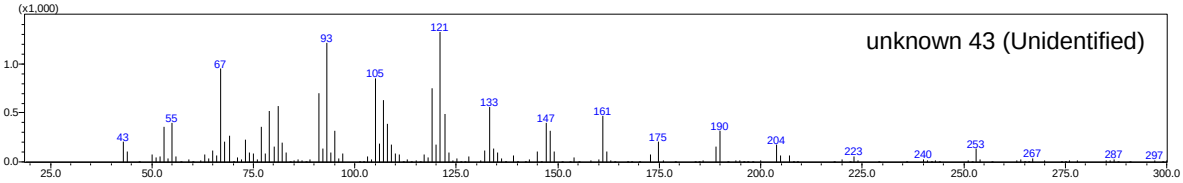
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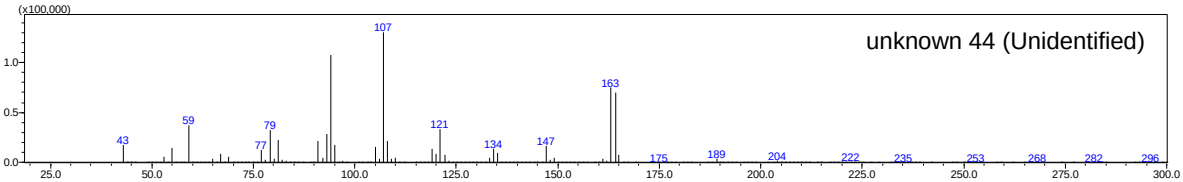
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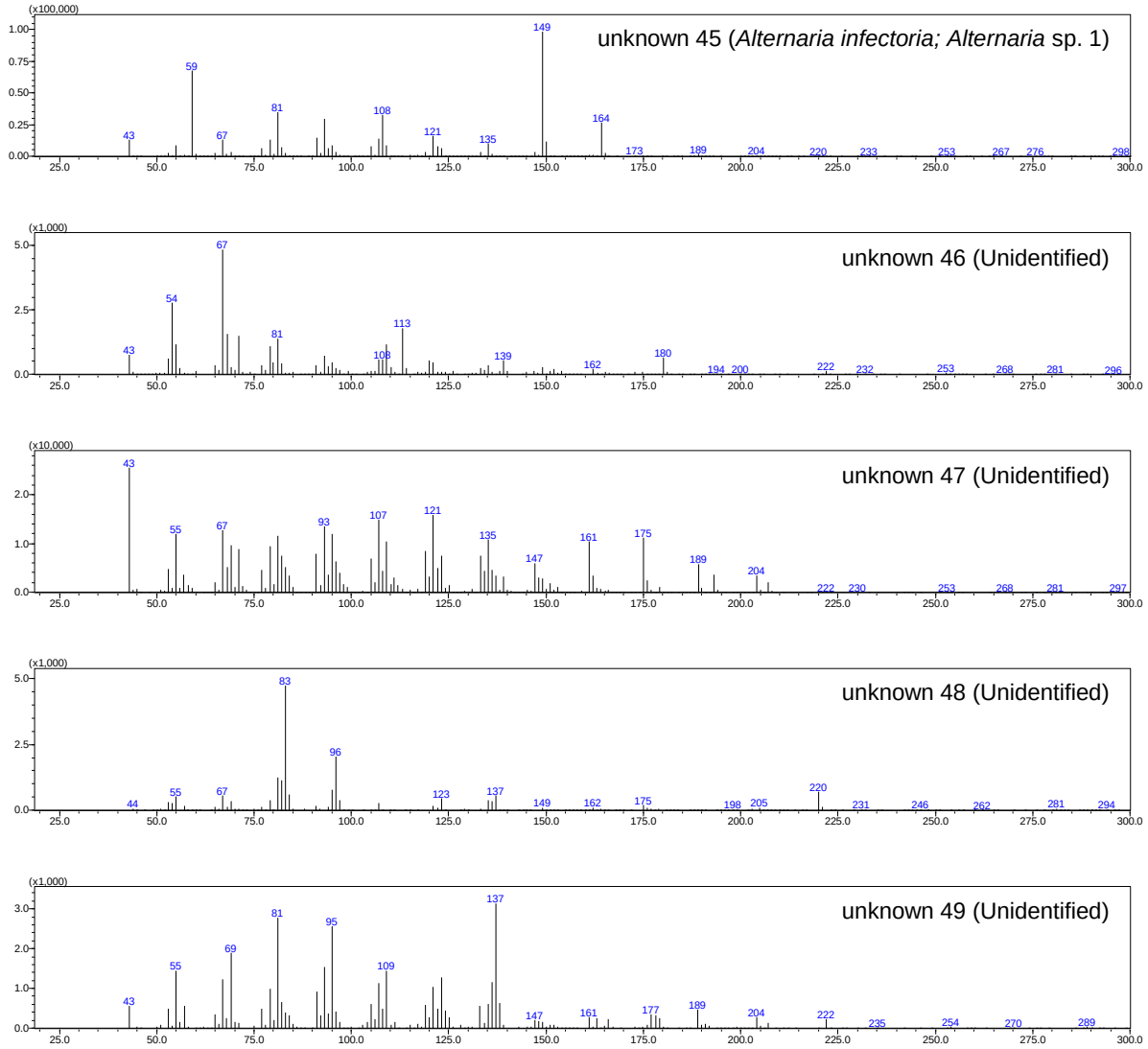

Figure S2: Mass spectra of unknown volatile organic compounds shown in Table S3. Background has been subtracted.

BUSCO version is: 3.0.2
The lineage dataset is: fungi_odb9 (Creation date: 2016-02-13, number of species: 85, number of BUSCOs: 290)
To reproduce this run: python /opt/software/bin/run_BUSCO.py -i /opt/software/packages/galaxy-dist/database/files/009/dataset_9623.dat -o busco_galaxy -l /opt/software/packages/busco/lineage/fungi_odb9/ -m transcriptome -c 11 -e 0.01 -z
#
Summarized benchmarking in BUSCO notation for file /opt/software/packages/galaxy-dist/database/files/009/dataset_9623.dat
BUSCO was run in mode: transcriptome

C:98.3%[S:93.1%,D:5.2%],F:1.4%,M:0.3%,n:290

285	Complete BUSCOs (C)
270	Complete and single-copy BUSCOs (S)
15	Complete and duplicated BUSCOs (D)
4	Fragmented BUSCOs (F)
1	Missing BUSCOs (M)
290	Total BUSCO groups searched

Figure S3: BUSCO analysis of the *Cladosporium* sp. *de novo* assembly. The BUSCO software tool (Afgan *et al.* 2018) was used to validate the completeness of the *de novo* assembly.

Reference

Afgan E, Baker D, Batut B, Van Den Beek M, Bouvier D, Čech M, Chilton J, Clements D, Coraor N, Grüning BA (2018) Nucleic acids research, 46 (W1), W537-W544.

13.3 Manuscript III

Table S1: Germination test of *Cladosporium* sp. spores for the lab experiment, the preference assay and the field experiment. To determine the germination rate of the spores, the spore solution was diluted to approximately 100 spores/mL. From this spore solution, 100 mL were plated on PDA media and germinated spores were counted.

experiment	replicate	germinated spores
Lab experiment	1	131
	2	130
	3	133
	4	134
	5	161
Preference assay	1	89
	2	75
	3	77
	4	90
Field experiment	1	70
	2	57
	3	63
	4	63

Table S2: Primers used in this study.

Name	fw	rev	Reference
Actin2	CCCATTGAGCACGGTATTGT	TACGACCACTGGCATACAGG	Ramirez-Carjaval *et al*. 2008
CladoITS	TGAAGAACGCAGCGAAATGC	GAAATGACGCTCGAACAGGC	This study

Table S3: A Relative endophyte abundance quantified with qPCR (relative expression $\Delta\Delta C_t$) in leaves of black poplar trees from the laboratory (lab) experiment, preference assay and field experiment (field) quantified with qPCR grown under different treatments: inoculated with *Cladosporium* sp. (fungus), uninoculated control (control), gypsy moth feeding on *Cladosporium* sp. inoculated plants (fungus + herbivory), and caterpillar feeding on uninoculated control plants (control + herbivory). The quantity of fungal genomic DNA (gDNA) amplified was normalized to the amount of plant gDNA. Mean ± SE. *P*-values of Welch-ANOVA for lab experiment (n = 4), student's t-test (n = 4-5), or Mann-Whitney-U-Test for field experiment (n = 10) are given. Fungal genomic DNA from the field experiment was quantified in leaves that were treated before with either spore or control solution in the lab and experienced natural damage in the field. **B** Stachydrine concentration (nmol/g dw) in black poplar

leaves from experiments conducted in the laboratory (lab) and field compared to levels of stachydrine in cultured fungus (mycelium). For the field experiment damaged (control + herbivory; fungus + herbivory) and undamaged (control; fungus) leaves from each tree were harvested separately. Mean ± SE are shown (lab experiment n = 4; mycelium n = 3; field experiment n = 10). **C** Statistical results of a two-way ANOVA (lab experiment) and a GLM (field experiment) on stachydrine concentrations of black poplar trees treated with the fungus (f), herbivory (h), and the interaction of both (f x h). For the GLM, concentrations were corrected for the amount of damage and the factor tree is included as random factor. **D** Stachydrine concentration of leaf material from the stachydrine preference assay. Leaves (8^{th} - 15^{th} leaf from apex) from 10 trees were coated with either a control solution (0.01% Silwet®Gold, UPL Deutschland GmbH, Brühl, Germany) or a stachydrine solution (45 nmol/g dw resp. 1.5 µg/mL fresh weight, 0.01% Silwet) (n = 5).

A

experiment	control	fungus	control + herbivory	fungus + herbivory	F/ Z-value	*p* - value
Lab	0.01 ± 0.002	1.26 ± 0.12	0.004 ± 0.004	1.45 ± 0.14	56.638	< 0.001
Preference assay			0.09 ± 0.013	1.07 ± 0.11	21.282	0.002
Field	0.08 ± 0.009	0.50 ± 0.09	-	-	-3.685	< 0.001

B

experiment	control	fungus	control + herbivory	fungus + herbivory	mycelium
Lab	1.82 ± 0.22	45.8 ± 8.03	1.03 ± 0.1	34.52 ± 8.53	-
Field	5.51 ± 0.84	18.76 ± 0.82	8.45 ± 0.40	27.53 ± 1.88	-
Mycelium	-	-	-	-	6047.78 ± 459.91

C

experiment	factor	df	*F/Chi²*-value	*P*-value
Lab	f	1	388.12	**< 0.001**
	h	1	6.79	0.061
	f x h	1	0.50	0.972
Field	f	1	36.71	**< 0.001**
	h	1	4.79	**0.028**
	f x h	1	3.44	0.063

D

treatment	nmol/g (DW)
control	0,53 ± 0.1
stachydrine	5.86 ± 0.24

Table S4: Influence of endophyte (fungus), gypsy moth caterpillar herbivory (control + herbivory), and both (fungus + herbivory) on concentrations on black poplar defense hormones, flavonoids, phenolic acids and phenylacetaldoxime in the laboratory experiment. Trees were inoculated with either endophytic spore solution or a control solution 15 d before caterpillar feeding. 15 gypsy moth caterpillars (4^{th} - 5^{th} instar) were allowed to feed for 48 h. Mean ± SE are shown (n = 4). n.d. not detected.

	control	**fungus**	**control + herbivory**	**fungus + herbivory**
Defense hormones (µg/g DW)				
JA	1.34 ± 0.14	1.15 ± 0.27	2.35 ± 0.5	3.79 ± 0.71
12-hydroxyjasmonic acid sulfate	14.28 ± 1.02	15.04 ± 1.57	17.17 ± 1.15	21.41 ± 1.75
JA-Ile	0.24 ± 0.04	0.11 ± 0.03	0.44 ± 0.06	0.65 ± 0.22
OH-JA-Ile	0.01 ± 0.001	0.007 ± 0.001	0.41 ± 0.57	0.66 ± 0.32
OH-JA	0.009 ± 0.0001	0.007 ± 0.001	0.43 ± 0.06	0.67 ± 0.32
COOH-JA-Ile	n.d.	n.d.	0.09 ± 0.01	0.14 ± 0.06
Flavonoids (mg/g DW)				
Rutin	3.72 ± 0.12	3.55 ± 0.49	2.93 ± 0.52	3.35 ± 0.3
Phenolic acids (µg/g DW)				
Gallic acid	0.59 ± 0.07	0.47 ± 0.11	0.70 ± 0.19	0.68 ± 0.16
Ferulic acid	26.6 ± 1.63	21.86 ± 2.52	28.12 ± 2.37	21.14 ± 1.74
Others				
Phenylacetaldoxime (ng/g DW)	23.65 ± 3.42	29.95 ± 5.32	370.61 ± 98.73	877.15 ± 396.57

Table S5: Statistical results of a two-way ANOVA or Kruskal Wallis test on concentrations of chemical compounds of black poplar trees treated with the fungus (f), herbivory (h), and the interaction of both (f x h) in the laboratory experiment. Trees were inoculated with either endophyte spore solution or a control solution 15 d before caterpillar feeding. Gypsy moth larvae were allowed to feed for 48 h.

	factor	df	*F*-value	*P*-value
Defense hormones (µg/g DW)				
JA	f	1	1.911	0.192
	h	1	16.397	0.002
	f x h	1	3.275	0.095
12-hydroxyjasmonic acid sulfate	f	1	1.354	0.267
	h	1	6.149	**0.029**
	f x h	1	3.921	0.071
JA-Ile	f	1	1.927	0.190
	h	1	22.356	**< 0.001**
	f x h	1	5.316	**0.040**
OH-JA-Ile		3	12.375	**0.006**
OH-JA		3	12.706	**0.005**
COOH-JA-Ile		3	-0.761	0.746
Flavonoids (mg/g DW)				
Rutin	f	1	0.105	0.751
	h	1	1.602	0.230
	f x h	1	0.594	0.456
Phenolic acids (µg/g DW)				
Gallic acid	f	1	0.310	0.588
	h	1	1.337	0.270
	f x h	1	0.137	0.717
Ferulic acid	f	1	7.792	**0.016**
	h	1	0.036	0.853
	f x h	1	0.280	0.606
Others				
Phenylacetaldoxime (ng/g DW)	f	1	1.125	0.310
	h	1	91.913	**< 0.001**
	f x h	1	0.015	0.905

Table S6: Influence of endophyte on concentrations on selected metabolites of black poplar trees in the field experiment. Trees were inoculated with either endophyte spore solution or a control solution 15 d before caterpillar feeding. For the field experiment damaged (control + herbivory; fungus + herbivory) and undamaged (control; fungus) leaves from one tree were harvested separately. Mean ± SE are shown (n = 10).

	control	fungus	control + herbivory	fungus + herbivory
Phytohormones (µg/g DW)				
Abscisic acid	0.60 ± 0.51	0.30 ± 0.16	0.62 ± 0.52	0.23 ± 0.10
Salicylic acid	5.98 ± 0.78	4.44 ± 1.36	3.61 ± 0.46	3.81 ± 0.60
Jasmonates	0.66 ± 0.21	0.94 ± 0.52	0.98 ± 0.18	1.17 ± 0.18
12-hydroxyjasmonic acid sulfate	60.1 ± 3.33	55.78 ± 7.61	69.53 ± 2.50	66.06 ± 2.92
Salicinoids (mg/g DW)				
Salicin	3.01 ± 0.26	3.08 ± 0.28	3.03 ± 0.38	3.59 ± 0.58
Salicortin	43.71 ± 2.26	46.99 ± 2.65	44.06 ± 1.88	43.57 ± 2.77
Homaloside D	19.05 ± 1.18	20.17 ± 1.16	19.20 ± 0.93	18.79 ± 1.00
Nigracin	1.34 ± 0.05	1.35 ± 0.08	1.23 ± 0.06	1.12 ± 0.04
6'-*O*-benzoylsalicortin	0.95 ± 0.15	1.23 ± 0.28	0.99 ± 0.10	0.94 ± 0.07
Flavonoids (mg/g DW)				
Rutin	2.29 ± 0.52	4.23 ± 1.51	2.27 ± 0.34	2.45 ± 0.40
Catechin	1.31 ± 0.19	1.11 ± 0.15	1.46 ± 0.19	1.41 ± 0.13
Procyanidin B1	0.41 ± 0.03	0.43 ± 0.03	0.39 ± 0.02	0.44 ± 0.04
Phenolic acids (µg/g DW)				
Caffeic acid	17.48 ± 2.15	79.13 ± 47.76	46.27 ± 18.25	51.41 ± 12.28
p-Coumaric acid	3.24 ± 0.80	2.73 ± 0.46	3.37 ± 1.15	4.64 ± 1.63
Gallic acid	0.07 ± 0.04	0.06 ± 0.02	0.11 ± 0.06	0.34 ± 0.20
Ferulic acid	1.96 ± 0.23	1.89 ± 0.25	2.04 ± 0.11	1.92 ± 0.12
Others				
Phenylacetaldoxime (ng/g DW)	5.57 ± 4.71	1.35 ± 0.43	64.31 ± 47.29	216.08 ± 182.92

Table S7. Parameters used in LC-MS/MS analysis of phytohormones and phenolic acids by LC-MS/MS on a triple quadrupole instrument (HPLC 1260 (Agilent Technologies)-QTRAP6500 (SCIEX)) in the negative ionization mode. Abbreviations: Q1, quadrupole 1; Q3, quadrupole 3; RT, retention time; RF, response factor; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision exit potential.

Q1 (Da)	Q3 (Da)	RT (min)	Compound	Internal std	RF	DP (V)	EP (V)	CE (V)	CXP (V)
136.93	93	5.9	SA	D4-SA	1.0	-30	-8	-24	-7
263.0	153.2	6.0	ABA	D6-ABA	1.0	-30	-12	-22	-2
209.07	59.0	7.2	JA	D6-JA	1.0	-30	-9	-24	-2
322.19	130.1	7.3	JA-Ile	D6-JA-Ile	1.0	-30	-4.5	-30	-4
338.1	130.1	6.0	OH-JA-Ile	D6-JA-Ile	1.0	-30	-4.5	-30	-4
225.1	59.0	4.4	OH-JA	D6-JA	1.0	-30	-9	-24	-2
352.1	130.1	5.65	COOH-JA-Ile	D6-JA-Ile	1.0	-30	-4.5	-30	-4
305.0	97.0	4.0	12-OH-JA sulfate	D6-JA	6.0	-30	-10	-60	-10
140.93	97.0	5.9	D4-SA			-30	-8	-24	-7
269.0	159.2	6.0	D6-ABA			-30	-12	-22	-2
215.0	59.0	7.2	D6-JA			-30	-9	-24	-2
214.0	59.0	7.2	D5-JA			-30	-9	-24	-2
328.19	130.1	7.3	D6-JA-Ile			-30	-4.5	-30	-4
327.19	130.1	7.3	D5-JA-Ile			-30	-4.5	-30	-4
163.0	118.9	4.7	*p*-coumaric acid	triF-methyl-CA	2.56	-30	-8	-20	-5
169.0	125.0	1.3	gallic acid	triF-methyl-CA	3.15	-30	-10	-18	-19
179.0	134.9	4.0	caffeic acid	triF-methyl-CA	1.62	-30	-8	-22	-5
193.1	133.9	5.0	ferulic acid	triF-methyl-CA	7.77	-30	-8	-22	-5
215.064	171.056	7.3	triF-methyl-CA			-30	-8	-18	-4

Table S8: Arthropods attracted during the field experiment. To monitor visiting insects, trees were observed four times per day (9 a.m., 12 a.m., 3 p.m., 6 p.m.) for nine days by two experimenters. Arthropods observed on the trees were classified at least to the order. Shown are the calculated sums of visiting arthropods over the whole experimental period.

Order	Suborder	Family	Control	Fungus
Araneae	unknown	unknown	49	61
Araneae Total			**49**	**61**
Coleoptera	Polyphaga	Cerambycidae	1	0
Coleoptera	Polyphaga	Chrysomelidae	12	2
Coleoptera	Polyphaga	Coccinellidae	2	5
Coleoptera	Polyphaga	Curculionidae	57	22
Coleoptera	unknown	unknown	83	67
Coleoptera Total			**155**	**96**
Dermaptera	unknown	unknown	4	0
Dermaptera Total			**4**	**0**
Diptera	Brachycera	Calliphoridae	0	2
Diptera	Brachycera	Syrphidae	1	0
Diptera	Nematocera	unknown	50	51
Diptera	unknown	unknown	77	79
Diptera Total			**128**	**132**
Ephemeroptera	unknown	unknown	8	2
Ephemeroptera Total			**8**	**2**
Hemiptera	Auchenorrhyncha	unknown	93	112
Hemiptera	Heteroptera	unknown	0	1
Hemiptera	Sternorrhyncha	unknown	1360	1728
Hemiptera Total			**1453**	**1841**
Hymenoptera	Apocrita	Formicidae	868	744
Hymenoptera	Apocrita	Ichneumonidae	54	46
Hymenoptera Total			**922**	**790**

Order	Suborder	Family	Control	Fungus
Ixodida	unknown	unknown	0	5
Ixodida Total			**0**	**5**
Lepidoptera	Glossata	Geometridae	4	7
Lepidoptera	Glossata	Noctuidae	7	1
Lepidoptera	unknown	unknown	18	17
Lepidoptera Total			**29**	**25**
Neuroptera	*Planipennia*	Chrysopidae	1	4
Neuroptera Total			**1**	**4**
Orthoptera	unknown	unknown	8	12
Orthoptera Total			**8**	**12**
Thysanoptera	unknown	unknown	26	32
Thysanoptera Total			**26**	**32**
unknown	unknown	unknown	16	36
unknown Total			**16**	**36**

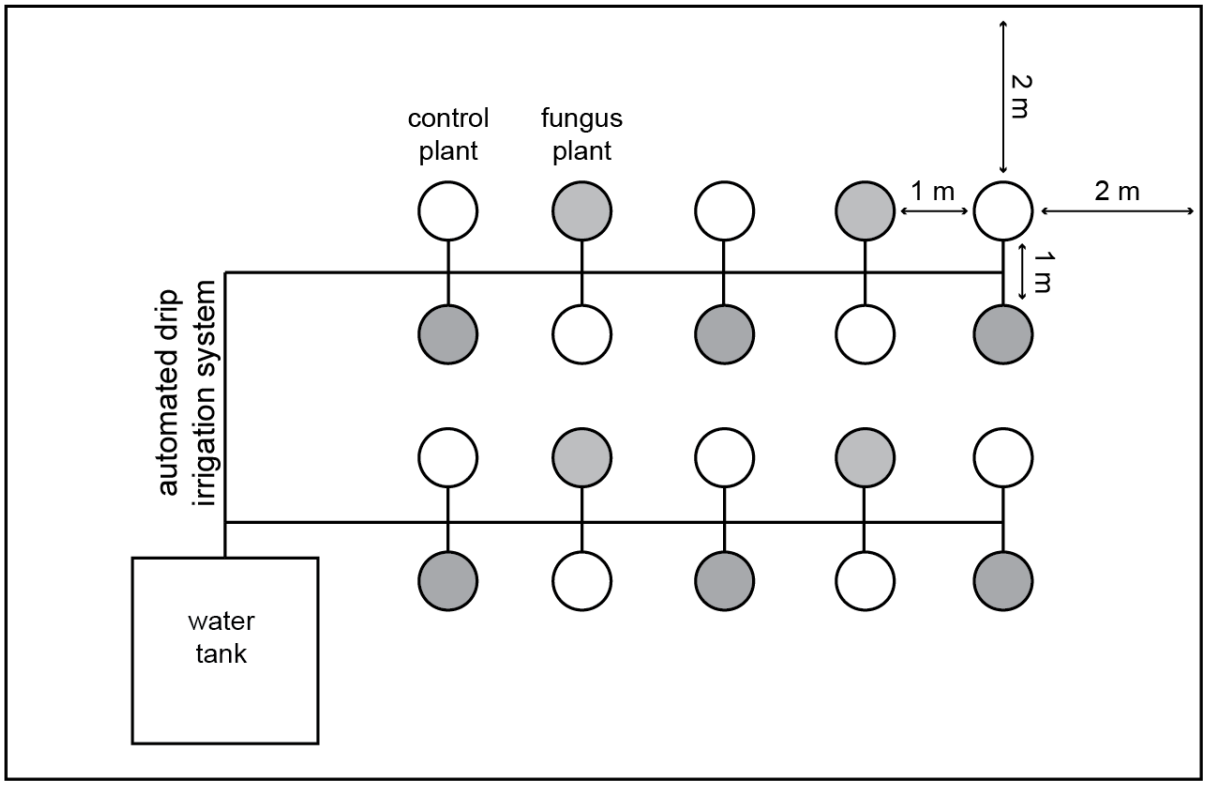

Figure S1: Planting scheme of the field experiment. Endophyte fungus-inoculated and uninoculated control trees were both transferred 15 dpi to a field site containing a natural stand of mature black poplar trees situated in a floodplain forest in northeastern Germany. Trees with pots were placed at fixed intervals of 1 m in a 4 x 5 grid. Plants were watered twice per day with an automated drip irrigation system and pots were fixed with tent pecks. A plastic fence that was installed at a 2 m distance from the experimental trees protected the trees against herbivory and trampling by large mammals.

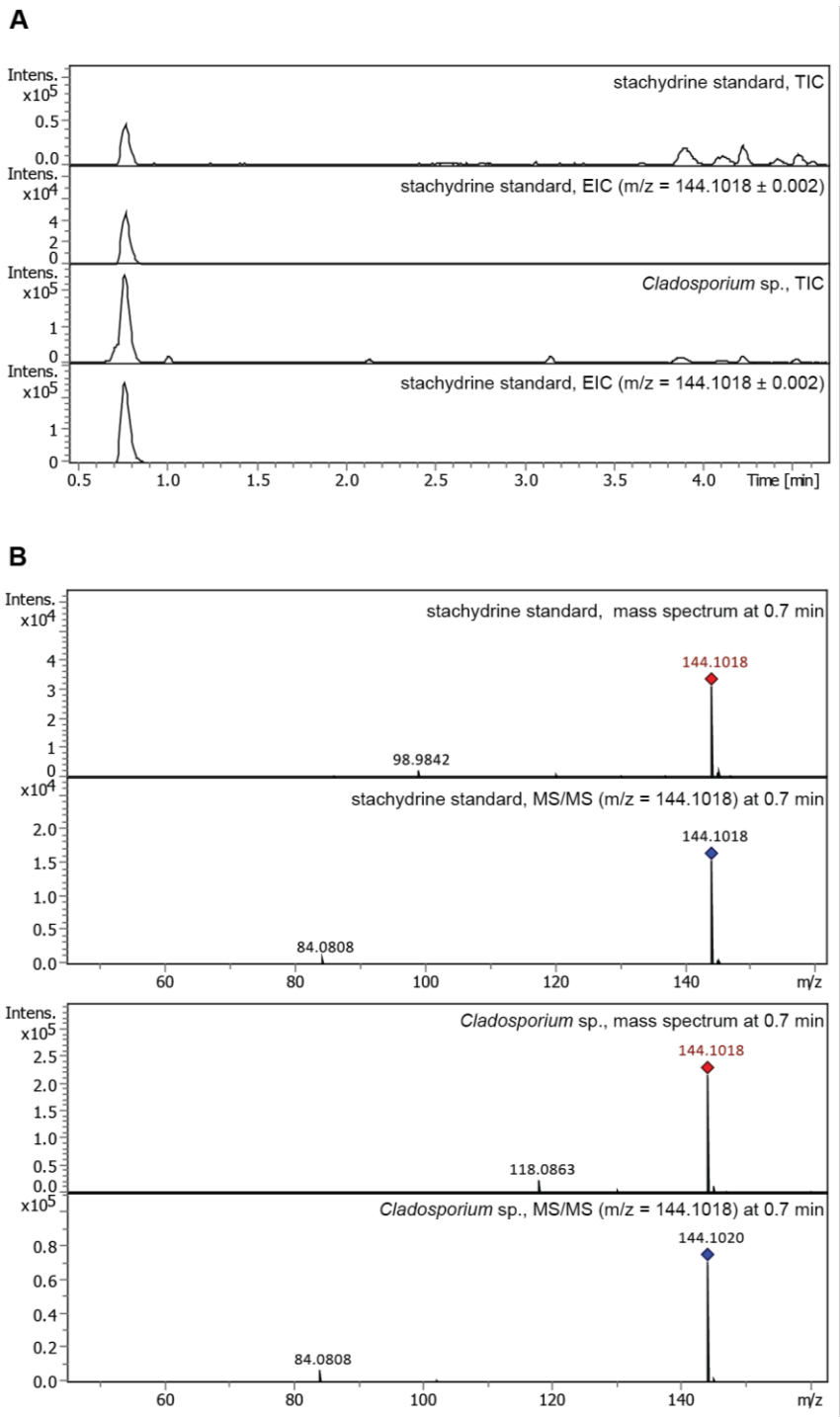

Figure S2: A Chromatogram and **B** mass spectra of stachydrine in fungal mycelium compared to an authentic standard at 1 µg/mL concentration. Fungal mycelium was harvested from fungal cultures, freeze-dried and extracted with methanol. Samples were measured in positive mode on a HPLC coupled to a Bruker timsToF mass spectrometer. TIC (total ion chromatogram), EIC (extracted ion chromatogram).

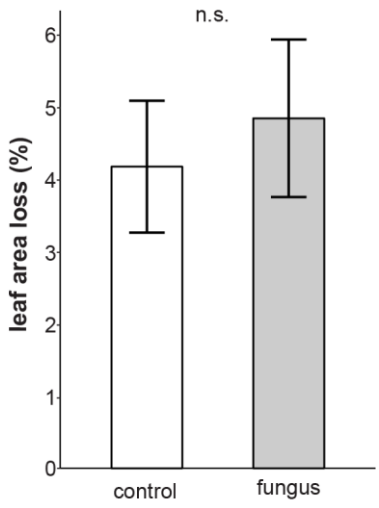

Figure S3: Herbivore damage to endophytic fungus-inoculated (fungus) and uninoculated (control) trees from the experiment conducted in the laboratory. To explore the effect of herbivory on control and endophyte treated plants, 15 gypsy moth caterpillars (4^{th}-5^{th} instar) were added to each plant on the treated part of the trees 15 dpi. After 48 h caterpillars were removed. Damage was determined by reconstructing the original leaf area in digital photographs taken of all leaves with an image editing software (Adobe Photoshop CS5, Adobe, San Jose, CA, USA). Leaf area loss is expressed in %. Mean ± SE (n = 4). Student's t-test (T = -0.471, p = 0.654).

References

Ramírez-Carvajal GA, Morse AM, Davis JM (2008) Transcript profiles of the cytokinin response regulator gene family in Populus imply diverse roles in plant development. *New Phytologist, 177*(1), 77-89.

13.4 Detailed author contributions

Manuscript I

Angaben zum Eigenanteil

gemäß der Durchführungsbestimmung zu § 8 Abs. 2 der Promotionsordnung vom 23.09.2019 der Fakultät für Biowissenschaften der FSU Jena, Anlage 1, Formular 2 (Freitext)

Friend or foe?

The role of leaf-inhabiting fungal pathogens and endophytes in tree-insect interactions

Franziska Eberl, Christin Uhe and Sybille B. Unsicker

Published in *Fungal Ecology* (2019), 38, 104-112, doi:10.1016/j.funeco.2018.04.003

Author contributions	Conceptualization: FE, **CU (35%)**, SBU
	Literature research: FE, **CU (40%)**, SBU
	Figure preparation: FE (100%)
	Table preparation: FE, **CU (30%)**
	Manuscript writing: FE, **CU (40%)**, SBU

The review was conceptualized by SBU, FE and CU. Literature research was carried out by FE, CU and SBU, while CU particularly focused on the literature based on endophyte research. After conceptualization Figure 1 was prepared by FE. FE and CU jointly worked on Table 1 and Table S1, CU focused particularly on endophytic fungi. The original draft was written by FE, CU and SBU and revised by all.

Manuscript II

Angaben zum Eigenanteil

gemäß der Durchführungsbestimmung zu § 8 Abs. 2 der Promotionsordnung vom 23.09.2019 der Fakultät für Biowissenschaften der FSU Jena, Anlage 1, Formular 2 (Freitext)

Volatile emission and biosynthesis in endophytic fungi colonizing black poplar leaves

Christin Walther, Pamela Baumann, Katrin Luck, Beate Rothe, Peter H. W. Biedermann, Jonathan Gershenzon, Tobias G. Köllner, and Sybille B. Unsicker

Published in *Beilstein J. Org. Chem.* (2021), 17, 1698-1711, doi:10.3762/bjoc.17.118

Author contributions	Conceptualization: **CW (30%)**, TGK, SBU
	Designed experiments: **CW (30%)**, TGK, SBU
	Performed experiments: **CW (75%)**, PB, KL, BR, SBU
	Data analysis and statistics: **CW (75%)**, PM, KL, TGK
	Data visualization: **CW(80%)**, TGK, JG
	Writing – original draft: **CW (90%)**, PM, TGK
	Writing – review and editing: **CW (50%)**, PM, PHWB, TGK, JG, SBU

The project was built on fundamental studies from SBU and PHWB. The conceptualization was conceived by CW, TGB and SBU. The experiments, particularly the volatile collection of endophytic fungi, were designed by CW, with contributions from PM, SBU. Experiments, particularly the identification of isolated endophytic fungi and the volatile collection and analysis, were performed by CW, with contribution from PM, KL, BR and SBU (Table 1-2, Table S1-S3, Figure 1, Figure 2b, and Figure S1-S2). The identification and characterization of TPS as well as the dendrogram analysis was done in collaboration with TGK and KL (Figure 2a, Figure 3, and Figure S3). The data were analyzed by CW with contributions from PM, KL, TGK. The draft of the manuscript was written by CW with contributions by PM and TGK. SBU, TGK, JG, PM, PHWB revised the manuscript and the draft was written in the final form by CW.

Manuscript III

Angaben zum Eigenanteil

gemäß der Durchführungsbestimmung zu § 8 Abs. 2 der Promotionsordnung vom 23.09.2019 der Fakultät für Biowissenschaften der FSU Jena, Anlage 1, Formular 2 (Freitext)

A fungal endophyte modifies leaf phytochemistry and shapes insect communities in poplar

Christin Walther, Beate Rothe, Michael Reichelt, Pamela Medina van Berkum, Jonathan Gershenzon, and Sybille B. Unsicker

In preparation

Author contributions	Conceptualization: **CW (50%)**, SBU and JG
	Designed experiments: **CW (70%)**, SBU
	Performed experiments: **CW (80%)**, BR, MR
	Data analysis and statistics: **CW (80%)**, MR, PMB
	Data visualization: **CW (90%)**, MR, PMB
	Writing – original draft: **CW (90%)**, MR, PMB
	Writing – review and editing: **CW (50%)**, MR, JG, SBU

The conceptualization was conceived by CW, JG and SBU. The experiments were designed by CW and SBU. Experiments were performed by CW, with contribution from BR and MR (Figure 1-7, Figure S1, Figure S3, and Table S1-S8). The identification and characterization of stachydrine was done in collaboration with MR (Figure 4a, Figure S2). The data were analyzed by CW with contributions from PMB (Figure 7), MR (Figure 4a, Figure S2, and Table S7). The draft of the manuscript was written by CW with contributions by PMB and MR. SBU, MR, JG revised the manuscript and the draft was written in the final form by CW.