Consequences of intra- and inter-plant communication for tree-herbivore interactions

DISSERTATION

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1. INTRODUCTION

1.1. Plant defense against insect herbivores

Since plants colonized terrestrial habitats 450 million years ago they have had to face numerous abiotic and biotic stresses. Due to their sessile nature, plants have had to find other strategies than flight to cope with abiotic stresses like wind, heat and drought. Additionally, plants have had to cope with biotic stresses, such as herbivores, pathogens and parasites. Fossil records show that arthropods have been feeding on plant organs almost from the beginning of their evolutionary history (Labandeira 2007). Therefore, plants have evolved physical defense mechanisms like wax layers, cuticles, hairs and thorns as well as an arsenal of chemical defense compounds to fight their antagonists. Herbivores, on the other hand, developed many strategies to cope with plant defenses, such as strong mandibles, detoxification, excretion and sequestration of toxic compounds as well as behavioral adaptations. Because of this strong reciprocal relationship, the co-evolution of plants and herbivores can be regarded as an ongoing evolutionary arms race (Rasmann 2014), which has led to the huge complexity of interactions we can observe today.

Plants produce a wide array of compounds to mediate interactions with their abiotic and biotic environment (Schoonhoven *et al*. 1998, Hartmann 2007). Those compounds are often thought to be non-essential for plant growth and reproduction under optimal conditions and are therefore referred to as secondary metabolites (Wink 2003). Some defense related secondary metabolites are present constitutively (at all times), whereas other compounds are only inducible by wounding or herbivore attack (Wu & Baldwin 2010). Most often the synthesis of constitutive defense compounds is believed to consume a large amount of resources (Theis & Lerdau 2003). Inducible defenses, on the other hand, include morphological and physiological changes as well as increased levels of secondary metabolites that are relocated or *de novo* synthesized after mechanical damage, pathogen or herbivore attack. Therefore, induced defenses require extra time until the defense is active against the attacker compared to a constitutive defense strategy. However, induced defenses are believed to be more cost efficient than constitutive defenses (Karban *et al*. 1997; Heil & Baldwin 2002; R. Zangerl 2003).

The costs of defense not only include the costs for their biosynthesis, transport and storage and value of included nutrients in limited supply (nitrogen or sulfur), but also indirect ecological costs. For example, toxic or deterrent compounds can have unwanted effects on

mutualists like pollinators. Additionally, investing in defense rather than growth might be a disadvantage in the presence of strong competition with neighboring plants (Strauss *et al*. 2002). Hypothetically, a plant should therefore only invest in defense when there is substantial risk of attack, the organ under attack is of high value and the benefits of defense outweigh the costs (McKey 1974). Unfortunately, natural systems are so complex that it is difficult to calculate these costs. A tree, for example, has to grow annually, which incurs an additional cost compared to non-perennial plants (Holopainen 2011). Also in recent years evidence has arisen that some secondary metabolites also co-function in primary metabolism. Besides being well known defense compounds, glucosinolates for example are believed to regulate ion channels in guard cells and are involved in the remobilization and storage of sulfur compounds. Such auxiliary roles of defense compounds could reduce costs and therefore the necessity of a trade-off between growth and defense (Neilson *et al*. 2013).

Direct plant defense

Constitutive and inducible defense compounds can directly affect the behavior or development of an herbivore by reducing digestibility or by being deterrent or toxic. There are multiple different classes of direct defense. Some compounds are simply toxic by themselves, for example pyrethins that are produced by chrysanthemums. These compounds interfere with ion channels in the nervous system of both insects and animals (Wolansky & Harril 2008). Small proteins known as proteinase inhibitors that can irreversibly bind proteases in the insect guts are not toxic *per se* but have a negative impact on feeding herbivores by preventing the digestion of consumed proteins. Direct defense compounds can also produce reactive oxygen species that have multiple effects, such as, damaging proteins or crosslinking metabolites.

Some direct defenses are stored in the plant as prototoxins that are enzymatically activated to toxins upon herbivory. The prototoxin and the catalytic enzyme are located in different cells or different compartments within a cell. This mechanism protects the plant against auto-toxicity. Classic examples of activated defenses are cyanogenic glycosides, which have been described for more than 2500 plant species (Zagrobelny *et al*. 2004). When cells are disrupted by herbivory, an enzyme cleaves off the sugar moiety. The aglycone formed then spontaneously releases HCN under basic pH conditions, which are most common in insect guts. HCN is a very potent toxin that interferes with the cytochrome c oxidases of the respiratory pathway (Leavesley *et al*. 2008).

With the exception of protease inhibitors, all of the above mentioned compounds are only found in herbaceous plants. Deciduous tree species produce a rich variety of phenolic compounds. The most widespread group of phenolic defense compounds are condensed

tannins. These are flavan-3-ol polymers that can precipitate proteins. However, their effect on herbivores is controversial. There are studies that found a negative connection between condensed tannins and herbivore performance and other studies that found no connection (reviewed in Barbehenn & Peter Constable 2011). Another phenolic defense are phenolic glycosides, which are enzymatically-activated *in vivo*. The aglycone is then oxidized to a quinone spontaneously or with the help of enzymes like peroxidases or polyphenoloxidases (Duffey 1996; Pourcel *et al*. 2007). Along with the formation of a quinone, reactive oxygen species can be produced as byproducts that cause oxidative stress (Barbehenn *et al*. 2003).

Plant defense *via* volatiles

Another widespread form of plant defense is the release of volatile organic compounds (VOCs). Plants can emit a vast amount of volatiles from different chemical classes. The biggest classes of VOCs are the terpenoids. They consist of one or more isoprene units, which each consist of five carbon atoms (C_5), and can therefore be further classified with regard to how many isoprene units are linked in one molecule. Isoprene itself is considered to be a hemiterpene (C_5). Other volatile terpenes consist of either two isoprene units, the monoterpenes (C_{10}), three isoprene units, called sesquiterpenes (C_{15}), or compounds with one extra carbon, called homoterpenes. The last step in the biosynthesis of most volatile terpenoids is catalyzed by terpene synthases. These often produce multiple products, leading to a very diverse group of compounds. They use geranyl diphosphate and farnesyl diphosphate as substrates to form mono- and sesquiterpenes respectively. Farnesyl diphosphate is biosynthesized from geranyl diphosphate, which in turn is formed by the condensation of isopentenyl pyrophosphate and dimethylallyl pyrophosphate units derived from the mevalonate pathway or the 2-methyl-erythritol 4-phosphate pathway. The C_{11} homoterpene 4,8-dimethyl-1,3,7-nonatriene (DMNT) derives from the conversion of farnesyl diphosphate into the sesquiterpene alcohol *E*-nerolidol (Maffei *et al*. 2011). Another widespread group of VOCs are the green leaf volatiles (GLVs). They are C_6 fatty acid derivatives, mainly isomers of hexenal, hexenol and hexenyl acetate. GLVs are rapidly emitted after leaf wounding because the fatty acid substrates meet with lipoxygenases and hydroperoxide lyases upon cell disruption (Maffei *et al*. 2011). In addition plants emit a variety of compounds from other chemical classes, such as aromatic compounds or those containing a nitrogenous group. The latter are usually emitted in rather small amounts compared to terpenes and GLVs.

The emission of VOCs is believed to be an evolutionarily ancient strategy because it is found throughout the plant kingdom and even primitive species like gingko emit plant volatiles (Van Den Boom *et al*. 2004). Volatiles can serve as protection against thermal and irradiation

stresses, and the massive release of these compounds can form aerosols that may enhance cloudiness in the atmosphere (Holopainen 2004). Additionally, volatiles play a major role in defense against insect herbivores (reviewed in Unsicker *et al*. 2009). Like many other defenses, VOCs are emitted constitutively but emission can also be induced upon mechanical or herbivore damage. Many plants show a different induction pattern upon herbivory compared to mechanical damage alone because of elicitors in herbivore saliva. It is even possible that volatiles are induced in undamaged parts of a plant under attack (Halitschke *et al*. 200; Dicke *et al*. 2003). Some compounds like GLVs are universally induced after herbivory in many herbaceous plants like clover, Arabidopsis or tobacco as well as in perennial plants like spruce, beech and poplar (Kigathi *et al*. 2009; Aharoni *et al*. 2003; Allmann & Baldwin 2010; Schmidt *et al*. 2011; Gossner *et al*. 2014; Clavijo McCormick *et al*. 2014b). Plant volatile emissions are very dynamic in general. During night time there is usually less emission than during the day (De Moraes *et al*. 1998; Clavijo McCormick *et al*. 2014a). But HIPV emissions can also vary over the course of herbivory and the time following herbivore removal (Hoballah & Turlings 2005; Scascighini, 2005; Loughrin *et al*. 1994; kugimiya *et al*. 2010). Even the type of herbivore species and the developmental stage of the attacker can have an impact on the emission pattern (Sabelis *et al*. 2007; McCormick *et al*. 2014a).

The emitted HIPVs can directly affect herbivores. It has been shown that both larvae and egg laying females are repelled by certain VOCs (L.Bernasconi *et al*. 1998; De Moraes *et al*. 2001; Rostás & Hilker 2002), and exposure to HIPVs can even lead to reduced larval growth. Von Mèrey *et al*. (2013) showed that *Spodoptera littoralis* caterpillars exposed to the volatiles of herbivore infested maize plants gained less weight compared to caterpillars that were exposed to constitutive maize volatiles. Additionally the emission of HIPVs can attract herbivore enemies like parasitoids or predators (Mumm & Dicke 2010; Clavijo McCormick *et al*. 2014b). Amo *et al*. (2013) even showed that birds (*Parus major*) are attracted to caterpillar-infested apple trees even when they cannot see the larvae nor the damage dealt to the leaves. The plant can be considered to be calling for help *via* volatiles. This form of defense, which is also known as indirect defense, is considered to be a form of mutualism. The plant obviously benefits because the herbivore enemies will reduce the predation pressure by killing the herbivores. The predators and parasitoids on the other hand benefit from the volatile cues of the plant as those make it easier to locate their prey. Some plants even offer extra floral nectars and food bodies to attract herbivore enemies (Arimura *et al*. 2005; Heil & Ton 2008).

However, it can be complicated to elucidate the biological role of plant volatiles since their function is not limited to defense alone. Most herbivores and ovipositing females use plant volatile emission to find and identify their host plant. More than 1700 volatile compounds are

reported to be emitted from flowers, bark, leaves and even the roots of a plant. In general flowers and fruits show a broader variety of emitted compounds, whereas leaves show the greatest total emission rates (Knudsen *et al*. 2006; Laothawornkitkul *et al*. 2009). With the help of volatiles, plants also attract insects and other organisms for pollination and seed dispersal. Some VOCs are toxic to microorganisms. These compounds can protect wounding sites from a secondary infection caused by pathogenic fungi or bacteria (Quintana-Rodriguez *et al.* 2018). Antibacterial volatiles can also help to shape the bacterial communities on flower petals and leaves, presumably to avoid the spread of pathogens that can be transmitted through pollinators (Junker *et al.* 2011). Additionally, volatiles can act as signals between parts of the same plant as well as neighboring plants (Heil & Silva Bueno 2007).

1.2. Inter- and intra-plant communication

When a plant is challenged by insect herbivores, it may drastically change its volatile emission profile both quantitatively and qualitatively to produce direct and/or indirect defenses, as discussed in the previous chapter. However, in recent years increasing evidence has arisen that the emitted HIPVs also serve as signaling cues between damaged and undamaged parts of the same plant as well as between neighboring plants. Plants or plant parts perceiving the volatile signal may then induce their own defenses or enter an alarmed state to respond rapidly to a future attack (Li *et al*. 2012). This phenomenon is known as “defense priming”.

There are multiple facets of defense priming. In general, it can be seen as a strategy for enhanced activation of existing inducible defense mechanisms. Priming begins with the perception of a primary stimulus, which can be a physical or chemical cue as well as the actual attack of a pathogen or an herbivore. Previously, it was assumed that exposure to a priming stimulus does not lead to any phenotypic changes in the plant. However, modern analytical techniques have revealed that physiological, metabolic, transcriptional or epigenetic modifications can occur upon priming (Mauch-Mani *et al*. 2017). In the event of a subsequent attack, a primed plant will show a stronger or faster defense response compared to a non-primed plant. This strategy should be even more cost efficient than immediate induction of defenses upon signal perception since only minimal fitness costs are associated with priming in comparison to an induced defense. Thus, in the event that no subsequent attack occurs, there is little investment to lose. But, if an attack occurs and the induction of defenses is more rapid and intense than in unprimed plants, fitness should be significantly increased (van Hulten *et al*. 2006; Kost, 2006; Douma, 2017). The duration of the primed state is not fully

known and most likely varies greatly among species, depending on the mechanism involved. Priming may even be passed onto the next generation *via* epigenetic changes (Rasmann *et al*. 2012; Luna *et al*. 2012).

Volatile-mediated defense priming

In plant defense against pathogens, priming is regarded as a sort of immunological memory and a crucial part of systemic acquired resistance (Hilker *et al*. 2016). However the mechanism of volatile-mediated priming in defense against insect herbivores is less well understood. It has been shown that plants or undamaged parts of the same plant can be primed by exposure to HIPVs emitted from a plant under herbivore attack. However, inter-plant signaling *via* volatiles most likely did not evolve as a form of active communication as it is difficult to see a benefit for a signaling plant except in a situation where it is surrounded by close kin. It is especially hard to identify a benefit for signaling plants when the receivers are of a different species. Tobacco plants for example become more resistant to herbivores when they were exposed to VOCs emitted from a mechanically wounded sage bush (Karban *et al*. 2003). In such cases, neighboring plants are most likely eavesdropping and interpreting the volatile cues for their own benefit as evidence for herbivores in the vicinity that could spread to their own tissues.

Evidence of volatile-mediated defense priming is scatterd throughout the plant kingdom. Priming is reported for many herbaceous plants like lima bean, Arabidopsis, maize and tobacco (Yi *et al.* 2009; Godard *et al*. 2008; Ton *et al*. 2007; Paschold *et al*. 2006) as well for woody plants like alder, birch and aspen (Tscharntke *et al*. 2001; Girón-Calva *et al*. 2014; Li *et al*. 2012). In trees inter- and intra-plant signaling can even occur at the same time. Girón-Calva *et al*. (2014) showed in birch trees that exposure to HIPVs leads to an increase in the emission of terpenes and aromatic compounds in undamaged neighbors and increased GLV emissions in undamaged branches on the same tree. Many VOCs are rather short-lived under natural conditions and can therefore not travel great distances, this is another argument for the ecological significance of intra-plant priming *versus* inter-plant priming (Frost *et al*. 2007; Li & Blande 2017). Additionally, it has been suggested that volatile signals are especially important for communication where there are vascular constraints, for example in plants with a complex architecture such as trees (Karban *et al*. 2006; Frost *et al*. 2007).

Despite the number of studies addressing volatile-mediated defense priming, there are many unanswered questions about the mechanisms involved, starting with the identity of the volatile compounds that serve as signals. It has been reported that GLVs can prime jasmonic acid (JA)-dependent signals in Arabidopsis (Engelberth *et al*. 2004) and the expression of genes related to direct defense in hybrid poplar (Frost *et al*. 2008). However, indole and

terpenoids seem to be essential for priming in maize (Ton *et al*. 2007; Erb *et al*. 2015). Additionally, very little is known about how plants can perceive VOCs. Only recently Nagashima *et al*. (2019) found that (*E*)-β-caryophyllene binds to a transcriptional co-repressor in tobacco cells thereby causing the repressor protein to be released from the gene. This study represents the first evidence of how volatile signals could be detected by plants. Furthermore there is little knowledge about how the initial volatile signal is transduced. After priming by non-volatile stimuli in defense against pathogens, signal transduction has been shown to occur through changes in cytosolic calcium, tricarboxylic acids, reactive oxygen species, amino acids and sugars as well as membrane depolarization, chromatin, transcriptional and posttranscriptional modifications and the accumulation of transcription factors (van den Burg & Takken 2009; Conrath 2011; Mauch-Mani *et al*. 2017; Hilker & Schmülling 2019). Future studies are needed to elucidate whether the signal transduction following a volatile cue functions in a similar manner.

There is also little information on the consequences of defense priming for herbivores. Many well replicated studies on multiple plant species have shown that plant-plant communication results in reduced amounts of plant damage presumably because of enhanced expression of defense related genes or the accumulation of defense metabolites like terpenes or protease inhibitors (Engelberth *et al*. 2004; Farag *et al*. 2005; Kessler *et al*.; also reviewed in Karban *et al*. 2014). However, only a few studies focused on the consequences for the feeding herbivore. For example, Morrell & Kessler (2017) showed that volatile communication between goldenrods increased herbivore movement and therefore decreased the amount of damage but, herbivore behavior did not differ between herbivore-induced and HIPV-exposed plants. A recent study conducted with willows showed that fast growing caterpillars performed better on control leaves than on leaves exposed to HIPVs, but slow growing caterpillars showed no difference in performance (Hughes *et al*. 2015). It therefore remains unclear how herbivores are generally affected by volatile mediated defense priming.

Phytohormone signaling in above- and belowground interactions

Another form of intra-plant communication is signaling *via* phytohormones. In contrast to volatile mediated-signaling by HIPVs, the role of phytohormones is well-established in the literature and one major phytohormone is the volatile gas ethylene. Plant hormones play an important role during multiple phases of growth and development as well as in tolerance to abiotic stresses and defense against pathogens and herbivores. Many phytohormones mediate signal transduction for multiple processes. Abscisic acid (ABA), for example, regulates stomatal closure, controls organ size and development and is vital for processes like seed and bud dormancy. But, additionally ABA is key to a plant´s tolerance to many

abiotic stresses including drought and salinity as well as cold and heat stress (Finkelstein 2013). The hormone salicylic acid (SA) is involved in defense against biotrophic pathogens, but also against piercing and sucking herbivores (Dempsey *et al*. 1999). Jasmonic acid (JA) and its derivatives mediate defense against both leaf chewing herbivores and necrotrophic pathogens (Wasternack & Hause 2013). Upon the detection of an attack *via* pathogen-associated or herbivore-associated molecular patterns, SA or JA are increased resulting in the upregulation of defense-related genes and the production of defense compounds like pathogenesis-related proteins, antimicrobial compounds or hydrogen peroxide in the case of SA, and trichomes, protease inhibitors (PIs) and VOCs in the case of JA.

Phytohormones can travel through the vascular system of the plant. Therefore, it is not uncommon that an herbivore attack can induce defenses in systemic undamaged parts of the plant (Heil & Ton 2008; Erb *et al*. 2012). Thus, defenses induced as a response to one attacker could potentially affect defense mechanisms against another attacker that feeds sequentially or simultaneously on distal parts of the same plant (Vos *et al*. 2013). In nature it is highly unlikely that only a single herbivore species will attack a plant. Therefore, inducible defenses against multiple attacking herbivores have been intensively studied in the past (Rodriguez-Saone *et al*. 2005; Poelmann *et al*. 2008; Ali & Agrawal 2014; Eberl *et al*. 2017). However, most of these studies focus only on plant-insect interaction happening aboveground (AG). Literature on belowground (BG) defenses and the interaction with AG defenses is sparse. And even less is known about BG-AG interactions for woody plant species. The results of studies investigating BG-AG interactions have not been uniform, likely due to the fact that most studies have been carried out on either *Brassica spp.* or maize plants, two plant groups with very different root architecture (recently reviewed in Papadopoulou & Dam 2016).

The damage dealt to plant tissue by a chewing herbivore may be accompanied by water loss (Aldea *et al*. 2005; Erb *et al*. 2009[a]). It is commonly known that ABA plays an important role for plant responses to wounding and drought stress (Nguyen *et al*. 2016). Furthermore, ABA is known to be systemically transported from roots to shoots (Jackson 1997). Therefore, it is likely that BG-AG interactions are mediated by ABA. However, *Brassica spp.* plants did not show signs of water stress in AG tissues after BG herbivory even though the loss of root biomass should have limited the plant's capacity to take up water (van Dam *et al*. 2005). Unfortunately, there are no studies to this point that measured changes in phytohormone levels in *Brassica* in the context of BG-AG interactions. For maize it has been shown that root herbivory can cause drought-like stress symptoms leading to the increase of ABA in roots as well as in AG tissues, but BG feeding had no effect on the levels of SA or JA and its derivatives (Erb *et al*.2009[a]; Erb *et al*. 2011). However, since phytohormones are known to interact with each other, the increase of ABA in leaves could potentially influence AG

signaling *via* other phytohormones. This does not seem to be the case for SA though. SA does not seem to play a signaling role in plant defenses induced by root herbivory (Erb *et al*. 2009[a]; Pieterse *et al*. 2012). Additionally, BG application of SA did not change the amount of AG defense metabolites in *Brassica* (van Dam *et al*. 2004). On the other hand, many studies hint at the importance of JA in BG-AG interactions (reviewed by Erb *et al*. 2008 and Soler *et al*. 2013).

Effects of belowground herbivory on aboveground tissues

Besides interfering with plant signaling pathways, root herbivory has more imminent consequences for a plant. A plant may lose a substantial amount of root biomass to herbivores, which will most likely hinder its capability to uptake water and nutrients from the soil. Therefore, BG herbivory can have a harsh impact on plant growth and development depending on the age of the plant and the severity of the attack (Tsunoda *et al*. 2014). Additionally, root feeding can affect the preference and performance of AG herbivores feeding on the same plant and also affect higher trophic levels (Bezemer & van Dam 2005; Rasmann & Turlings 2007). When root herbivory is accompanied by drought stress, a plant may undergo osmotic adjustments with proteins being mobilized into free amino acids and polymeric carbohydrates being hydrolyzed into free sugars (Rosa *et al*. 2009; Hummel *et al*. 2010; Bowne *et al*. 2012). The released compounds can then form a hydration shell around fragile proteins to prevent their deformation (Hajlaoui *et al*. 2010). Unfortunately for the plant, an increase of available sugars and amino acids in the leaves can be exploited by AG feeding insects. Ximénez-Embún *et al*. (2016), for example, reported that caterpillars dealt more damage to drought stressed tomatoes in comparison to well-watered plants. The increased levels of available nitrogen in drought stressed shoots can also be beneficial for the performance of AG feeding insects (Brown & Gange 1990; Poveda *et al*. 2003).

Due to the increased attractiveness of drought stressed leaves to AG herbivores, plants may induce defenses AG when they are challenged by root herbivory. For example it has been reported that genes annotated as encoding protease inhibitors (PI) are upregulated in AG tissues following BG herbivory (Erb, 2011; Nguyen *et al*. 2016). Furthermore, classic defense compounds like phenolic compounds in *Brassica* (van Dam, 2005; Jansen *et al*. 2008) and chlorogenic acid and benzoxazinoids in maize (Erb *et al*. 2009[a]) seem to be universally induced upon root damage. The effect of root feeding on AG volatile emissions, on the other hand, is not well understood. Researchers have found increasing or decreasing emissions of VOCs (Soler *et al*. 2007; Tariq *et al*. 2013) as well as no changes in AG emissions after root herbivory (Rasmann & Turlings 2007). As mentioned earlier, almost all studies investigating

BG-AG interactions have been carried out with herbaceous plants. Therefore, it remains almost completely unknown how woody species react to root herbivory.

1.3. Study organisms

To study the effects of intra- and inter-plant communication on plant tissues and their herbivores, I used young black poplar trees (*Populus nigra*) and caterpillars of the gypsy moth (*Lymantria dispar*).

Within the family Salicaceae, the genus *Populus* consists of 25 to 35 species that are commonly referred to as poplar, cottonwood and aspen. Black poplars are deciduous plants that are native to Europe, Asia and northwest Africa. They can grow between 20 to 30 m in height and up to 1.5 m in diameter. Both pollen and seeds of these dioecious trees are dispersed through wind. *Populus nigra* is a key species in riparian ecosystems that are amongst the most endangered habitats in Europe (Krause *et al*. 2011). As a pioneer species, it serves important ecosystem functions by stabilizing river banks (Stettler *et al*. 1996). Poplars are cultivated for multiple uses like the production of fiber, timber, animal food and biofuel. But, they are also planted as windbreaks or protection against soil erosion. Additionally, they are used in phytoremediation since they have a great potential to detoxify soils contaminated with heavy metals (Robinson *et al*. 2000).

Black poplar is well suited as a model organism because it is a fast growing tree species that can be cultivated monoclonally from stem cuttings. Additionally the genome of the closely related *Populus trichocarpa* was the first fully sequenced tree genome (Tuskan *et al*. 2006). Poplars are known to mount a wide array of direct defenses against herbivores including VOCs, phenolics and defensive proteins like PIs (Philippe & Bohlmann 2007). HIPVs of poplar have also been reported to serve as indirect defenses by attracting parasitoid wasps (Clavijo McCormick *et al*. 2014[b]). As it is typical for boreal trees, the leaves of *P. nigra* show high levels of condensed tannins, which can make up to 20 % and more of leaf dry mass (Donaldson *et al*. 2006). Condensed tannins are thought to serve as direct defenses against mammalian herbivores through protein precipitation. However their effect on insect herbivores is still unclear and controversial (reviewed in Barbehenn & Peter Constable 2011). Other well-known phenolic defense compounds of the Salicaceae family are the salicinoids. More than 20 different salicinoid compounds are described for poplar (Boeckler *et al*. 2011). They are known to affect the behavior and fitness of feeding insects (Lindroth 1991; Ruuhola *et al*. 2001; Osier & Lindroth 2006) and even mammals (Diner *et al*. 2009).

One of the natural enemies of *P. nigra* is the gypsy moth caterpillar (*Lymantria dispar,* Linnaeus 1758). It belongs to the family of the Erebidae and consists of three subspecies: the Asian gypsy moth, *L. dispar asiatica*, the Japanese gypsy moth, *Lymantria dispar japonica*, and the European gypsy moth, *Lymantria dispar dispar*. Here, *Lymantria dispar* is used as a synonym for the European gypsy moth. In 1869 the European gypsy moth was introduced to the USA by a French entomologist, who wanted to crossbreed *L. dispar* with silkworms. Since then the gypsy moth has become the most devastating forest pest in eastern North America. It can feed on over 500 different tree and shrub species, including poplar, birch and oak, and is listed in the top 100 of the most destructive species of the world. Since the females of *L. dispar* cannot fly, the larvae choose feeding and oviposition sites. They can disperse over long ranges up to 50 km by a phenomenon known as ballooning, where the presence of hairs and production of silk threads allow the larvae to be blown by wind (Global Invasive Species Database 2019).

1.4. Aim of the thesis

Although it has long been known that plants communicate internally among their tissues and organs *via* phytohormones traveling through the vascular system, the discovery that volatile compounds besides ethylene also play an important role in intra-plant signaling has come much more recently. Still, literature on the volatile signaling of woody and perennial plant species is relatively sparse. Moreover, most studies focus solely on the plant response to volatiles with only a few trying to elucidate the effects on the herbivore. When it comes to, belowground and belowground-aboveground interactions, both phytohormone-mediated and volatile-mediated communication have been neglected, especially in woody plants.

The aim of my study was therefore to investigate different aspects of intra- and interplant communication in trees and the resulting effects on feeding herbivores. First of all, I wanted to know how tree roots respond to herbivore damage and whether belowground herbivory influences aboveground defense mechanisms. Second, I wanted to learn more about volatile-mediated defense priming in trees and whether priming has negative consequences for feeding insects.

1. Introduction

2. OVERVIEW OF MANUSCRIPTS

This thesis is based on the following manuscripts.

2.1. Manuscript I

The occurrence and formation of monoterpenes in herbivore-damaged poplar roots

Nathalie D. Lackus, Sandra Lackner, Jonathan Gershenzon, Sybille B. Unsicker & Tobias G. Köllner

Published in *Scientific Reports* (2018), Volume 8: 17936; doi: 10.1038/s41598-018-36302-6

Summary

In this study, we investigated the formation and emission of volatile organic compounds from roots of two poplar species, *Populus nigra* and *Populus trichocarpa*, upon belowground herbivory by larvae of the common cockchafer (*Melolontha melolontha*). We showed that damaged poplar roots released a mixture of monoterpenes, which are biosynthesized by terpene synthases in the root tissue. Three of the respective terpene synthases could be identified and characterized *in vitro*. Additionally, two compounds, 1,8-cineole and β-pinene, showed antimicrobial activity against the oomycete *Phytophthora cactorum*, which is a devastating pest for many fruit crop species. This indicates that herbivore-induced root volatiles can act as defenses against secondary pathogen infection upon root wounding.

Author Contributions

Conceived project:	SL (10 %), NDL, JG, SBU, TGK
Designed experiments:	SL (30 %), NDL, SBU, TGK
Biochemical characterization:	NDL
Fungal assays:	SL (100 %)
Data analysis:	SL (30 %), NDL, TGK
Manuscript writing:	SL (10 %), NDL, TGK

2. Overview of the Manuscripts

2.2. Manuscript II

Aboveground phytochemical responses to belowground herbivory in poplar trees and the consequences for leaf herbivore preference

Sandra Lackner, Nathalie D. Lackus, Christian Paetz, Tobias G. Köllner & Sybille B. Unsicker

Submitted to *Plant, Cell and Environment* (2019).

Summary

In this study, we investigated the effects of root herbivory by larvae of the common cockchafer (*Melolontha melolontha*) on the leaf phytochemistry of black poplar (*Populus nigra*) and how this influences the generalist caterpillars of the gypsy moth (*Lymantria dispar*). We showed that belowground herbivory did not affect aboveground defenses such as volatiles, protease inhibitors or salicinoids. But, root herbivory caused drought stress-like symptoms in leaves, resulting in an increase of abscisic acid and proline. Gypsy moth caterpillars showed a preference for leaves from root-damaged trees, most likely due to the phagostimulatory properties of proline. This study demonstrates that trees rely on vascular intra-plant signaling from roots to shoots and that belowground herbivores can influence behavior and possibly performance of aboveground herbivores *via* phytochemical changes in the common host tree.

Author Contributions

Conceived project:	SL (30 %), NDL, TGK, SBU
Designed experiments:	SL (30 %), SBU
Performed experiments:	SL (30 %), SBU
Chemical analysis:	SL (80 %), SBU
NMR analysis:	CP
Data analysis:	SL (100 %)
Manuscript writing:	SL (80 %), NDL, CP, SBU

2. Overview of the Manuscripts

2.3. Manuscript III

Volatile mediated defense priming in black poplar.

Minor changes can cause major differences

Sandra Lackner, Thomas Fabisch, Heiko Vogel, Beate Rothe, Jonathan Gershenzon & Sybille B. Unsicker

In preparation for *Ecology Letters*.

Summary

In this study, we investigated whether exposure to herbivore-induced plant volatiles changes black poplar´s (*Populus nigra*) leaf chemistry and how this might affect feeding gypsy moth caterpillars (*Lymantria dispar*). Black poplar trees showed no measurable phenotypical changes after exposure to plant volatiles. However, upon subsequent herbivore attack salicin levels increased compared to non-exposed leaves. This indicates that volatile exposure primed the salicinoid defense compounds. Furthermore, gypsy moth caterpillars avoided primed leaves and showed increased mortality and decreased performance when being forced to feed on primed leaves. We argue that this is due to the priming of the salicinoids. These results suggest that volatile-mediated defense priming leads to increased herbivore resistance of black poplar trees.

Author Contributions

Conceived project:	SL (60 %), JG, SBU
Designed experiments:	SL (70 %), TF, SBU
Performed experiments:	SL (90 %), TF
Chemical analysis:	SL (80 %), BR
Bioinformatical analysis:	HV
Data analysis:	SL (90 %), TF
Manuscript writing:	SL (90 %), JG, SBU

2. Overview of the Manuscripts

3. MANUSCRIPT I

The occurrence and formation of monoterpenes in herbivore-damaged poplar roots

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The occurrence and formation of monoterpenes in herbivore-damaged poplar roots

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Volatiles are often released upon herbivory as plant defense compounds. While the formation of volatiles above-ground has been intensively studied, little is known about herbivore-induced root volatiles. Here, we show that cockchafer larvae-damaged roots of *Populus trichocarpa* and *P. nigra* release a mixture of monoterpenes, including (−)-α-pinene, (−)-camphene, (−)-β-pinene, *p*-cymene, and 1,8-cineole. Three terpene synthases, PtTPS16 and PtTPS21 from *P. trichocarpa* and PnTPS4 from *P. nigra*, could be identified and characterized *in vitro*. PnTPS4 was found to produce 1,8-cineole as sole product. PtTPS16 and PtTPS21, although highly similar to each other, showed different product specificities and produced γ-terpinene and a mixture of (−)-camphene, (−)-α-pinene, (−)-β-pinene, and (−)-limonene, respectively. Four active site residues were found to determine the different product specificities of the two enzymes. The expression profiles of *PtTPS16*, *PtTPS21*, and *PnTPS4* in undamaged and herbivore-damaged poplar roots generally matched the emission pattern of monoterpenes, indicating that monoterpene emission in roots is mainly determined at the gene transcript level. Bioassays with *Phytophtora cactorum* (Oomycetes) revealed inhibitory effects of vapor-phase 1,8-cineole and (−)-β-pinene on the growth of this important plant pathogen. Thus herbivore-induced volatile monoterpenes may have a role in defense against pathogens that cause secondary infections after root wounding.

The production and emission of volatiles in response to herbivory is a well-studied phenomenon that has been described in a multitude of plant species. The released volatiles can fulfill different functions in direct and indirect plant defense, including the deterrence of herbivores and the attraction of herbivore enemies[1]. Moreover, such volatiles can act as signals in intra- and inter-plant communication where they warn other plant parts or neighboring plants against impending herbivore attacks[2,3]. Since herbivore-induced volatiles can be toxic to microorganisms, they have also been discussed as phytoanticipins, which may protect the wounding site by inhibiting secondary infections caused by phytopathogenic bacteria or fungi[1,4,5].

While the formation and biological roles of volatiles emitted from herbivore-damaged above-ground organs have been intensively investigated during the last three decades, our knowledge about herbivore-induced root volatiles is still limited[6,7]. Maize (*Zea mays*) roots have been shown to release the sesquiterpene (*E*)-β-caryophyllene after damage by larvae of the root beetle *Diabrotica virgifera virgifera*[8,9]. (*E*)-β-Caryophyllene rapidly diffuses through the soil and can attract entomopathogenic nematodes able to attack and kill the beetle larvae. A mixture of four sesquiterpenes emitted from herbivore-damaged roots of Citrus trees (*Citrus paradise x Poncirus trifoliata*) was also shown to be attractive for entomopathogenic nematodes[10]. Herbivory of cockchafer larvae (*Melolontha melolontha*) on roots of apple trees (*Malus x domestica*) resulted in the emission of the monoterpene camphor[11], while oak roots damaged by *M. hippocastani* larvae emitted a volatile mixture comprising 1,8-cineole, 1-octen-3-ol, octan-3-one, and the aromatic compound anisole[12]. Several *Brassica* species such as *B. nigra*, *B. juncea*, and *B. napus*, however, were shown to release mainly sulfur-containing volatiles derived from glucosinolate breakdown after infestation with the cabbage root fly (*Delia radicum*)[13,14].

Herbivore-induced volatile blends are often dominated by mono- and sesquiterpenes, which are produced through the action of a specific class of enzymes called terpene synthases (TPS). Plant terpene synthases have mainly been described in seed plants[15], but also in a few non-seed plants including lycophytes and mosses[16,17]. They catalyze a magnesium ion-dependent conversion of the ubiquitous precursors geranyl diphosphate (GPP),

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	P. trichocarpa				*P. nigra*			
	ctr	herb	*P*-value	t-value	ctr	herb	*P*-value	t-value/ *T-value*
α-pinene	83.9 ± 34.3	149.3 ± 77.1	0.765	0.305	25.8 ± 9.2	78.1 ± 40.9	0.677	−0.426
camphene	52.6 ± 13.9	118.5 ± 31.6	0.061	−2.042	38.2 ± 11.8	666.4 ± 233.6	≤0.001	*37.00*
β-pinene	108.5 ± 27.9	207.9 ± 48.5	0.085	−1.854	31.3 ± 5.3	74.8 ± 21.9	0.058	−2.065
p-cymene	108.7 ± 31.9	200.6 ± 84.1	0.458	−0.764	NA	NA	NA	NA
1.8-cineole	258.4 ± 47.2	577.3 ± 150.0	0.048	2.168	101.3 ± 20.3	117.5 ± 22.7	0.578	−0.570
unidentified MT1	NA	NA	NA	NA	0.0 ± 0.0	20.1 ± 8.0	0.038	*48.00*
unidentified MT2	NA	NA	NA	NA	24.7 ± 3.6	30.5 ± 6.0	0.388	−0.892
unidentified MT3	NA	NA	NA	NA	82.9 ± 29.9	104.0 ± 33.9	0.625	−0.5

Table 1. Emission of volatile monoterpenes from undamaged (ctr) and *Melolontha melolontha*-damaged (herb) roots of *Populus trichocarpa* and *P. nigra*. Emission levels are displayed as means ± SE in pg g^{-1} h^{-1} fresh weight (n = 8). *P*-values are based on the results from Student's t-tests or from Mann-Whitney Rank Sum Tests between control and herbivore treatments. MT: monoterpene, NA: not detected.

farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) into the different mono-, sesqui-, and diterpene skeletons, respectively, and thus determine the chemical nature of the formed terpenes[15]. Due to their reaction mechanisms, which include the formation of highly reactive carbocation intermediates, many terpene synthases have broader product specificity and produce mixtures of terpenes[15]. The exchange of single amino acids in the active site of TPS proteins often results in dramatic shifts in product specificity[15]. The opposite stereospecificity of two closely related maize terpene synthases TPS4 and TPS5, for example, is determined by only four amino acid differences in their active sites[18].

During the last years, trees of the genus *Populus* have been established as model organisms to study the biochemical basis of terpene formation in trees. The genome of the western balsam poplar *Populus trichocarpa* contains 38 *TPS* gene models and about half of them have been characterized in previous studies[19–21]. Gene expression analysis and *in vitro* enzyme characterization revealed that the complex terpene blend emitted from gypsy moth caterpillar-damaged *P. trichocarpa* leaves can be fully explained by the enzyme activities of eight terpene synthases[19,20]. Four other TPS enzymes of *P. trichocarpa* could be characterized as diterpene synthases likely involved in the formation of non-volatile compounds in leaves and roots[21]. The biological relevance of the remaining 26 *TPS* genes in *P. trichocarpa*, however, is still unclear.

In the present study we performed volatile collection and gas chromatography-mass spectrometry analysis to identify and compare the volatile blends emitted from *M. melolontha*-damaged roots of the two poplar species *P. trichocarpa* and *P. nigra*. Heterologous expression of *TPS* genes in *Escherichia coli* and gene expression analysis using qRT-PCR allowed us to identify terpene synthases involved in monoterpene formation in *P. trichocarpa* and *P. nigra* roots. *In vitro* bioassays with the root pathogen *Phytophthora cactorum* and different volatile terpenes indicated that 1,8-cineole and (−)-β-pinene might be involved in plant defense against this generalist oomycete.

Results

Root herbivory leads to elevated monoterpene emission in poplar. As part of our ongoing research on volatile-mediated defenses in poplar, we analyzed the volatiles emitted from undamaged and *M. melolontha* (cockchafer)-damaged roots of *P. trichocarpa*, a species native to North America, and *P. nigra*, an indigenous European species. Trees were grown in sand-filled pots and a push-pull system was used to collect root volatiles from air pumped through the soil (Supplementary Fig. S1). Volatile analysis was conducted with gas chromatography-mass spectrometry (GC-MS). Root damage caused by the larvae was monitored after the experiment by measuring the total root biomass. For *P. trichocarpa*, cockchafer herbivory led to a significantly reduced root mass in comparison to undamaged controls (Supplementary Fig. S2). For *P. nigra*, however, we could not observe significant differences, although a slight trend of root biomass reduction in the grub treatments was visible (Supplementary Fig. S2).

Undamaged roots of both poplar species released considerable amounts of monoterpenes, including (−)-α-pinene, (−)-camphene, (−)-β-pinene, and 1,8-cineole (Table 1). Two further monoterpenes, *p*-cymene and an unidentified compound with a molecular mass of 136, could only be detected in the volatile bouquet of *P. trichocarpa*. Herbivory significantly increased the emission of 1,8-cineole from *P. trichocarpa*, while (−)-camphene and an unidentified monoterpene were significantly induced in herbivore-damaged roots of *P. nigra* (Table 1). Beside monoterpenes, both species also constitutively emitted the aromatic compounds benzaldehyde, benzyl alcohol, and salicylaldehyde, however, emission of these compounds was not influenced by the herbivore treatment (Supplementary Table S1). Considering that the plastics pots and the moist sand in the volatile collection system both might adsorb plant-released volatiles, our quantification of constitutive and herbivore-induced root volatiles in *P. trichocarpa* and *P. nigra* is likely an underestimation of the total volatile release.

To measure the potential accumulation of volatile compounds in the roots, we extracted plant material with hexane and analyzed the extracts using GC-MS. While the aromatic volatiles benzaldehyde, benzyl alcohol, and salicylaldehyde accumulated in root material collected from *P. trichocarpa* and *P. nigra*, monoterpenes (camphene) could only be detected in the extracts of *P. nigra* (Supplementary Table S2). Interestingly, salicylaldehyde and benzaldehyde showed significantly increased accumulation after cockchafer herbivory, although their emission rates were not influenced by the treatment as already mentioned above (Supplementary Tables S1 and S2).

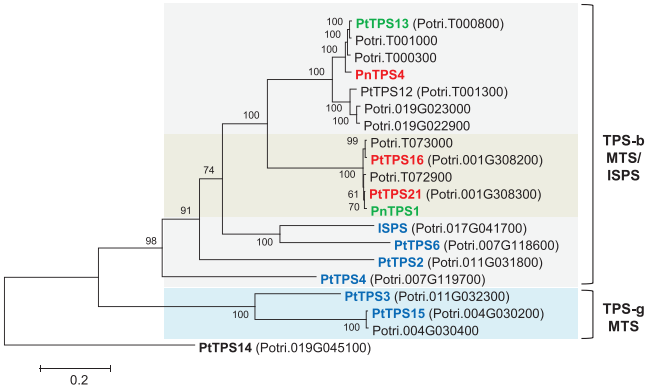

Figure 1. Dendrogram (maximum likelihood tree) of putative monoterpene synthases of *Populus trichocarpa* and *P. nigra* cloned in this study (red), their previously identified orthologues (green), other previously characterized *P. trichocarpa* TPS-b and TPS-g enzymes (blue), and further putative monoterpene synthases found in the *P. trichocarpa* genome version 3.0 (black). Bootstrap values (n = 1000) are shown next to each node. ISPS, isoprene synthase; MTS, monoterpene synthase; TPS-b and -g represent TPS subfamilies. Scale bar under the tree indicates number of substitutions per site.

Identification and characterization of three monoterpene synthases in *P. trichocarpa* and *P. nigra*. The recently identified terpene synthase PnTPS1 produces camphene, α-pinene, β-pinene, and limonene and has been shown to be involved in herbivore-induced monoterpene formation in the leaves of *P. nigra*[22]. Another monoterpene synthase, the 1,8-cineole synthase PtTPS13, was reported to be expressed in herbivore-damaged leaves of *P. trichocarpa*[20]. Since products of both enzymes were found in the volatile bouquets of herbivore-induced *P. trichocarpa* and *P. nigra* roots (Table 1), we hypothesized that these or similar enzymes are also involved in the formation of root terpenes. To identify potential orthologues of *PnTPS1* and *PtTPS13* in *P. trichocarpa* and *P. nigra*, respectively, we amplified the open reading frames from cDNA made from *M. melolontha*-damaged root material. Amplification and cloning were successful and the resulting genes were designated *PtTPS21* (*P. trichocarpa* genome version 3.0 accession, Potri.001G308300) and *PnTPS4*, respectively, according to the poplar TPS nomenclature initiated in previous studies[19,20,22]. Sequencing of several amplicons revealed another *P. trichocarpa* gene with 97% nucleotide similarity to *PtTPS21* that was designated *PtTPS16* (Potri.001G308200). A sequence comparison and dendrogram analysis of predicted monoterpene synthase genes in the *P. trichocarpa* genome showed that *PtTPS16* and *PtTPS21* were part of a gene cluster comprising four members highly similar to each other (Fig. 1).

Heterologous expression of the N-terminal truncated open reading frames of the identified sequences lacking their putative signal peptides and subsequent enzyme assays confirmed monoterpene synthase activity for all tested enzymes. As expected, PtTPS21 and PnTPS4 had the same activity as their putative orthologues, PnTPS1 and PtTPS13, respectively, and produced camphene, α-pinene, β-pinene, and limonene (PtTPS21) and 1,8-cineole and a few minor products (PnTPS4) (Fig. 2). Interestingly, PtTPS16, although highly similar to PtTPS21, showed different product specificity and produced γ-terpinene together with a mixture of minor monoterpene products (Fig. 2). Chiral analysis of PtTPS21 enzyme products showed that all of the produced monoterpenes were exclusively formed as (−)-enantiomers (Fig. 3). When tested with the sesquiterpene precursor (*E,E*)-FPP, PnTPS4 showed activity and produced a mixture of sesquiterpens including (*E*)-α-bergamotene, (*E*)-β-farnesene, (*E,E*)-α-farnesene, sesquiphellandrene, (*Z*)-α-bisabolene, and nerolidol (Supplementary Fig. S3). However, since PnTPS4 was found to possess a signal peptide that targets the protein to the plastids (Supplementary Fig S4), it functions most likely as monoterpene synthase in planta. In contrast to PnTPS4, PtTPS16 and PtTPS21 were not active with (*E,E*)-FPP. The diterpene substrate (*E,E,E*)-GGPP was not accepted by the tested enzymes.

Homology modeling of PtTPS16 and *in vitro* mutagenesis of active site residues. To identify the amino acid residues that determine the observed differences in product specificity of PtTPS16 and PtTPS21, we performed homology modeling of the three-dimensional structure of PtTPS16 using the crystal structure of (+)-limonene synthase from *Citrus sinensis*[23] as a template. Visualization of the resulting model and an amino acid sequence comparison of PtTPS16 and PtTPS21 revealed four amino acid substitutions within the active site pockets of the two proteins (Fig. 4; Supplementary Fig. S5). *In vitro* mutagenesis of the single residues isoleucine 335, valine 441, and valine 483 in PtTPS16 revealed no or only marginal changes in product specificity of the resulting mutant enzymes PtTPS16 I335V, PtTPS16 V441I, and PtTPS16 V483L, while a mutation of threonine 336 to asparagine led to an enzyme able to produce camphene, α-pinene, β-pinene, and limonene in addition to γ-terpinene (Fig. 5). Different combinations of single amino acid changes finally showed that the quadruple

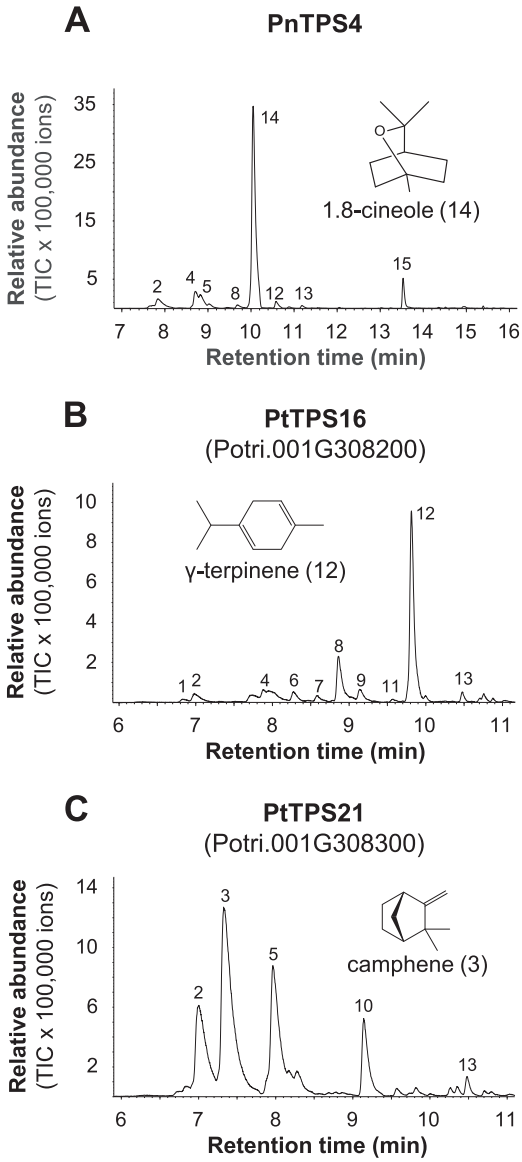

Figure 2. Biochemical characterization of the newly identified poplar root terpene synthases PnTPS4 (**A**), PtTPS16 (**B**), and PtTPS21 (**C**). The genes were heterologously expressed in *E. coli* and partially purified proteins were incubated with GPP as substrate. Enzyme products were analyzed using GC-MS. 1, α-thujene; 2, α-pinene*; 3, camphene*; 4, sabinene; 5, β-pinene*; 6, myrcene; 7, α-phellandrene; 8, α-terpinene*; 9, β-phellandrene; 10, limonene*; 11, ocimene; 12, γ-terpinene*; 13, terpinolene; 14, 1.8-cineole; 15, fenchyl alcohol. Compounds marked with * were identified by comparison of retention time and mass spectrum to those of authentic standards. Others were identified by database comparisons.

mutant PtTPS16 I335V, T336N, V441I, V483L had a product specificity highly similar to PtTPS21, although there were still minor quantitative differences in the product profiles of the mutant and PtTPS21 (Fig. 5).

Gene expression analysis of terpene synthases in poplar roots. The expression levels of *P. trichocarpa PtTPS13*, *PtTPS16*, and *PtTPS21* and *P. nigra PnTPS4* and *PnTPS1* were measured in undamaged and

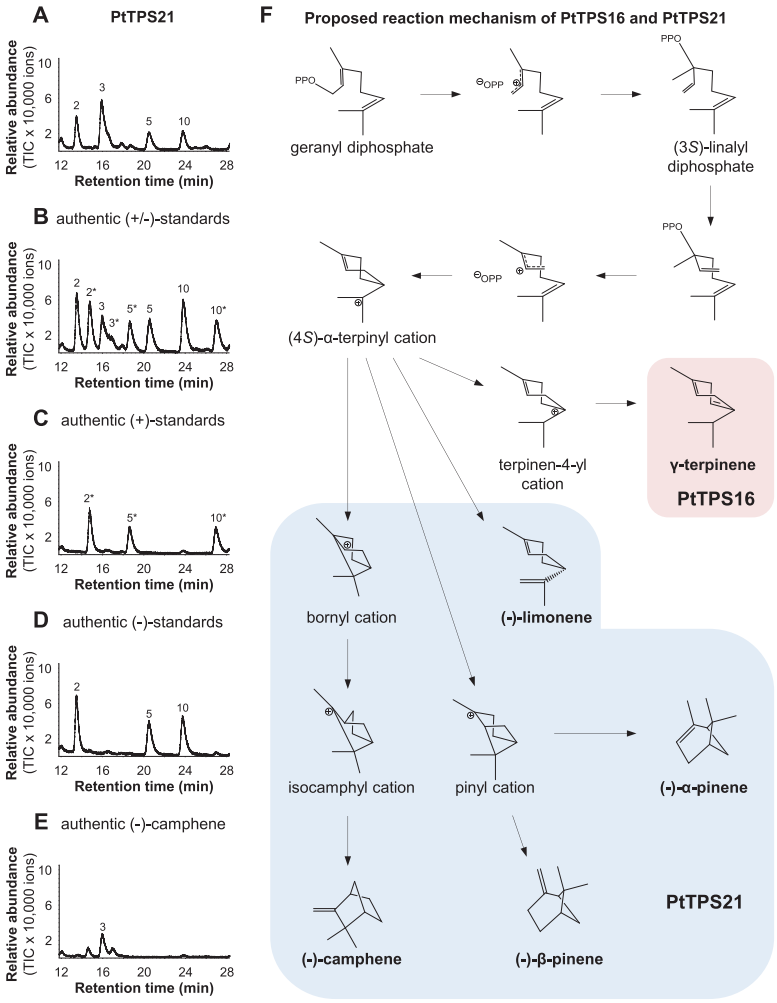

Figure 3. Stereochemical analysis of PtTPS21 enzyme products (**A**–**E**) and proposed reaction mechanism of PtTPS16 and PtTPS21 (**F**). 2, (−)-α-pinene; 3, (−)-camphene; 5, (−)-β-pinene; 10, (−)-limonene, 2*, (+)-α-pinene; 3*, (+)-camphene; 5*, (+)-β-pinene; 10*, (+)-limonene.

M. melolontha-damaged roots using qRT-PCR. The 1,8-cineole synthase genes *PtTPS13* and *PnTPS4* were less expressed in undamaged roots than the other genes, but showed significant upregulation upon root herbivory (Fig. 6). Expression of *PnTPS1* was also significantly induced by the herbivore treatment. *PtTPS21* and *PtTPS16* showed a trend towards higher expression levels in damaged-roots, although it was not significant when compared to expression levels in undamaged control roots (Fig. 6).

1,8-Cineole reduces the growth of the plant pathogen *Phytophthora cactorum in vitro*. *Phytophthora cactorum* (Oomycetes) is a widespread plant pathogen that can infest numerous plant species including crops and trees[24]. Infection by this pathogen often results in root rot and causes massive yield losses or even plant death. To prove the hypothesis that herbivore-induced root monoterpenes might play a role in protecting wounded roots against soil-borne pathogens, we tested the influence of volatile 1,8-cineole, (−)-β-pinene, and (−)-limonene on the growth of *P. cactorum in vitro*. 2-Phenylethanol, a common plant volatile known to have antifungal activity[25], was also included into the experiment. While 2-phenylethanol, (−)-limonene, and mineral oil as negative control had no influence on the growth of *P. cactorum*, 1,8-cineole and (−)-β-pinene significantly reduced the growth of this pathogen when present in the headspace (Fig. 7).

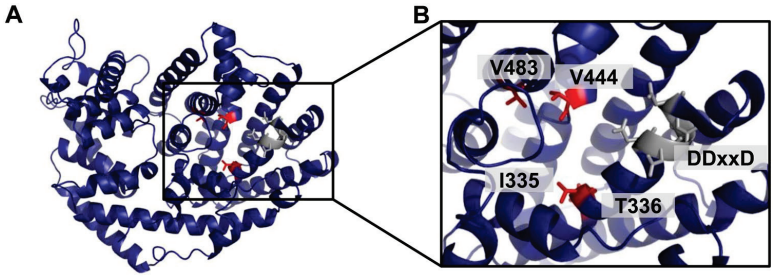

Figure 4. Structure model of PtTPS16. Models of N-terminal truncated PtTPS16 (**A**) and the active site of PtTPS16 (**B**) are shown. The conserved DDxxD motif is displayed in gray and the four amino acid residues that differ between the active sites of PtTPS16 and PtTPS21 are depicted in red.

Discussion

The emission of volatile organic compounds upon herbivory is part of a complex defense strategy that has been evolved as consequence of the ongoing evolutionary arms race between plants and their enemies. Both, the diverse biological roles of induced volatiles in plant defense as well as the biochemical basis of volatile formation have been intensively investigated in the past. However, the majority of this research focused on above-ground volatiles and thus our knowledge about root volatiles is still limited. Above-ground volatile blends are often dominated by mono- and sesquiterpenes that can fulfill diverse functions in plant-herbivore interactions[1]. Here we could show that undamaged and herbivore-damaged roots of the two poplar species *P. trichocarpa* and *P. nigra* produce and release a mixture of monoterpenes together with a few aromatic compounds (Table 1; Supplementary Table S1). These findings together with previous studies on maize, citrus, oak, and apple[8,10–12] indicate that monoterpenes are also common, major components of herbivore-induced root volatile blends.

Interestingly, although emitted as volatiles, (−)-α-pinene, (−)-β-pinene, *p*-cymene, and 1,8-cineole could not be detected in root tissues of the investigated poplar trees. This suggests de novo biosynthesis, but also an efficient transport of the terpenes from the site of their formation to the rhizosphere that prevents any detectable accumulation in the plant. Root volatile emission without detectable tissue accumulation has also been reported in Arabidopsis, where the 1,8-cineole synthase AtTPS-Cin was found to be exclusively expressed in roots. While the enzyme product could not be detected in root extracts[26], 1,8-cineole is indeed one of the major components of the Arabidopsis root volatile blend[27]. The 1,8-cineole synthase gene *AaTPS* in *Artemisia annua* is also expressed in roots but its product was not found in pentane extracts made from the respective tissues[28]. Although one cannot exclude a conversion of 1,8-cineole into another product, it is tempting to speculate that *A. annua* roots emit 1,8-cineole as shown for *Populus* and *Arabidopsis*.

Beside monoterpenes, poplar roots released considerable amounts of aromatic compounds including salicylaldehyde, benzaldehyde, and benzyl alcohol (Supplementary Table S1). In contrast to the volatile terpenes that showed no accumulation, the aromatic compounds accumulated in the roots (Supplementary Table S2). While their emission was not significantly influenced by the treatments, accumulation of salicylaldehyde and benzaldehyde was significantly increased upon herbivory. Thus one may hypothesize that both aldehydes are formed as degradation products of preformed salicinoids, a class of Salicaceae-specific phenolic glycosides derived from salicyl alcohol[29]. Or, the formation of these aromatic compounds might be independently induced by herbivory.

The 1,8-cineole synthase PtTPS13 and the camphene synthase PnTPS1 from *P. trichocarpa* and *P. nigra*, respectively, have recently been shown to be involved in herbivore-induced monoterpene formation in poplar leaves[20,22]. Since 1,8-cineole and camphene were also part of the poplar root volatile blend (Table 1), we tested the expression of *PtTPS13* and *PnTPS1* in undamaged and herbivore-damaged roots of *P. trichocarpa* and *P. nigra*. Moreover, we amplified and characterized the putative orthologues of *PtTPS13* and *PnTPS1*, *PnTPS4* and *PtTPS21*, respectively, and included them into gene expression analysis. The qRT-PCR experiment revealed that all analyzed genes were expressed in roots and showed significant induction (*PtTPS13, PnTPS4, PnTPS1*) or at least a trend for higher expression (*PtTPS21*) upon herbivory (Fig. 6). With one exception, the gene expression levels matched the emission of the respective major enzyme products, indicating that the terpene synthases contribute to monoterpene formation in *P. trichocarpa* and *P. nigra* roots. The 1,8-cineole synthase PnTPS4, however, was strongly upregulated upon herbivory, although 1,8-cineole was constitutively released from *P. nigra* roots (Fig. 6). This discrepancy might be explained by a conversion of 1,8-cineol to another terpenoid specifically upon herbivory in *P. nigra*. Interestingly, *P. trichocarpa* possesses two other *TPS* genes (Potri.T00100 and Potri.T000300) with high similarity to *PtTPS13* and *PnTPS4* (Fig. 1)[20]. Because amplification of Potri.T00100 and Potri.T000300 in *P. trichocarpa* failed, we speculate that they are not expressed in this species. However, their potential orthologues might be expressed in *P. nigra* roots and could be responsible for the constitutive 1,8-cineole emission observed from this organ.

Using primers specific for the (−)-camphene synthase *PtTPS21*, we amplified a further gene designated as *PtTPS16* from *P. trichocarpa* root cDNA. Despite a high sequence similarity of about 97% to PtTPS21, PtTPS16 had different product specificity and produced mainly γ-terpinene (Supplementary Fig. S3; Fig. 2). *PtTPS16* was found to be expressed in *P. trichocarpa* roots, but its product γ-terpinene could neither be detected in the root volatile blend nor in root extracts. Since γ-terpinene has been reported as a biosynthetic precursor for *p*-cymene

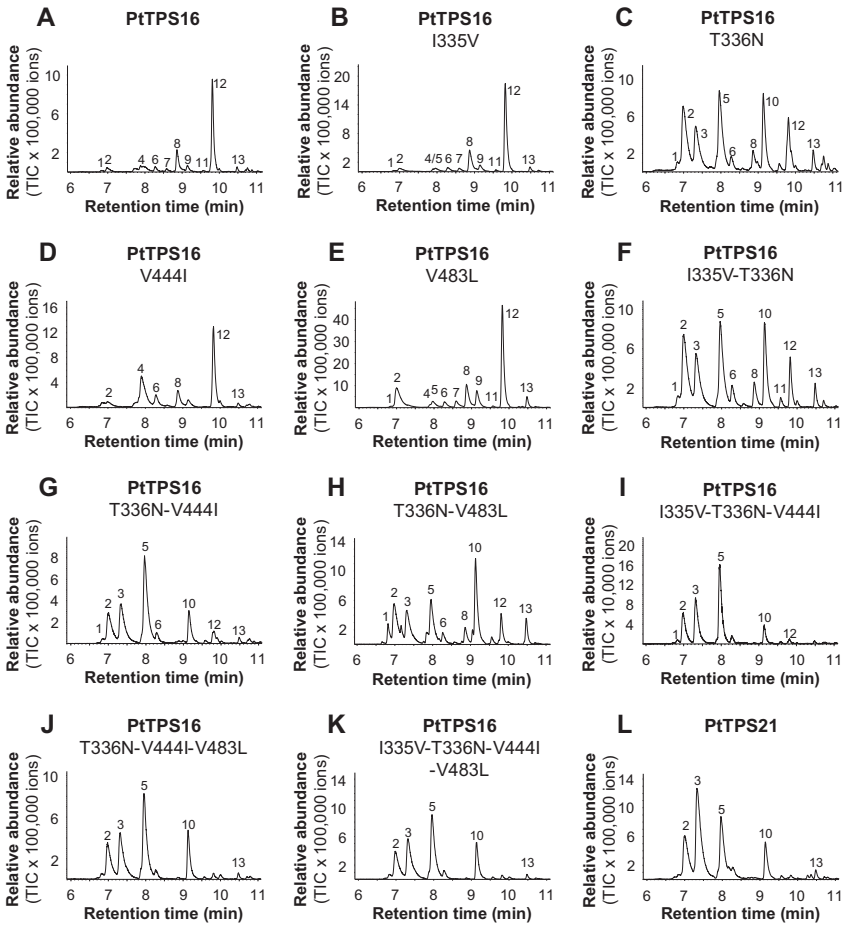

Figure 5. Biochemical characterization of PtTPS16 mutants generated using *in vitro* mutagenesis. GC-MS chromatograms representing the product spectra of wild type PtTPS16 (**A**), wild type PtTPS21 (**L**), and the different PtTPS16 mutants (**B**–**K**) are shown. Amino acid changes (one letter code) and their positions relative to the PtTPS16 sequence are indicated in the name of the mutants. The genes were heterologously expressed in *E. coli* and partially purified proteins were incubated with GPP as substrate. Enzyme products were analyzed using GC-MS. 1, α-thujene; 2, α-pinene*; 3, camphene*; 4, sabinene; 5, β-pinene*; 6, myrcene; 7, α-phellandrene; 8, α-terpinene*; 9, β-phellandrene; 10, limonene*; 11, ocimene; 12, γ-terpinene*; 13, terpinolene. Compounds marked with * were identified by comparison of retention time and mass spectrum to those of authentic standards. Others were identified by database comparisons.

in thyme (*Thymus vulgaris*)[30] and *p*-cymene was found as one of the major root volatiles in *P. trichocarpa*, it is conceivable that *P. trichocarpa* also metabolizes γ-terpinene into this oxidized monoterpene. The absence of *p*-cymene in *P. nigra* could likely be explained by the loss of the respective *PtTPS16* orthologue or loss of its expression in this species (Fig. 6).

Terpene synthases catalyze complex reactions, which are usually initiated by the metal ion-mediated cleavage of the diphosphate group from the substrate. The resulting carbocation is highly reactive and can undergo a series of cyclizations and rearrangements such as hydride and methyl shifts. A final elimination of a proton or addition of water terminates the reaction (Fig. 3)[15]. Product specificity of terpene synthases is believed to be a consequence of the three-dimensional contour of the active site that restricts the conformations of the substrate and/or reactive cationic intermediates[31,32]. It has been shown that small structural changes in the active site caused by single amino acid mutations often dramatically alter the product specificity of terpene synthases (e.g.[21,33,34]). Indeed, when compared to each other, the active sites of PtTPS16 and PtTPS21 differed only in four amino acid residues (Fig. 4; Supplementary Fig. S5), and a series of site-directed mutagenesis experiments with PtTPS16

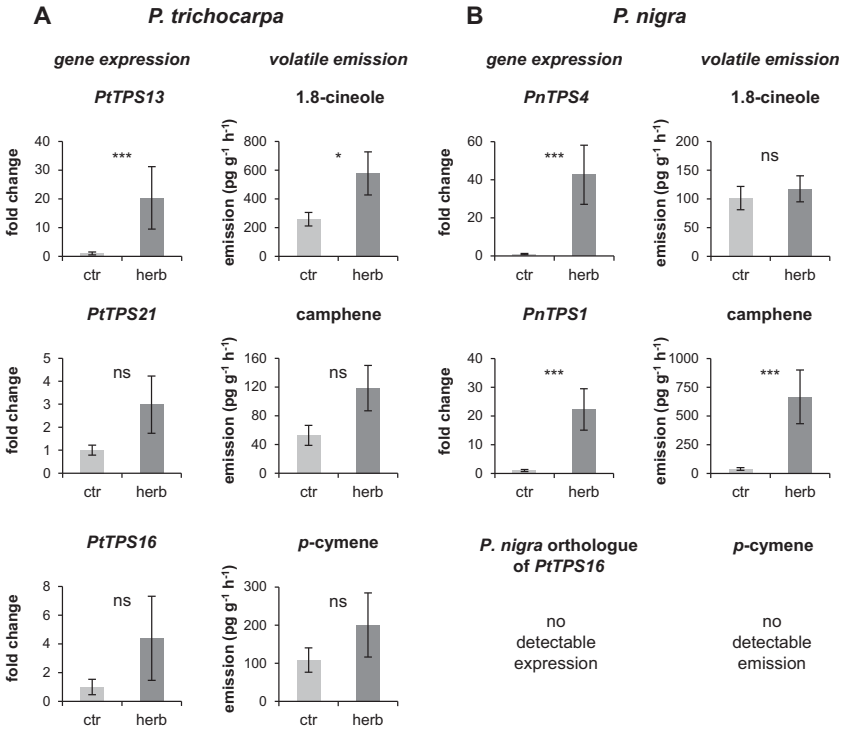

Figure 6. Expression of poplar root *TPS* genes and emission of major TPS products. Expression of *TPS* genes was analyzed using qRT-PCR. *TPS* expression and emission of monoterpenes are displayed for *M. melolontha*-damaged (herb) and undamaged (ctr) roots from *P. trichocarpa* and *P. nigra*. Means ± SE are shown (n = 8). Asterisks indicate statistical significance in Student's t-tests or from Mann-Whitney Rank Sum Tests. *PtTPS13* ($P \leq 0.001$, T = 36.00); *PtTPS21* ($P = 0.209$, T = 42.00); *PtTPS16* ($P = 0.195$, T = 55.00); *PnTPS4* ($P \leq 0.001$, T = 36.00); *PnTPS1* ($P \leq 0.001$, T = 36.00); *P. trichocarpa*: 1.8-cineole ($P = 0.048$, t = 2.168); camphene ($P = 0.061$, t = −2.042); *p*-cymene ($P = 0.458$, t = −0.764); *P. nigra*: 1.8-cineole ($P = 0.578$, t = −0.570); camphene ($P \leq 0.001$, T = 37.00).

revealed that all of them are important for the interconversion of PtTPS16 into PtTPS21 (Fig. 5). However, the largest effect on product specificity was observed for the single mutant PtTPS16 T336N, suggesting that the threonine-asparagine polymorphism at position 336 in PtTPS16/21 mainly determines the different product outcome of the two enzymes. Notably, the product profiles of the PtTPS16 quadruple mutant and the PtTPS21 wild type were not completely identical but still showed minor quantitative differences. It is thus likely that amino acid residue substitutions near the active site also influence the backbone and/or side chain conformation of active site residues and thus contribute to the fine tuning of TPS product specificity. Such effects have already been observed in mutagenesis experiments conducted with the 5-*epi*-aristolochene synthase from tobacco[35,36].

In general, wounding caused by herbivory or other mechanical stresses leads to favorable infection sites for microbial pathogens, providing them with nutrients and facilitates their entry into the plant tissue[37]. Many antimicrobial compounds have been described in plants, and among them are a variety of volatile mono- and sesquiterpenes[5]. Thus, we hypothesize that the increased emission of 1,8-cineole, (−)-camphene, and (−)-β-pinene from herbivore-damaged poplar roots is part of a defense reaction against soil-borne pathogens. Indeed, 1,8-cineole has been shown to have antimicrobial activities against a wide range of bacteria and fungi[38,39]. Moreover, its emission is induced by *Pseudomonas syringae* in Arabidopsis[27]. Our data showed that 1,8-cineole and (−)-β-pinene are also toxic for an oomycete root pathogen. Bioassays with *P. cactorum*, a common pathogen with a wide host range including many tree species, showed significantly reduced growth when it was cultivated in the presence of vapor-phase 1,8-cineole or (−)-β-pinene (Fig. 7). Other tested volatiles such as 2-phenylethanol and (−)-limonene, however, had no effect on *P. cactorum* growth, although they have been described as antimicrobial compounds in previous studies[25,39]. Our data suggest specific roles of single monoterpenes in different root-pathogen interactions.

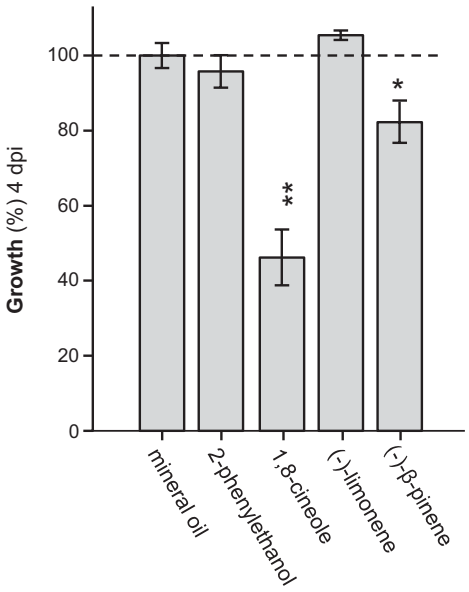

Figure 7. The effect of volatile monoterpenes on the growth of *Phytophthora cactorum* (Oomycetes). Mycelial growth of *P. cactorum* was measured in the presence of vapor-phase 2-phenylethanol, 1,8-cineole, (−)-limonene, and (−)-β-pinene. Pathogen growth in response to the individual compounds was compared to pathogen growth when exposed to pure mineral oil. The area of *P. cactorum* mycelium for the control treatment was set at 100% and growth under the influence of the different volatile compounds is shown relative to the mineral oil control. Means ± SE are shown (n = 5). Asterisks indicate significant differences (Student's t-tests. 2-phenylethanol ($P = 0.452$, $t = -0.797$); 1,8-cineole ($P = 0.001$, $t = -6.6$); (−)-limonene ($P = 0.166$, $t = 1.524$); (−)-β-pinene ($P = 0.023$, $t = -2.792$)).

Beside a potential function in pathogen defense, poplar root monoterpenes also might directly influence insect attackers. 1,8-Cineole and limonene, for example, have been shown to have insecticidal activity against the American wheat weevil *Rhyzopertha dominica* and the red flour beetle *Tribolium castaneum*[40] and it is conceivable that they are toxic for *M. melolontha* and other beetles as well. Moreover, root volatiles may influence the behavior of the beetle larvae. In bioassays with pure compounds, Eilers could show that γ-terpinene and benzaldehyde were both repellent for *M. melolontha* larvae while camphene and α-pinene attracted the larvae[41]. Because benzaldehyde, camphene, and α-pinene are all present in the root volatile blend of poplar, the combined effect of the blend on the beetle larvae is unclear. How the behavior of *M. melolontha* is influenced by the complete poplar volatile blend and whether there are potential synergistic effects among single root volatiles should be addressed in future studies.

Recent research showed that root volatiles play also roles in indirect plant defense. Herbivore-damaged roots of maize and citrus trees, for example, release volatile sesquiterpenes that attract entomopathogenic nematodes able to infest the attacking beetle larvae[8,10]. Whether herbivore-induced poplar monoterpenes can attract nematodes is still unclear. However, it has been shown that infection of *M. melolontha* larvae with nematodes of the genera *Heterorhabditis* and *Steinernema* increased larval mortality[42], and thus the attraction of nematodes by poplar root volatiles might provide an efficient defense against herbivorous beetles.

Material and Methods

Plants and insects. Western balsam poplar (*Populus trichocarpa*, clone Muhle-Larsen, P&P Baumschule, Eitelborn, Germany) and black poplar (*P. nigra*, clone f41)[43] trees were propagated from monoclonal stem cuttings and grown under summer conditions in the greenhouse (24 °C, 60% rel. humidity, 16 h/8 h light/dark cycle) in sand (Klasmann-Deilmann, Geeste, Germany), until they reached about 0.5 m in height.

Cockchafer larvae (*Melolontha melolontha*) were collected from meadows near Mespelbrunn/Spessart (Germany). Insects were reared individually in 200 ml plastic beakers filled with a mix of potting soil and grated carrots in a wine cooler operating in the dark at 12 °C.

Volatile collection. For volatile measurements, pots with single trees were completely enclosed in PET bags ("Bratschlauch", Toppits, Minden, Germany) by fixing one end of the bags to the stem of the tree with a cable binder and closing the other end also with a cable binder. Volatiles were collected using a dynamic push-pull system as shown in Supplementary Fig S1. Air flow was maintained in the system through teflon tubes. Charcoal

filtered air was pumped into the bags at a flow of 1 l min^{-1}. A portion of the aspirated air (flow: 0.6 l min^{-1}) was withdrawn with a 2nd pump and passed through a cooling trap (a glass bottle that was cooled down to 9 °C) to remove air humidity. A filter packed with 30 mg Poropak (ARS, Inc., Gainsville, USA) was used to adsorb the volatile compounds. Volatiles were collected for 68 h. After the collection, the volatile compounds were desorbed by eluting the filter with 200 µl dichloromethane containing nonyl acetate as an internal standard (10 ng μl^{-1}). Samples were stored at −20 °C until gas chromatography analysis. During the experiment, all plants were watered daily by injecting 30 ml of tap water into the sand through a tiny hole in the plastic bag that was tightly closed after watering.

For the herbivore treatment, two *M. melolontha* larvae (3d instar) were buried in each pot. Larvae were allowed to feed throughout the duration of the experiment. Volatile collections of sand-filled pots without trees and volatile collections of *M. melolontha* larvae were performed as negative controls. Root material was harvested and weighed immediately at the end of the volatile collection, flash-frozen with liquid nitrogen and stored at −80 °C until further sample preparation.

Hexane extraction of root tissue. To determine terpene accumulation in poplar roots, 100 mg of root powder was extracted in a GC glass vial with 400 µl hexane including 10 ng/uL nonyl acetate as internal standard. The extracts were shaken for one hour at 300 rpm and incubated over night at room temperature. After centrifugation for 10 min at 5,000 x g, the supernatant was analyzed using GC.

RNA extraction and reverse transcription. Total RNA was isolated from ground plant tissue using an InviTrap Spin Plant RNA kit (Stratec, Berlin, Germany) according to manufacturer's instructions. RNA concentration and purity were assessed using a spectrophotometer (NanoDrop 2000c, Thermo Scientific, Wilmington, DE, USA). RNA was treated with DNaseI (ThermoFisher Scientific, https://www.thermofisher.com) 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).

Isolation of TPS genes. Open reading frames (ORF) encoding the N-terminal truncated versions of PtTPS16 (Δ42), PtTPS21 (Δ42), and PnTPS4 (Δ42) lacking the putative signal peptides predicted with the programs ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) and TargetP (http://www.cbs.dtu.dk/services/TargetP/) (Supplementary Fig. S4) were amplified from cDNA made from herbivore-damaged root material with the primers listed in Supplementary Table S3. The PCR products obtained were inserted into the expression vector pET100/D-TOPO® (ThermoFisher Scientific, https://www.thermofisher.com) (*PtTPS16* and *PtTPS21*) or pASK-IBA7 (IBA-GmbH, Göttingen, Germany) (*PnTPS4*) and the cloned genes were fully sequenced.

***In vitro* mutagenesis.** For site-directed mutagenesis, 100 ng pET100/D-TOPO® vector containing the N-terminal truncated ORF of *PtTPS16* was used as template in a mutagenesis PCR (18 cycles, Phusion® High-Fidelity DNA Polymerase (ThermoFisher Scientific), according to manufacturer's instructions. The primers used contained the desired mutations and their sequences are given in Supplementary Table S3. After PCR, the plasmid template DNA was digested with *Dpn*I and the reaction mixture was inserted and amplified in *E. coli* TOP10 (Invitrogen). The mutagenized constructs were fully sequenced before expression.

Heterologous expression and TPS enzyme assays. The *E. coli* strain BL21 Star™ (DE3) (ThermoFisher Scientific) was used for expression of *PtTPS16/21* while *PnTPS4* was expressed in *E. coli* TOP10 (Invitrogen). Cultures were grown at 37 °C, induced at an $OD_{600} = 0.6$ with 1 mM IPTG (*PtTPS16/21*) or 200 ug l^{-1} anhydrotetracycline (*PnTPS4*) 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 used for enzyme assays.

To determine the catalytic activity of the different terpene synthases, enzyme assays were performed in a Teflon-sealed, screw-capped 1 ml GC glass vial containing 40 µl of the bacterial extract and 60 µl assay buffer with 10 µM substrate (GPP or (*E,E*)-FPP) and 10 mM $MgCl_2$. A solid phase microextraction (SPME) fiber consisting of 100 µm polydimethylsiloxane (SUPELCO, Belafonte, PA, USA) was placed into the headspace of the vial for 60 min incubation at 30 °C. For analysis of the adsorbed reaction products, the SPME fiber was directly inserted into the injector of the gas chromatograph (see below). Potential diterpene synthase activity was tested in assays with 50 µM (*E,E,E*)-GGPP as substrate. Assays were overlaid with 100 µl hexane and incubated for 60 minutes at 30 °C. Two microliters of the hexane phase were injected into the gas chromatograph. To determine the chirality of the produced monoterpenes, assays were set up as described above, containing 50 µM GPP as substrate, and overlaid with 100 µl hexane. After incubation for 60 min at 30 °C, the hexane phase was collected and analyzed using chiral gas chromatography-mass spectrometry (GC-MS).

GC-MS analysis of volatiles and enzyme products. Qualitative and quantitative analysis of root volatiles and terpene accumulation was conducted using an Agilent 6890 Series gas chromatograph coupled to an Agilent 5973 quadrupole mass selective detector (interface temp, 250 °C; quadrupole temp, 150 °C; source temp, 230 °C; electron energy, 70 eV) or a flame ionization detector (FID) operated at 300 °C, respectively. The constituents of the volatile bouquet were separated using a ZB5 column (Phenomenex, Aschaffenburg, Germany, 30 m × 0.25 mm × 0.25 µm) and He (MS) or H_2 (FID) as carrier gas. The sample (2 µL) was injected without split at an initial oven temperature of 45 °C. The temperature was held for 2 min and 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 comparison of retention times and mass spectra to those of authentic standards

obtained from Fluka (Seelze, Germany) and Sigma-Aldrich (St. Louis, MO, USA), or by reference spectra in the Wiley and National Institute of Standards and Technology libraries.

TPS enzyme products were analyzed and identified using GC-MS as described above for poplar root volatiles. The sample (SPME) was injected without split at an initial oven temperature of 45 °C. The temperature was held for 2 min, then increased to 130 °C with a gradient of 7 °C min^{-1}, and further increased to 300 °C with a gradient of 100 °C min^{-1} and a hold of 2 min.

Chiral GC-MS analysis was performed using a Rt™-βDEXsm-column (Restek, Bad Homburg, Germany). The temperature was first held at 45 °C for 28 min and then increased to 200 °C with a gradient of 100 °C min^{-1} and a hold for 2 min. Enantiomers were identified using authentic standards obtained from Fluka, Sigma-Aldrich, and Merck (Darmstadt, Germany).

Gene expression analysis (qRT-PCR). cDNA was prepared as described above and diluted 1:10 with water. qPCR primers for poplar *TPS* genes were designed having a Tm ≥ 60 °C, a GC content between 50–58%, and a primer length of 22–24 nucleotides (Supplementary Table S3). The amplicon size was between 110 to 140 base pairs. Expression analysis of *PtTPS13* was performed with primers previously published[20]. The specificity and efficiency of the primers were confirmed by agarose gel electrophoresis, melting curve analysis, standard curve analysis, and by sequence verification of cloned PCR amplicons. *Ubiquitin* was used as a reference gene[44]. The expression of *PtTPS13* was analyzed using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Stratagene, Carlsbad, CA, USA) with the following PCR conditions: Initial incubation at 95 °C for 3 min followed by 40 cycles of amplification (95 °C for 5 sec, 60 °C for 10 sec). Expression of *PtTPS16*, *PtTPS21*, *PnTPS1*, and *PnTPS4* was analyzed using HiDi DNA Polymerase (Genaxxon Bioscience GmbH, Ulm, Germany) and Green DNA Dye (Genaxxon Bioscience GmbH) as dye using the following PCR conditions: Initial incubation at 95 °C for 3 min followed by 40 cycles of amplification (95 °C for 15 sec, 60 °C for 10 sec, 72 °C for 30 sec). For all measurements, plate reads were taken at the end of the extension step of each cycle and data for the melting curves were recorded at the end of cycling from 60 °C to 95 °C. All samples were run on the same Bio-Rad CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratory, Hercules, CA, USA) in an optical 96-well plate. Eight biological replicates were analyzed in triplicate.

***Phytophthora cactorum* cultivation and bioassays.** *Phytophthora cactorum* (Oomycetes) was obtained from the Leibniz Institut DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). The generalist root pathogen was grown *via* mycelial inoculation in Petri dishes containing tomato juice medium. A 1.5 l quantity of medium contained 300 ml tomato juice ("Bio" quality from Netto supermarket), 4.5 g $CaCO_3$ (Roth, Karlsruhe, Germany), and 11.25 g agar-agar, filled to full volume with triple distilled water (adjusted to pH 7.2) at room temperature.

For bioassays, the inoculum was punched out with a cork borer (4 mm diameter) and placed at the center of fresh Petri dishes containing 25 ml of tomato juice medium. Small PCR tubes containing either 20 µl of 1,8-cineole (Sigma-Aldrich), (−)-limonene (Sigma-Aldrich), (−)-β-pinene (Sigma-Aldrich), 2-phenylethanol (Sigma-Aldrich), or mineral oil were placed on the very edge of the Petri dishes. Petri dishes were sealed and stored in an incubator at room temperature. Pathogen growth was monitored daily by taking a digital photograph that was later analyzed using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA, USA). Mycelial growth in the control treatment, where the pathogen was just exposed to mineral oil in the PCR tubes, was set at 100% and growth under the influence of the different volatile compounds is shown relative to the control. The fourth replicate of the *2*-phenylethanol treatment was contaminated and was therefore not considered for analyses.

Sequence analysis and phylogenetic tree construction. An alignment of all *P. trichocarpa TPS-g* and *TPS-b* genes and characterized *TPS* genes from *P. nigra* was constructed using the MUSCLE (codon) algorithm (gap open, −2.9; gap extend, 0; hydrophobicity multiplier, 1.2; clustering method, UPGMB) implemented in MEGA6[45]. Tree reconstruction was done with MEGA6 using a maximum likelihood algorithm (model/method, Tamura 3-paramter model; substitutions type, nucleotide; rates among sites, gamma distributed with invariant sites (G + I); gamma parameters, 5; gaps/missing data treatment, partial deletion; site coverage cutoff, 95%). A bootstrap resampling analysis with 1000 replicates was performed to evaluate the tree topology.

TPS structure modeling. A model of the three dimensional structure of PtTPS16 was generated using the Swiss-Model Server (https://swissmodel.expasy.org/). For modeling, the poplar TPS sequence was fitted to the template structure of (+)-limonene synthase from *Citrus sinensis*[23]. The resulting model was visualized with the program Swiss-PdbViewer3.7 (https://spdbv.vital-it.ch/).

Statistical analysis. Throughout the manuscript, data are presented as means ± SE. To compare volatile emissions, expression of *TPS*, and loss of root biomass in undamaged and herbivore-damaged *P. trichocarpa* and *P. nigra* roots, Student's t-tests or Mann-Whitney Rank Sum Tests were performed with SigmaPlot 11.0 for Windows (Systat Software Inc., https://systatsoftware.com). Whenever necessary, the data were log transformed to meet statistical assumptions such as normality and homogeneity of variances. For statistical analysis of *P. cactorum* bioassays, a student's t-test was carried out in SPSS 20 (IBM, Armonk, NY, USA). Normality distribution and heterogeneity of variances was tested.

Accession numbers. Sequence data for genes in this article can be found in the GenBank under the following identifiers: *PtTPS16* (MH541838), *PtTPS21* (MH541839), *PnTPS4* (MH541837).

Data Availability

All data generated or analyzed during this study are included in the main text or supplement of this published article.

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Author Contributions
T.G.K., N.D.L., S.B.U., and J.G. designed research. N.D.L. and S.L. carried out the experimental work. N.D.L., S.L., T.G.K., and S.B.U. analyzed data. T.G.K. wrote the manuscript. All authors read and approved the final manuscript.

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4. MANUSCRIPT II

Aboveground phytochemical responses to belowground herbivory in poplar trees and the consequences for leaf herbivore preference

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4. Manuscript II

Title: Aboveground phytochemical responses to belowground herbivory in poplar trees and the consequence for leaf herbivore preference

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Abstract

Belowground herbivory can influence aboveground herbivore performance and food preference *via* changes in plant chemistry. Most evidence for this phenomenon comes from studies in herbaceous plants but studies on woody plants are scarce. Here we investigated whether and how belowground herbivory on black poplar (*Populus nigra*) trees by *Melolontha melolontha* larvae influences the feeding preference of *Lymantria dispar* (gypsy moth) caterpillars. In a food choice assay caterpillars preferred to feed on leaves from trees that had experienced attack by belowground herbivores. Therefore, we investigated the effect of belowground herbivory on the phytochemical composition of *P. nigra* trees alone and in combination with aboveground feeding by *L. dispar* caterpillars. Belowground herbivory did not increase systemic aboveground tree defenses like volatile organic compounds, protease inhibitors and salicinoids. Jasmonates and salicylic acid were also not induced by belowground herbivory in leaves but abscisic acid concentrations drastically increased together with a few free amino acids, i.e. proline. Leaf coating experiments with amino acids suggest that proline is responsible for the caterpillar feeding preference *via* putative phagostimulatory properties. This study shows that belowground herbivory in poplar can modify the feeding preference of aboveground herbivores *via* phytochemical changes as a consequence of root to shoot signaling.

Keywords: Abscisic acid (ABA), belowground-aboveground interaction, induced resistance, *Lymantria dispar*, *Melolontha melolontha*, proline, Salicaceae, water stress.

Introduction

The role of belowground (BG) herbivory for aboveground (AG) plant defense chemistry and associated effects on insect herbivores and higher trophic levels has gained increasing attention (reviewed by Bezemer and van Dam, 2005; van Dam, 2009; van Dam and Heil, 2011). BG herbivory can shape AG arthropod community structure in individual plants (Johnson *et al.*, 2013) and influence plant species composition in more complex communities (Stein *et al.*, 2010). Furthermore, BG herbivore damage affects growth (Tsunoda *et al.*, 2014) and the nutritional content of the AG plant tissues (Blossey and Hunt-Joshi, 2003). There is a rather general pattern in herbaceous species that BG herbivory positively effects AG piercing and sucking insects such as aphids and spider mites (Johnson *et al.*, 2012 and references therein; Johnson *et al.*, 2013; Hoysted *et al.*, 2017; Kammerhofer *et al.*, 2015), but negatively affects AG chewing insects (Bezemer *et al.*, 2003; Bakhtiari *et al.*, 2018; Erb *et al.*, 2011; van Dam *et al.*, 2005).

Plant chemical defense responses to insect attack are mediated by the hormones salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA). While JA and ABA are often induced systemically in AG tissues following BG herbivory (Erb *et al.*, 2011; Erb *et al.*, 2008; Soler *et al.*, 2013), salicylic acid (SA) plays a minor role in BG-AG interactions (Erb *et al.*, 2009; Erb *et al.*, 2011; Pieterse *et al.*, 2012; Soler *et al.*, 2013). An increase in plant defense hormones following root herbivory can lead to the production of secondary metabolites such as glucosinolates, benzoxazinoids, phenolics, and alkaloids in systemic AG tissues (Papadopoulou and van Dam, 2016 and references therein). This defense induction can ultimately affect the preference and performance of AG herbivores (Brown and Gange, 1990; Poveda *et al.*, 2003; Rasmann and Turlings, 2007).

Primarily herbaceous plants, and here, mostly crop species, have been studied in the context of BG-AG interactions and herbivore induced defenses (reviewed by Papadopoulou and van Dam, 2016). In long-lived woody plants like trees, the consequence of BG herbivory on AG phytochemistry and insect herbivore preference and performance has so far attained only little attention (but see Huang *et al.*, 2015; Li *et al.*, 2016; Huang *et al.*, 2014). Differences in defense response to BG herbivory between herbaceous plants and trees are very likely, considering the life histories of these plants. Trees are long-lived and often large-sized which makes them generally more apparent to insects than annual herbaceous plants with shorter life times (Lämke and Unsicker, 2018). Herbivorous insects with very short life cycles in comparison to trees can adapt to the tree defenses within only a few generations. Hence, Haukioja and Koricheva (2000) argue that trees should exhibit more tolerance to insect herbivory than herbaceous plants due to different recovery potentials. Tree tolerance and resistance traits were so far mainly studied in response to AG herbivory and our knowledge on the role of BG herbivory is scarce (Zvereva and Kozlov, 2012).

Here, we investigated BG-AG interactions in black poplar (*Populus nigra*). Trees within the genus *Populus* possess a large diversity of anti-herbivore defense compounds that are either constitutively present or induced upon insect herbivore attack (Philippe and Bohlmann, 2007). Salicinoids, a group of two-component phenolics unique to the Salicaceae, are repellent and/or toxic for specifically generalist insect herbivores (Boeckler *et al.*, 2011; Boeckler *et al.*, 2016 and references therein). The results from studies investigating whether or not these constitutively present phenolics are also inducible by insect herbivores are inconsistent (e.g. Boeckler *et al.*, 2013; Rubert-Nason *et al.*, 2015). Systemic induction of salicinoids upon insect herbivore feeding was reported in a few previous studies (Stevens and Lindroth, 2005; Rubert-Nason *et al.*, 2015) and one study also investigated the effect of AG defoliation on root salicinoids (Stevens *et al.*, 2014). However, to our knowledge, the role of BG herbivory on AG salicinoid patterns in poplar trees is unknown.

Poplar trees also induce a number of defense-related proteins such as polyphenol oxidases, endochitinases, and Kunitz-type protease inhibitors (PI) (Philippe and Bohlmann, 2007) upon insect feeding. Kunitz-type PIs are a well-studied group of defense related proteins in poplars (Haruta *et al.*, 2001; Major and Constabel, 2006; Talyzina and Ingvarsson, 2006) and genes of the Kunitz type PI gene family are among the most highly upregulated genes after insect herbivory or methyl jasmonate treatment (Christopher *et al.*, 2004; Major and Constabel, 2007). Simulated AG herbivory with methyl jasmonate resulted in root induction of the trypsin inhibitor gene *PtdTI3* suggesting systemic shoot to root signaling (Major and Constabel, 2007).

Additionally poplar trees also produce a large number of volatile organic compounds (VOCs) specifically when they are under attack by herbivores (Arimura *et al.*, 2004; Brilli *et al.*, 2009; Clavijo Mccormick *et al.*, 2014b). The constitutive and herbivore-induced emission of poplar VOCs, as well as their biosynthesis and their role in direct and indirect defense have been intensively studied in the past (Clavijo McCormick *et al.*, 2014a; Clavijo Mccormick *et al.*, 2014b; Danner *et al.*, 2011; Eberl *et al.*, 2017; Irmisch *et al.*, 2014; Unsicker *et al.*, 2015; Lackus et al., 2018). However, so far no study investigated the consequences of BG herbivory on AG VOC emission in poplar.

Here, we studied the single and combined effects of above- and belowground herbivory on the phytochemistry of young *P. nigra* trees and tested whether BG herbivory by larvae of the beetle *Melolontha melolontha* (cockchafer) has an effect on the feeding preference of generalist gypsy moth (*Lymantria dispar*) caterpillars. Under controlled laboratory conditions, we first investigated whether caterpillars discriminate between black poplar leaves from root-infested (BG herbivory) *versus* non-infested trees. Due to a significant preference of *L. dispar* for leaves from BG infested trees, we then investigated the phytochemical profiles of leaves from trees that experienced a) BG herbivory by *M. melolontha*, b) AG herbivory by *L. dispar*

caterpillars, c) a combination of BG and AG herbivory and d) no herbivory (controls). We measured volatile organic compounds, salicinoids, protease inhibitor activity, defense hormones, free sugars and free amino acids to elucidate which of these primary and secondary metabolites could be responsible for caterpillar food preference.

Our results show that BG herbivory alone did not induce major defenses such as VOCs, salicinoids or protease inhibitor activity in AG tissues of *P. nigra*. Upon AG caterpillar damage, leaves responded, as already previously described, with an induction of major volatile groups and protease inhibitor activity. Although there was no induction of defense compounds by BG herbivory alone, beetle larvae feeding substantially increased the amount of ABA and proline in *P. nigra* leaves. A food choice assay in which we offered leaf discs of undamaged trees, that were either coated or uncoated with different amino acids, to *L. dispar* caterpillars suggests, that the originally observed preference for leaves of BG infested trees is most likely due to higher proline concentrations.

Material and Methods

Plants and insects

Populus nigra (black poplar) trees were cultivated from cuttings of one genotype growing in a common garden near Jena, Germany. 60 trees were rooted in 2-liter pots filled with pure sand and maintained in the greenhouse under summer conditions (24 °C, 60 % relative humidity, 16 h /8 h light cycle) for four month until the start of the experiment. By then, the trees were around 1 m tall. 24 hours before the experiment started, the trees were acclimatized in a climate chamber (humidity: 60 %, day/night temperature: 20 °C/16 °C; 16 h light). Due to limited space, the experiment was split in three blocks (three time points) with 20 trees in each, consisting of an equal number of replicates for each treatment. Time between the start of the experiments in the first block and the third block was four weeks.
Cockchafer (*Melolontha melolontha*) larvae (grubs), collected from meadows in Germany and Switzerland (Huber *et al.*, 2016), were reared individually in 200 ml plastic cups, filled with a mix of potting soil and grated carrots and kept in a wine cooler at 13 °C and 70 % humidity. Gypsy moth (*Lymantria dispar*) caterpillars were hatched from egg batches and reared on artificial wheat germ diet (MP Biomedical, Eschwege, Germany) in a climate chamber (25 °C, 60 % humidity, 14:10 L:D period) until they entered the experiment in 4th instar.

Experimental root and shoot herbivory

The 60 trees used for this experiment were split in three blocks with 20 individuals each. Half of the 20 trees in each block were induced with one *M. melolontha* larva (**grub**). The grub was allowed to feed on the roots for 6 days. After four days six 4th instar *L. dispar* caterpillars were released AG on 10 of 20 trees and allowed to feed for 40 hours. Each experimental block thus consisted of five replicates of four different treatments: non-damaged trees (**control**), trees with BG herbivory by *M. melolontha* (**grub**), trees with AG *L. dispar* caterpillar infestation (**caterpillar**), and trees with combined AG- BG infestation by both herbivores (**grub + caterpillar**, **Fig. S1**).

Food choice experiments with Lymantria dispar caterpillars

To investigate whether *L. dispar* caterpillars discriminate between leaves of trees previously infested by *M. melolontha* BG and non-infested control trees, food choice experiments were performed in modified 90 mm Petri-dishes (**Fig. 1B**). Four leaf discs (16 mm in diameter) of each of the two treatments (grub and control) were alternately stuck on pins glued to the petri dish equidistantly 3 cm around the middle of the dish. One 2nd instar *L. dispar* caterpillar (starved overnight) was then released in the center of the petri dish and allowed to feed for

24 h. Altogether 20 caterpillars were tested in each treatment. Thereafter, leaf discs were photographed and leaf area loss was determined by reconstructing the leaf blades with Adobe Photoshop CS4 (Adobe, San Jose, CA, USA).

Volatile collection and analysis

Six days after the onset of the experiment (6 days of grub feeding and 40 hours of caterpillar feeding), PET bags were installed on the poplar foliage and volatiles in the headspace were collected for 4 h with Poropak traps (Alltech, Florida, USA), as described in (Clavijo Mccormick *et al.*, 2014b). After the volatile collections, traps were eluted twice with 100 µl dichloromethane, containing an internal standard (nonyl acetate, concentration, 10 ng × μl^{-1}; Sigma Aldrich, Seelze, Germany). For identification of compounds, 2 µl of the eluate was injected splitless into a gas chromatograph (6890 series, Hewlett-Packard, Agilent Technologies, Santa Clara, CA, USA) equipped with a 30 m × 250 µm × 0.25 µm DB5-MS column (WicomGmbH, Heppenheim, Germany) coupled to a quadrupole mass spectrometer (5973 series, Hewlett-Packard, Agilent Technologies, Santa Clara, CA, USA) (in short GC/MS). The injector was held at 230 °C with helium used as carrier gas at 1 ml/min. The oven temperature of the GC/MS was held at 50 °C for 3 minutes after injection and then heated up to 95 °C at a rate of 4 °C/min. Afterwards, the oven temperature was increased to 145 °C with a 15 °C/min gradient and then to 180 °C with a 10 °C/min gradient. Finally, the oven temperature was kept stable for 3 min at 300 °C. Mass spectra were recorded (transfer line temperature: 230 °C, source temperature: 230 °C, quadrupole temperature: 150 °C, ionization energy: 70 eV, mass range: 40-500 *m/z*). Compounds were identified by comparing their mass spectra to authentic standards and three libraries (Wiley275, NIST, ADAMS). For quantification, the samples were separated with the same GC method as described above with hydrogen as the carrier gas. Afterwards the samples were analyzed with a flame ionization detector (FID, 9200 Hydrogen detector, Packard, Agilent Technologies, Santa Clara, CA, USA) operating at 300 °C. Absolute amounts of all compounds were calculated based on the relation of their FID peak area and the area of the internal standard according to the “effective carbon number (ECN) concept” (Scanlon and Willis, 1985).

Leaf harvest

Right after volatile collection, leaves of each tree were cut and photographed to determine leaf area and experimental leaf area loss by caterpillar herbivory. Then leaf midribs were cut and discarded. Pooled leaf halves were transferred to 5 ml vials and then immediately flesh frozen in liquid nitrogen. After lyophilization, the samples were stored at -20 °C until further analysis.

Protease inhibitor analysis via radial diffusion assay

Before the actual extraction, 10 mg ground leaf material was extracted with 400 µl HEPES-buffer (25 mM, pH 7.2, containing 3 % PVPP, 2 % PVP, 0.8 Triton X100 and 1mM EDTA). A 3 mm metal ball was added to the extracts. Then extracts were shaken for 8 minutes in a paint shaker before centrifugation for 10 minutes at maximum speed, 4 °C. The supernatant was used for analysis.

To identify PI activity, a radial diffusion assay was performed (modified from (Jongsma *et al.*, 1993; Van Dam *et al.*, 2001). A 1.8 % plant agar gel was prepared with HEPES-KOH (25 mM, pH 7.2) buffer containing 2 µl trypsin (0.2 mg/ml) per ml gel. With a 4 mm diameter cork borer, wells were punched in the gel 2 cm apart. The wells were filled with the leaf extracts or a standard trypsin inhibitor from soybean (Sigma Aldrich, Seelze, Germany). The loaded gel was incubated for 22 hours at 4 °C. After incubation, the gel was washed once with HEPES-KOH buffer (25 mM, pH 7.2, containing 10 mM $CaCl_2$) and stained with a freshly prepared staining solution (72 mg Fast Blue B Salt in 90 ml HEPES-KOH, 25 mM (pH 7.2) combined with 60 mg N-acetyl-DL-phenylalanine β-naphtyl ester (Sigma Aldrich, Seelze, Germany) in 10 ml DMF). Gel and staining solution were incubated for 30 to 90 minutes at 37 °C. When a sufficient coloring was achieved, the staining solution was poured of, the gel rinsed with water, and then photographed for later analysis with Adobe Photoshop (San José, CA, USA). Concentration of trypsin inhibition was calculated by comparisons to standard inhibitor. A standard Bradford assay (Biorad, Hercules, CA, USA) was performed to calculate protein content in each sample.

Salicinoid analysis

Phenolic compounds were extracted in parallel to phytohormones (see below). As internal standard, 0.8 mg/mL phenyl-β-glucopyranoside was added additionally to the internal phytohormone standards. The raw extracts of 2x200 µL separated during phytohormone extraction were combined and 400 µl of Milli-Q H_2O was added before measuring the analytes using HPLC/UV. 20 µl analyte was injected onto a chromatographic column (EC 250x4.6 mm NUCLEODUR Sphinx RP, 5 µm, Macherey Nagel, Germany) connected to a pre-column (C18, 5 µm, 4x3 mm, Phenomenex, USA). The mobile phases consisting of two solvents, solvent A (Milli-Q H_2O) and solvent B (acetonitrile), were run with solvent B in gradient mode. The time/concentration (min/%) of the gradient was set to 0/14; 22,00/58; 22,10/100; 25,00/100; 25,10/14; 30,00/14 with a constant flow rate of 1 ml/min. The column oven temperature was set to 25 °C. The signal was detected with Photo Diode Array (PDA) and Evaporative Light Scatter (ELSD) detectors (Varian, USA). Using these settings, the

retention times (RT) of the compounds of interest were: 5.10 min (salicin), 10.20 min (salicortin), and 15.20 min (homaloside D). One more unidentified salicinoid with a retention time at 18.50 min was isolated for subsequent structure elucidation by nuclear magnetic resonance spectroscopy (NMR). NMR spectral data were acquired using an Avance III HD 500 MHz spectrometer equipped with a 5 mm TCI cryoprobe (Bruker Biospin, Rheinstetten, Germany). Data acquisition and processing was accomplished using Bruker TopSpin software suite, ver. 3.5. Standard pulse programs as implemented in TopSpin were used. The NMR-sample was measured in MeOH-d_4.

Phytohormone and free sugar analysis

Phytohormones were extracted from 10 mg of freeze-dried *P. nigra* leaf material. 1 ml of methanol (MeOH) containing internal standards of labeled phytohormones (40 ng/ml jasmonic acid (D2-JA), abscisic acid (D6-ABA), salicylic acid (D4-SA), and 8 ng/ml JA-13C6-Ile) was added to each sample in a 96 well plate (Mironic, Lelystad, the Netherlands). Afterwards, the suspension was homogenized by shaking in a paint shaker together with one steel ball for 1 min before centrifuging it at 2,057 g for 1 min. 400 µl of the supernatant was taken out and transferred to a new 96 well plate. The remaining pellet was suspended with another 1 ml of MeOH without internal standards and the procedure (shaking and centrifuging) was repeated. Again, 400 µl were taken out and combined with the first 400 µl extract, to retrieve in sum 800 µl of extract per sample. Phytohormones were analyzed via high performance liquid chromatography (HPLC - Agilent 1100 Varian ELSD, Varian, USA) coupled to a MS/MS system (API 5000 LC/MS/MS System, AB Sciex, USA). The analytes were injected onto a chromatographic column (XDB-C18, 1.8 µm, 4.6x50 mm, Agilent, USA) connected to a pre-column (C18, 5 µm, 4x3 mm, Phenomenex). The injection volume was set to 2 µl. Two solvents, solvent A (0.05 % formic acid in H_2O) and solvent B (acetonitrile) were used. Solvent B was injected in a gradient mode driving the following gradient (time in min/concentration of solvent B in %): 0.00/5, 0.50/5, 9.50/58, 9.52/100, 11.00/100, 11.10/5 and 14.00/5. The constant flow rate was set to 1100 µl/min. The temperature of the column oven was set to 25 °C.

Phytohormones were ionized in negative electrospray ionization (ESI) mode and chromatograms were analyzed using the software Analyst 1.6 (AB Sciex, MA, USA). The peak integration was performed automatically by the software after adjusting the peak areas manually. The quantification was realized by comparing the peak areas of the samples to the peak area of the internal standards. Soluble sugars were measured from the same raw extracts, but diluted 1:10, that were used for phytohormone analysis as described in (Madsen *et al.*, 2015).

Amino acid analysis

Free amino acids were analyzed from the same raw extracts as used for phytohormone analysis. The raw extracts were diluted 1:10 with water containing an isotopically labeled amino acid mix (^{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 extracts were measured with high performance liquid chromatography (HPLC - Agilent 1200, Agilent Technologies, Waldbronn, Germany) coupled to a triple-quadrupole mass spectrometer (API 5000 LC/MS/MS System, AB Sciex, USA). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 mm × 4.6 mm, 1.8 µm, Agilent Technologies, Germany). Two solvents, solvent A (H_2O) and solvent B (acetonitrile) were used. Solvent B was injected in a gradient mode driving the following gradient (time in min/concentration of solvent B in %): 0.00/3, 1.00/3, 2.70/100, 3.00/100, 3.10/3, 6.00/3. The constant flow rate was set to 1100 µl/min. The temperature of the column oven was set to 25 °C. Analytes were ionized in negative electrospray ionization (ESI) mode. Multiple reactions monitoring (MRM) was used to monitor analyte parent ion → product ion: MRMs were chosen as in (Jander *et al.*, 2004) except for Arg (*m/z* 175 → 70) and Lys (*m/z* 147 → 84). Analyst 1.5 software (AB Sciex, Darmstadt, Germany) was used for data acquisition and processing. Individual amino acids in the sample were quantified by the respective ^{13}C, ^{15}N-labeled amino acid internal standard, except for tryptophan, and asparagin: tryptophan was quantified using ^{13}C, ^{15}N-Phe applying a response factor of 0.42 and asparagine was quantified using ^{13}C, ^{15}N-Asp applying a response factor of 1.0.

Caterpillar food choice with enhanced amino acid concentrations

To experimentally enhance the concentration of selected amino acids in poplar leaves, 20 µl of an amino acid-ethanol solution was pipetted onto 16 mm leaf discs and allowed to dry before the discs were offered to the caterpillars (method adapted from Ximénez-Embún *et al.*, 2016).

In a first preference assay where only proline was tested, the concentration applied to the leaf surfaces was based on mean *in planta* leaf proline concentration measured after 6 days of root herbivory by one *M. melolontha* grub (~500 nmol/g or 14.39 µg/g fresh weight). Control leaf discs were treated with ethanol only. Proline-coated leaf discs and control leaf discs were placed in Petri-dish arenas and a food choice experiment with 3rd instar *L. dispar* caterpillars feeding for 48 hours was performed as described above (**Fig. 1B**) with the exception that the caterpillars were not starved *prior* to the experiment.

In a second assay, proline and three additional amino acids, leucine, alanine, and tryptophan, were tested in the same way. Here, amino acid concentrations applied to the leaf surfaces

were based on the medians of *in planta* amino acid concentrations measured after 6 days of root herbivory by one *M. melolontha* grub (**Table S3**). Alanine was pre-dissolved in a 0.05 formic acid solution as it was not soluble in ethanol. To evaluate whether the amino acid concentrations in the artificially coated leaf discs yielded the desired increase, bulk samples of 5 leaf discs were freeze dried, extracted, and amino acid concentrations were analyzed as described above.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics version 20.0 (SPSS, Chicago, IL, USA). All data were checked for statistical assumptions such as normal distribution and heterogeneity of variances. Whenever necessary, log transformation (analyte concentration data) or *arcus sinus* transformation (percent leaf area loss) was applied. In case of simple two group comparisons, t-tests, Mann Whitney U-tests, or related samples Wilcoxon rank tests were performed. ANOVA followed by Tuckey *post hoc* comparison or Welch-ANOVA followed by Games-Howell *post hoc* testing was performed in case of normally distributed data with homogeneous variances. In case of non-parametric data, Kruskal-Wallis tests followed by Dunn´s post hoc tests were carried out.

Results and Discussion

Lymantria dispar caterpillars prefer leaves from trees that experienced BG herbivory

In a food choice assay, *L. dispar* caterpillars preferred to feed on leaf discs from *P. nigra* trees that were previously infested BG with a *M. melolontha* larva over discs from non-damaged control trees (Mann-Whitney U-test: $U = 4.021$ $p \leq 0.001$**, Fig.1A**). A positive effect of BG herbivory on AG insect herbivore preference has been observed in recent studies in herbaceous plant species (Huberty and Denno, 2004; Soler *et al.*, 2005) mostly for sucking insects (Hol *et al.*, 2013; Hoysted *et al.*, 2017; Kutyniok and Muller, 2012). However, in the majority of recent studies on interactions between chewing insects and crops, BG feeding negatively affected AG herbivores *via* systemically induced defense induction (Bakhtiari *et al.*, 2018; Johnson *et al.*, 2012; Anderson *et al.*, 2011).

To understand the molecular mechanisms underlying the preference of *L. dispar* caterpillars for black poplar leaves from BG infested trees, we characterized and compared the leaf phytochemistry including volatile emission, protease inhibitor activity, and salicinoid accumulation in uninfested trees, in trees infested either BG with one *M. melolontha* larva or AG with *L. dispar* caterpillars, and in trees infested with both herbivores.

BG herbivory did not induce AG VOC emission in Populus nigra trees

We identified 44 different VOCs in the headspace of *P. nigra* trees in the four experimental treatments (**Table S1**). These VOCs fall into six major groups, namely green leaf volatiles, monoterpenes, sesquiterpenes, aromatic compounds, nitrogenous compounds, and the homoterpene (*E*)-4,8-Dimethyl-1,3,7-nonatriene (*E*-DMNT) (**Fig. 2, Table 1**). The emission of green leaf volatiles, monoterpenes, aromatic and nitrogenous compounds, and *E*-DMNT significantly increased after AG caterpillar feeding and after combined BG-AG attack (**Fig. 2, Table 1**). There was also a trend for an increase in sesquiterpene emission upon caterpillar feeding alone and in combination with BG herbivory; however, this was non-significant (**Fig 2C**).

BG herbivory by *M. melolontha* alone did not induce systemic AG VOC emission (**Fig. 2**). In a few recent studies in maize, apple, and *Brassica* species, BG herbivory led to an induction of AG volatiles (Rasmann *et al.*, 2005; Neveu *et al.*, 2002; Soler *et al.*, 2007; Abraham *et al.*, 2015) but there are also examples where BG herbivory did not induce AG VOCs (Rasmann and Turlings, 2007; review by Papadopoulou and van Dam, 2016 and references therein). When *P. nigra* was attacked by BG and AG herbivores, the emission of green leaf volatiles, sesquiterpenes, and DMNT was slightly reduced (non-significantly) (**Fig 2**). Numerous studies in the past have shown that green leaf volatiles, DMNT, and sesquiterpenes function as attractants for higher trophic level predators and parasitoids (Brodmann *et al.*, 2008; Kappers *et al.*, 2005; Schnee *et al.*, 2006). A reduction in VOC emission by BG herbivory

could negatively affect the indirect tree defense properties, as this was shown for a number of herbaceous plants (e.g. Tariq *et al.*, 2013; Bezemer and van Dam, 2005; Holopainen and Gershenzon, 2010). We argue, however, that BG feeding by *M. melolontha* would not alter the efficacy of *L. dispar* parasitoids in finding their hosts since the most important volatiles for parasitoid attraction in poplar, namely nitrogenous compounds (Clavijo McCormick *et al.*, 2014a), are not affected by BG feeding (**Fig 2E**).

The VOC emission patterns from *P. nigra* did not explain the observed *L. dispar* caterpillar preference for leaves from BG infested trees. We therefore measured protease inhibitor activity, as these are considered efficient anti-herbivore poplar defenses.

AG- but not BG herbivory increases leaf protease inhibitor activity

L. dispar caterpillar feeding significantly increased the trypsin inhibitor activity in leaves of *P. nigra* (**Fig. 3**). The activity increase in caterpillar-infested leaves was more than two-fold in comparison to that in non-infested control trees. PIs are efficient defense proteins against insect herbivores (Howe and Jander, 2008; Zhu-Salzman and Zeng, 2015). In the artificial diet of *Eurygaster integriceps,* PIs had a negative impact on nymph developmental time, adult weight and survival (Saadati and Bandani, 2011). The consumption of artificial diet containing only 1 % soybean trypsin inhibitor (of total dietary protein) resulted in a 40 % reduction of trypsin activity in the guts of this hemipteran pest (Saadati and Bandani, 2011). Major and Constabel (2006) showed that the PI genes in hybrid poplar (*Populus trichocarpa x P. deltoids*) belong to the most strongly induced genes after mechanical wounding and insect herbivory. Kunitz-type PIs in *Populus trichocarpa x P. deltoides* strongly inhibited proteases in mid-gut extracts of the lepidopteran generalist *Malacosoma disstria* (Major and Constabel, 2008). Recent studies in tomato and maize report a systemic induction of PI activity upon BG herbivory (Arce *et al.*, 2017; Erb *et al.*, 2011). Although non-significant, we observed a trend for slightly higher PI activity in leaves of *P nigra* trees that were infested BG with *M. melolontha* larvae (**Fig. 3**). Future studies will reveal whether such slight induction in PI activity by BG herbivory is detrimental for leaf chewing insects in poplar trees.

Since the PI activity was not significantly higher after BG herbivory it cannot serve as an explanation for the previously observed *L. dispar* caterpillar preference for leaves from BG infested trees. It is also still unclear whether *L.* dispar caterpillars are able to taste the presence of PIs.

Leaf salicinoid concentrations in P. nigra were slightly affected by BG and AG herbivory

Concentrations of four different salicinoids in *P. nigra* leaves namely salicin, salicortin, homaloside D, and 6'-*O*-benzoylsalicortin were measured. The latter compound is first described here as a salicinoid in *P. nigra*. Details on the NMR identification of 6'-*O*-

benzoylsalicortin are given in the Supplemental material (***Fig. S4-S8***). Among all salicinoids, salicortin was the most abundant compound (**Fig. 4B**). Salicin, salicortin, and 6'-*O*-benzoylsalicortin concentrations were not affected by any treatment. However, there was a trend for an increase in foliar salicin concentrations in the grub treatment (**Fig. 4A**). 6'-*O*-benzoylsalicortin shows a similar trend and additionally an increase after combined BG-AG attack. Details of the statistical results are shown in **Table 1**. Homaloside D concentrations significantly differed between the four treatments and post-hoc comparisons revealed that the combined above- and belowground herbivore treatment (grub + caterpillar) significantly differed from the control trees but not from the grub and caterpillar treatments (**Fig. 4C**). AG herbivory by *L. dispar* did not lead to an increase in salicinoid levels in damaged leaves. This is coherent to the findings of Boeckler *et al.* (2013). However, Rubert-Nason *et al.* (2015) reported an induction of salicinoids in *P. tremuloides* leaves attacked by gypsy moth and an even higher induction in systemic leaves of the herbivore treatment.

The salicinoid patterns from *P. nigra* in our experiment did not explain the observed *L. dispar* caterpillar preference for leaves from BG infested trees. On the contrary, the slight but non-significant increase after BG feeding would hint at the opposite outcome of the food choice experiment. We therefore searched for changes in nutritional values in leaves like available carbon and nitrogen sources.

Phytohormone, sugar, and amino acid measurements in P. nigra leaves suggest water stress symptoms inflicted by BG herbivory.

We measured three major defense hormones in leaves of black poplar, namely salicylic acid (SA), abscisic acid (ABA), and jasmonic acid (JA) and its derivatives (JA-Ile-1, JA-Ile-2, cis-12-oxo-phytodienoic acid, OH-JA, OH-JA-Ile and COOH-JA-Ile presented here together as jasmonates). SA levels were not influenced by any of the treatments (**Fig. 5A**) in contrast to ABA and jasmonates. There was a significant increase in ABA concentrations in *P. nigra* leaves following BG herbivory (2-fold) but only moderately after aboveground caterpillar feeding (**Fig. 5B**). Combined BG-AG herbivory resulted in an even more pronounced ABA induction in the leaves (3-fold) (**Fig. 5B**). The jasmonates were significantly induced 4-fold after caterpillar feeding compared to the control (**Fig. 5C**, details of the statistical results are shown in **Table 1**) and there was a non-significant trend for higher systemic JA levels in leaves of *P. nigra* that received BG herbivory (**Fig. 5C, Table 1**).

Coherent to results from studies in herbaceous plants, BG herbivory in *P. nigra* did not have an impact on SA levels in leaves (Erb *et al.*, 2009; Erb *et al.*, 2011). It was argued before that SA does not act as a signal in systemic defense induction following BG herbivory (Erb *et al.*, 2009; Pierre *et al.*, 2012; Pieterse *et al.*, 2012) although SA could affect BG and/or BG-AG defense mechanisms by interactions with other phytohormones (Pieterse *et al.*, 2012). It has

long been known that ABA can be transported from roots to shoots *via* the vascular system (Jackson, 1997 and references therein). However, ABA is also *de novo* synthesized in AG foliage following BG herbivory, as a study by (Erb *et al.*, 2011) in maize plants showed. Whether the systemic increase in ABA in *P. nigra* following BG *M. melolontha* herbivory is due to vascular transport or *de novo* biosynthesis awaits further elucidation. JA is a major signal in plant inducible defenses against chewing insects (Bruce and Pickett, 2007; De Vos *et al.*, 2005; Erb *et al.*, 2012). We observed jasmonate induction following aboveground caterpillar herbivory and this is consistent to numerous recent studies in herbaceous and woody plant species (Nabity *et al.*, 2013; Boeckler *et al.*, 2013; Clavijo Mccormick *et al.*, 2014b; Eberl *et al.*, 2017). The overall phytohormone patterns in our study are comparable to results from studies by (Erb *et al.*, 2009; Erb *et al.*, 2011) in maize plants that were infested BG with larvae of the beetle *Diabrotica virgifera virgifera*. In these studies, BG feeding also substantially increased ABA levels, whereas SA, JA, JA-Ile, and OPDA levels were not systemically induced in leaves.

Root herbivory often goes hand in hand with water stress due to the loss of the fine root system and thus a decrease in the overall water uptake (Erb *et al.*, 2011; Blossey and Hunt-Joshi, 2003 and references therein). In our experiment, there was also substantial loss of fine roots in *M. melolontha* larva infested black poplar trees. When grubs are allowed to feed longer on the poplar trees, this will result in leaf desiccation and leaf fall (S. B. Unsicker, personal observation). A drastic increase of ABA in aboveground plant tissues is a consequence of water stress in plants (Seki *et al.*, 2007) often accompanied by an increase of free amino acids and free sugars in aboveground tissues (Masters *et al.*, 1993; reviewed by Blossey and Hunt-Joshi, 2003). To cope with water stress, many plants perform an osmotic adjustment, where carbohydrates are mobilized into free sugars and proteins into amino acids (Bowne *et al.*, 2012; Brown and Gange, 1990; Hummel *et al.*, 2010; Poveda *et al.*, 2003; Rosa *et al.*, 2009). The formed low-molecular weight compounds act as osmolytes and can protect fragile proteins from degradation by forming a hydration shell around them (Hajlaoui *et al.*, 2010).

To test, whether BG herbivory also influences the pool of free amino acids and sugars in *P. nigra,* we measured these compounds in leaf material collected from the differently treated trees. The experimental treatments had no effect on fructose, trisaccharide and tetrasaccharide concentrations in black poplar leaves. However, glucose and sucrose concentrations were significantly lower in the caterpillar treatment as compared to the controls. Sucrose concentrations in the combined treatment (caterpillar + grub) were also significantly lower than in non-infested control trees (**Table 2**). In contrast to the other free sugars the pentasaccharides significantly increased after combined BG and AG herbivory.

Additionally we observed a non-significant trend of increased tri-, tetra- and pentasaccharide concentrations after BG feeding alone (**Table 2**).

Five out of the 18 analyzed amino acids (**Table S2**), glutamine, proline, serine, threonine, and tryptophan, showed a significant response to the experimental treatments (**Fig. 6**). Caterpillar feeding alone significantly decreased concentrations of proline, serine, and threonine, whereas tryptophan was significantly increased in the caterpillar treatment (**Fig. 6)**. The combined caterpillar + grub treatment showed the highest concentrations of glutamine, proline and tryptophan as compared to the non-damaged controls. In contrast, serine and threonine concentrations significantly decreased in the combined caterpillar + grub treatment. Proline was the only amino acid that showed a significant increase in the grub treatment as compared to the control treatment (**Fig. 6)**. Total leaf protein concentrations did not differ between the four treatments (**Fig. S3**).

Higher proline levels in leaves of BG damaged trees likely explain the caterpillar preference

When we analyzed the experimental leaf area loss in both caterpillar treatments (caterpillar and caterpillar + grub), we found significantly lower damage in the combined treatment with AG and BG herbivory than in the treatment with only caterpillar feeding (**Fig. S2,** Mann Whitney U test: $U = 42.00$; $p < 0.01$). This observation together with the initial observation that *L. dispar* caterpillars prefer feeding on leaves from trees that were infested BG with a *M. melolontha* larva lead us to speculate that *L. dispar* consume less biomass from these leaves as their nutritional requirements are more easily met there. The BG treated trees showed signs of water stress with significantly increased ABA levels in the foliage and increased levels of e.g. proline, an amino acid considered to be a reliable marker for drought stress in herbaceous plants (Ximénez-Embún *et al.*, 2016; Yamada *et al.*, 2005) that most likely exists in woody plants as well. However, in herbaceous plants under drought stress stronger increases (3 to 9-fold) as what we observed in *P. nigra* in proline concentrations have been shown (Delauney & Verma, 1993). Proline is reported to have an impact on several developmental processes in plants like flowering, pollen- and seed production, and root growth (reviewed by Kavi Kishor & Sreenivasulu, 2014). Proline can be transported to the roots through the phloem *via* specific transporters (Lee *et al*., 2009). It is conceivable that the rather minor increase of proline we observed in leaves of *P. nigra* upon BG herbivory is due to an increased transport of proline to the damaged roots to aid compensatory growth.

We argue that our data support the “plant stress hypothesis” (Joern and Mole, 2005), which predicts a better performance and higher abundance of herbivorous insects on plants suffering from abiotic stresses such as drought. Insect preference, however, does not necessarily reflect the performance as was shown in a recent study by Gutbrodt *et al.* (2011). There, *Pieris brassicae* caterpillars chose well-watered over drought-stressed *Alliaria*

petioloata plants but they performed better on drought stressed plants. In our example, the data on caterpillar performance is missing and further studies have to reveal whether BG herbivory by *M. melolontha* larvae also positively affects *L. dispar* performance and fitness.

The concentrations of all putative anti-herbivore defense metabolites we measured in *P. nigra* leaves cannot explain the initially observed preference of *L. dispar* caterpillars for leaves from *P. nigra* trees infested BG with a *M. melolontha* larva. The only metabolite that could explain caterpillar feeding preference for leaves of the "grub" treatment was the amino acid proline. This compound is also known to be a phagostimulant for leaf chewing insects (e.g. Ximénez-Embún *et al.*, 2016; Meyer *et al.*, 2006; Behmer and Joern, 1994). Proline concentrations in *P. nigra* leaves in the BG treatment with *M. melolontha* herbivory were significantly higher in comparison to those in non-damaged control trees (**Fig. 6**). We therefore performed a choice assay with proline-supplemented leaf discs and solvent- control discs to investigate whether proline could be responsible for the preference of *L. dispar* caterpillars exhibited for leaves from trees suffering BG herbivory. Indeed, *L. dispar* caterpillars inflicted twice as much damage on proline-supplemented leaf discs than on the controls (Mann-Whitney U-test $p = 0.013$, **Fig. 7A**).

To test whether other amino acids also have phagostimulatory effects, the choice assay was repeated. Proline was again included into the experiment as well as alanine, leucine and tryptophan because these showed the most prominent changes between control and grub treatment (**Fig. 7B**). The caterpillars fed significantly more leaf material only when the discs were supplemented with proline. None of the other tested amino acids had a significant effect on caterpillar preference. Proline and also alanine have been reported to trigger a response in a phagostimulatory neuron of caterpillars of the arctiid moth (Bernays, Chapman & Singer, 2000). We did not observe a preference for alanine coated leaf discs in *L. dispar* caterpillars, which is most likely due to the fact that phagostimulatory effects of single amino acids are herbivore species specific (Agnihotri, Roy & Joshi 2016). Our results suggest that the change in concentration of a single amino acid can be responsible for the initially observed caterpillar preference for leaves from trees with BG herbivory by one *M. melolontha* larva. Furthermore, this might also explain the significantly higher leaf area loss in the caterpillar treatment as compared to the combined herbivore treatment (grub+caterpillar). The leaves that were affected by BG feeding had a higher nutritional value compared to control leaves. Thus, caterpillars feeding on control leaves might have to ingest more tissue to reach their nutrient intake target. Future studies will have to show whether this will also result in a fitness benefit for the caterpillars.

Conclusion

Besides putative signs of water stress as a consequence of BG *M. melolontha* larva feeding (induction of ABA and certain amino acids), there was no pronounced systemic induction of typical anti-herbivore poplar defense metabolites (VOCs, PIs and Salicinoids) or defense signaling hormones (JA and SA) in leaves of young *P. nigra* trees. Only in the combined treatment with simultaneous BG herbivory and AG feeding by *L. dispar* caterpillars, a few defense related metabolites were increased namely the salicinoid homaloside D and the monoterpene alcohol linalool. The non-essential amino acid proline, however, significantly accumulated in leaves of trees suffering from BG herbivory. When *L. dispar* caterpillars had the choice between black poplar leaf discs coated with proline and leaf discs without this amino acid, they preferred the first, supporting previous observations where this compound has been reported to be a phagostimulant. Our results provide a first insight into the defense chemistry of AG-BG interactions in poplar trees and comprise a basis for future chemical ecological studies in trees under more complex, natural conditions.

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The authors have no conflict of interest to declare.

4. Manuscript II

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Tables

Table 1: Statistical results of the Kruskal Wallis test for VOCs, phytohormones, and salicinoids in black poplar leaves after belowground damage by a cockchafer grub, aboveground damage by gypsy moth caterpillars**,** and a combination of both herbivores compared to non-damaged control plants. Bold letters indicate statistically significant differences. GLV = green leaf volatiles, MT = monoterpenes, ST = sesquiterpenes, n = 12-15.

Analyte	**H**	**p**
GLV	**32.281**	**< 0.001**
MT	**13.077**	**0.004**
ST	7.311	0.63
E-DMNT	**39.009**	**< 0.001**
Aromatics	**21.707**	**< 0.001**
Nitrogenous	**40.076**	**< 0.001**
SA †	1.352	0.717
ABA	**22.04**	**≤0.001**
Jasmonates	**33.802**	**≤0.001**
Salicin	4.722	0.193
Salicortin	5.313	0.15
Homaloside D	**10.825**	**0.013**
6'-*O*-benzoylsalicortin	5.753	0.124

† One outlier in the grub treatment was removed from the dataset.

Table 2: Soluble sugar concentrations after belowground damage by a cockchafer grub, aboveground damage by gypsy moth caterpillars and a combination of both herbivores compared to non-damaged control plants in µg g^{-1} DW. Bold p values indicate significant differences based on Kruskal-Wallis tests. Shown is the mean ± SEM; n = 13-15.

	control	**grub**	**caterpillar**	**grub + caterpillar**	**p-value**
Glucose	7.06±0.74^{a}	6.33±1.49^{ab}	3.94±0.50^{b}	7.12±1.40^{ab}	**0.04**
Fructose	2.78±0.32	3.16±0.93	2.17±0.17	3.14±0.44	0.198
Sucrose	20.09±0.34^{a}	19.51±0.59^{ab}	17.98±0.31^{b}	17.39±0.72^{b}	**0.001**
Trisaccharide	0.88±0.18	1.23±0.28	0.99±0.19	1.70±0.31	0.186
Tetrasaccharide	0.60±0.19	1.25±0.40	0.71±0.21	1.43±0.35	0.193
Pentasaccharide†	0.004±0.002^{a}	0.016±0.007^{ab}	0.006±0.002^{ab}	0.024±0.007^{b}	**0.022**

†Pentasaccharide concentration given in µg ml^{-1}

Figures

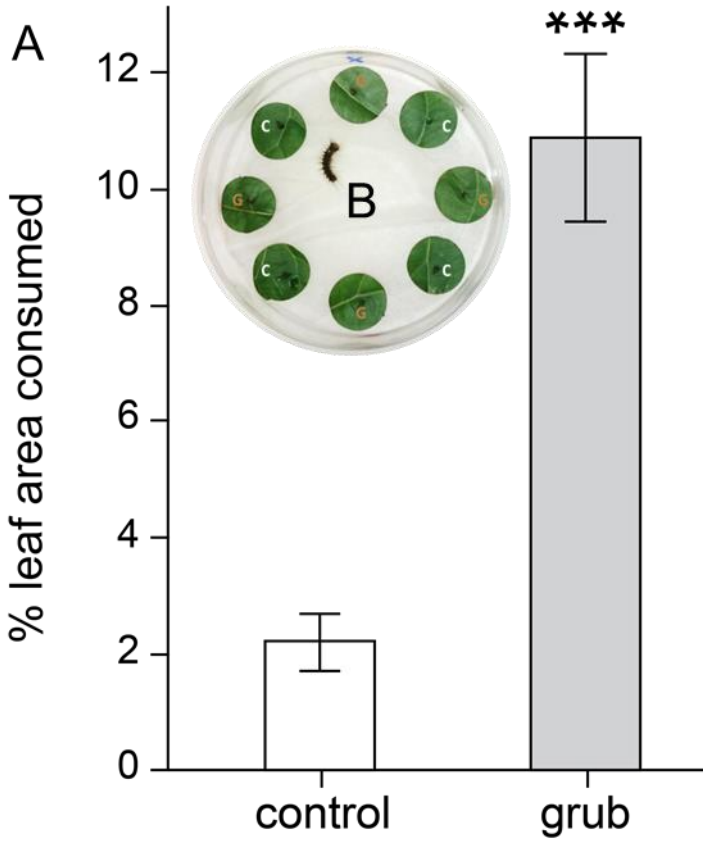

Figure 1A: Damage caused by *Lymantria dispar* caterpillars to black poplar leaf discs after belowground damage by one *Melolontha melolontha* larva (grub) compared to non-damaged control plants (control). Asterisks indicate significant differences between treatments based on a related-samples Wilcoxon signed rank test (W-value = 3.921; $p \leq 0.001$). Bars represent means ± SEM; n = 20. **Figure 1B: Arena used for caterpillar food choice assays.** A 90 mm petri dish was modified with simply office pins and padded with moist filter paper. The cut 2 cm² leaf discs were stuck upon the pins in an alternating fashion. Afterwards one caterpillar per petri dish was released in the middle of the arena for one day.

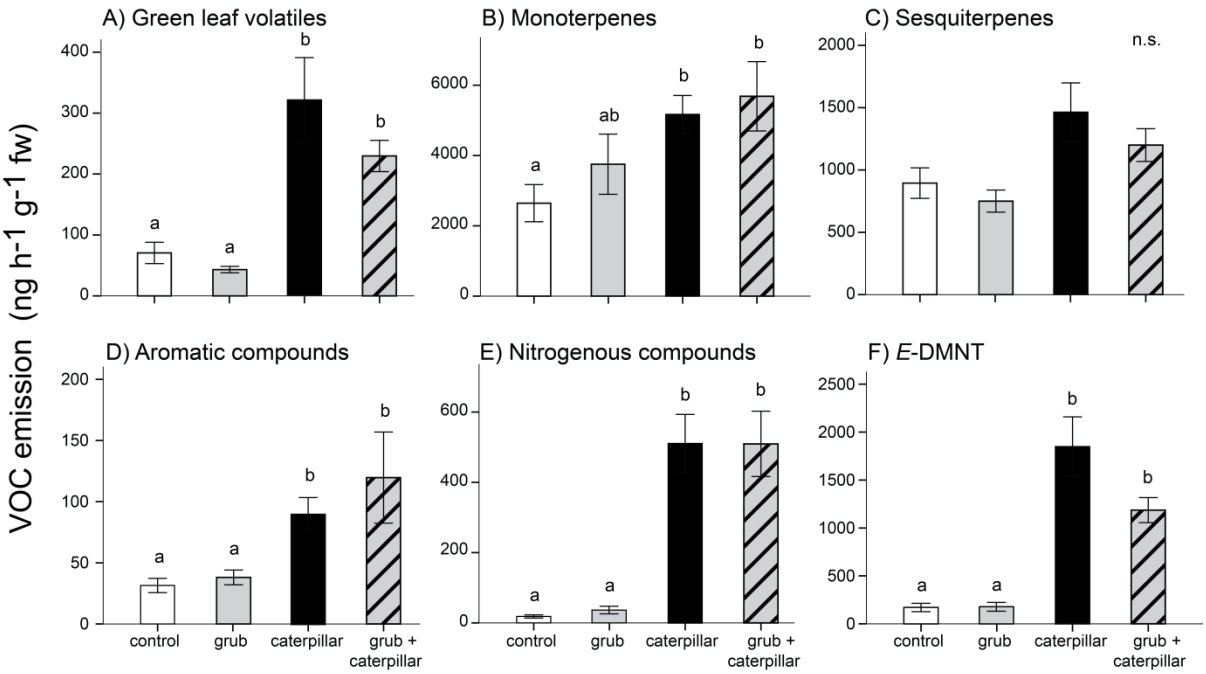

Figure 2: Green leaf volatile (A), monoterpene (B), sesquiterpenes (C), aromatic compound (D), nitrogenous compound (E) and *E*-DMNT (F) emissions from black poplar leaves after belowground damage by one *Melolontha melolontha* larva (grub), aboveground damage by *Lymantria dispar* caterpillars (caterpillar), and a combination of both herbivores (grub + caterpillar) as compared to non-damaged control plants (control). Different letters indicate significant differences between treatments based on a Kruskal Wallis test (A: $p < 0.001$, B: $p = 0.004$, C: $p = 0.63$, D: $p < 0.001$, E: $p < 0.001$ and F: $p < 0.001$) with Dunn´s post hoc test. Details of the statistical results are shown in Table 1. Bars represent means ± SEM; n = 13-15.

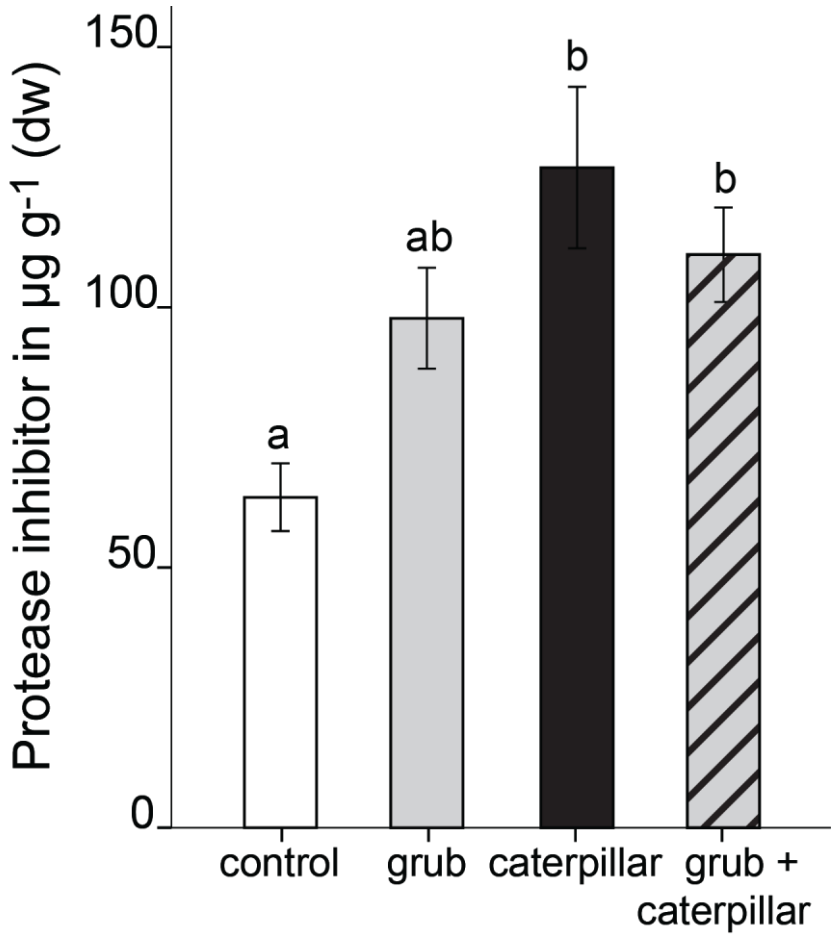

Figure 3: Protease inhibitor concentration in black poplar leaves after belowground damage by one *Melolontha melolontha* larva (grub), aboveground damage by *Lymantria dispar* caterpillars (caterpillar), and a combination of both herbivores (grub + caterpillar) as compared to non-damaged control plants (control). Different letters indicate significant differences between treatments based on an ANOVA ($F_{3, 53} = 9.187$, $p < 0.001$) with Tuckey´s post hoc test. The data was log transformed before all analyses. Bars represent means ± SEM; n = 13-15.

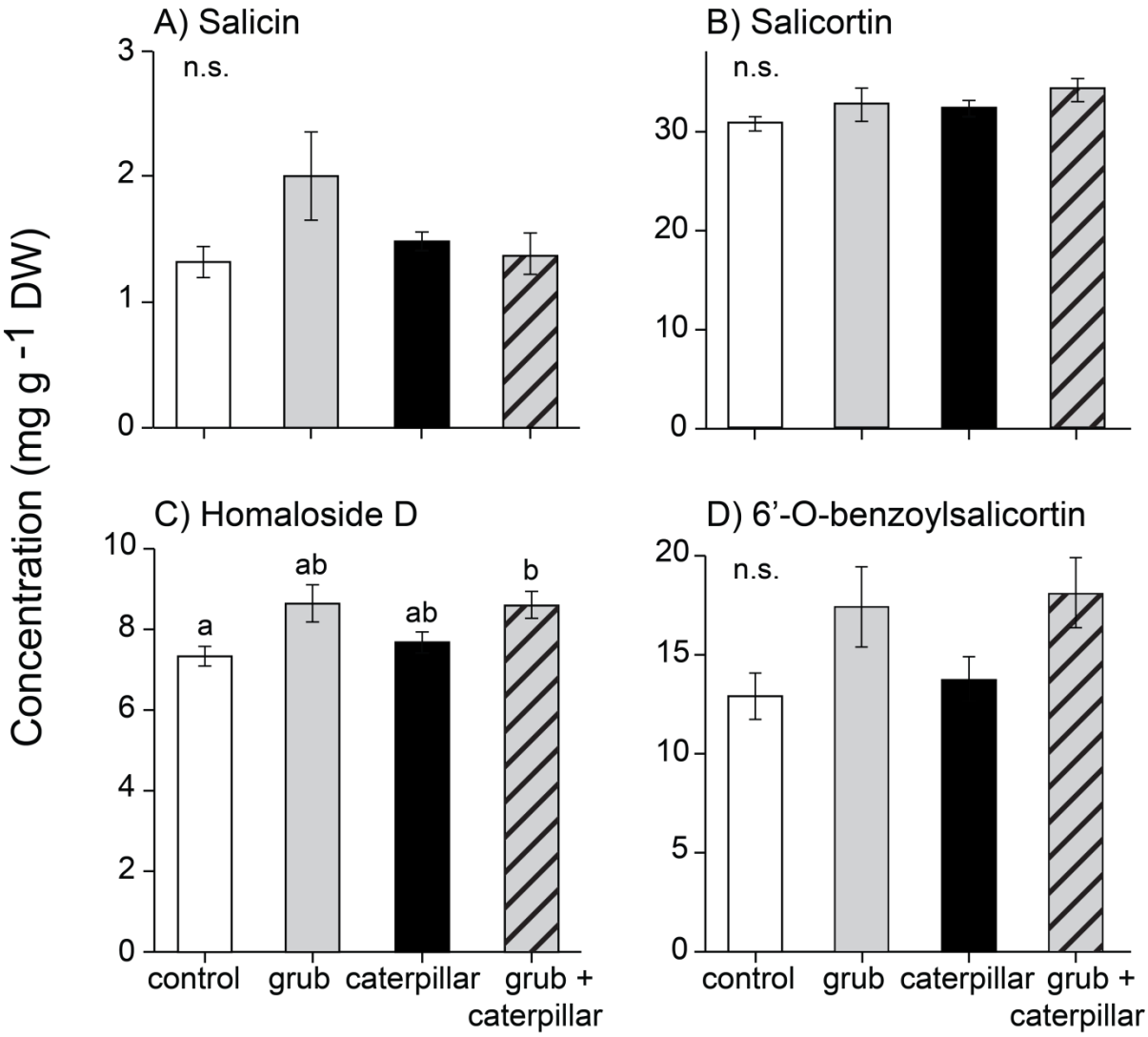

Figure 4: Salicin (A), salicortin (B), homaloside D (C) and 6'-*O*-benzoylsalicortin (D) concentrations in black poplar leaves after belowground damage by one *Melolontha melolontha* larva (grub), aboveground damage by *Lymantria dispar* caterpillars (caterpillar), and a combination of both herbivores (grub + caterpillar) as compared to non-damaged control plants (control). Different letters indicate significant differences between treatments based on a Kruskal Wallis test (A: p = 0.193, B: p = 0.15, C: p = 0.013, D: p = 0.124) with Dunn´s post hoc test. Details of the statistical results are shown in Table 1. Bars represent means ± SEM; n = 13-15.

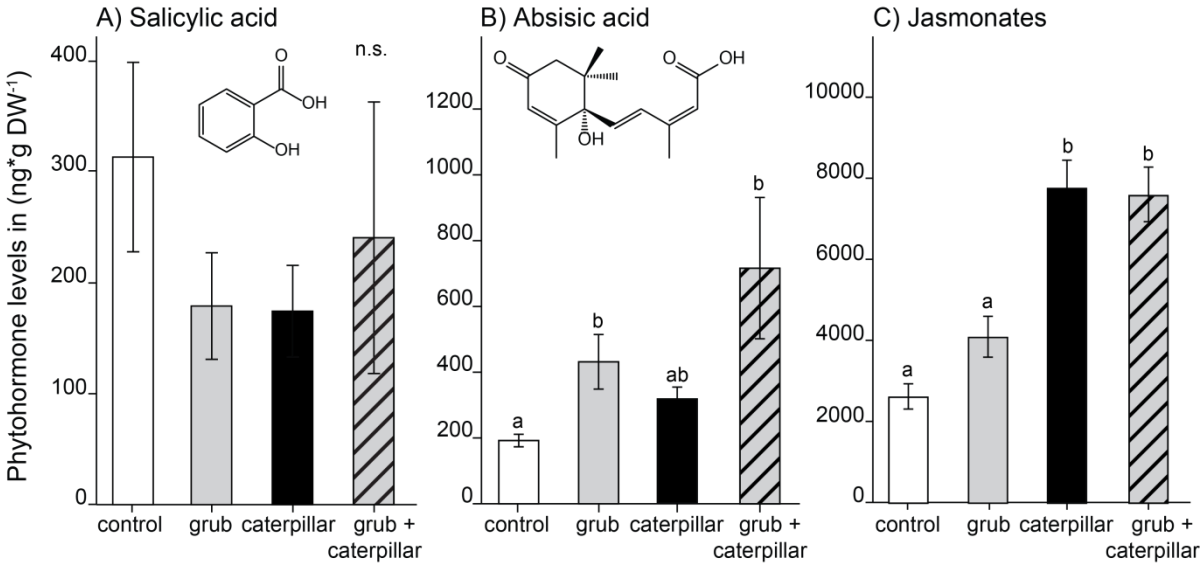

Figure 5: Salicylic acid (A), abscisic acid (B) and jasmonates (C) concentrations in black poplar leaves after belowground damage by a *Melolontha melolontha* larva (grub), aboveground damage by *Lymantria dispar* caterpillars (caterpillar), and a combination of both herbivores (grub + caterpillar) as compared to non-damaged control plants (control). The group jasmonetes consist of JA and its derivatives (JA-Ile-1, JA-Ile-2, cis-12-oxo-phytodienoic acid, OH-JA, OH-JA-Ile and COOH-JA-Ile). Different letters indicate significant differences between treatments based on a Kruskal Wallis test (A: $p = 0.717$, B: $p \leq 0.001$, C: $p \leq 0.001$) with Dunn´s post hoc test. Details of the statistical results are shown in Table 1. Bars represent means ± SEM; n = 12-15.

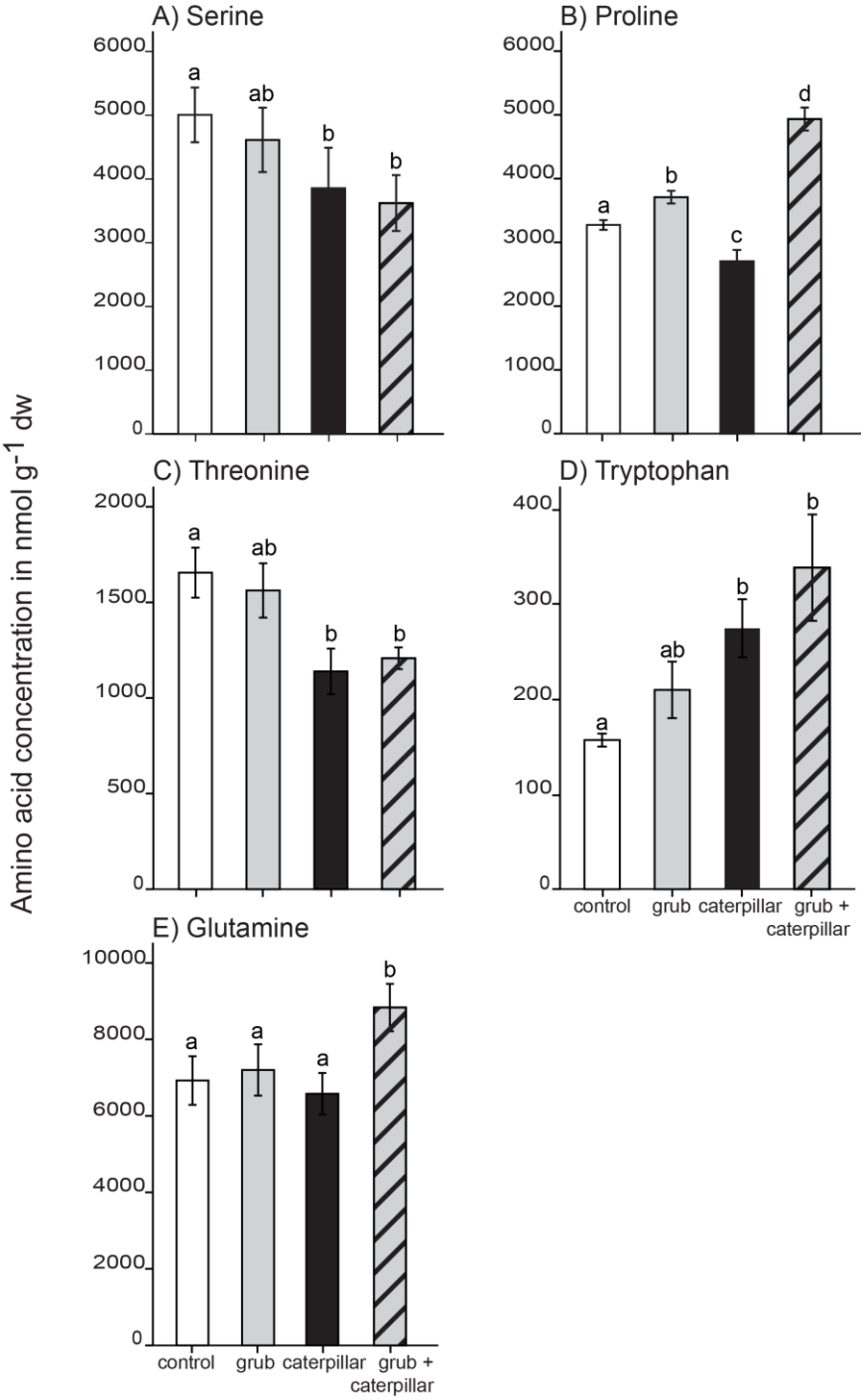

Figure 6: Serine (A), proline (B), threonine (C), tryptophane (D) and glutamine (E) concentrations in black poplar leaves after belowground damage by one *Melolontha melolontha* larva (grub), aboveground damage by *Lymantria dispar* caterpillars (caterpillar), and a combination of both herbivores (grub + caterpillar) as compared to non-damaged control plants (control). Different letters indicate significant differences between treatments based on a Welch ANOVA (A: $p = 0.032$, B: $p \leq 0.001$, C: $p = 0.002$, D: $p \leq 0.001$, E: $p = 0.007$) with Games-Howell post hoc test. Details of the statistical results are shown in Table S2. Bars represent means ± SEM; n = 14 – 15.

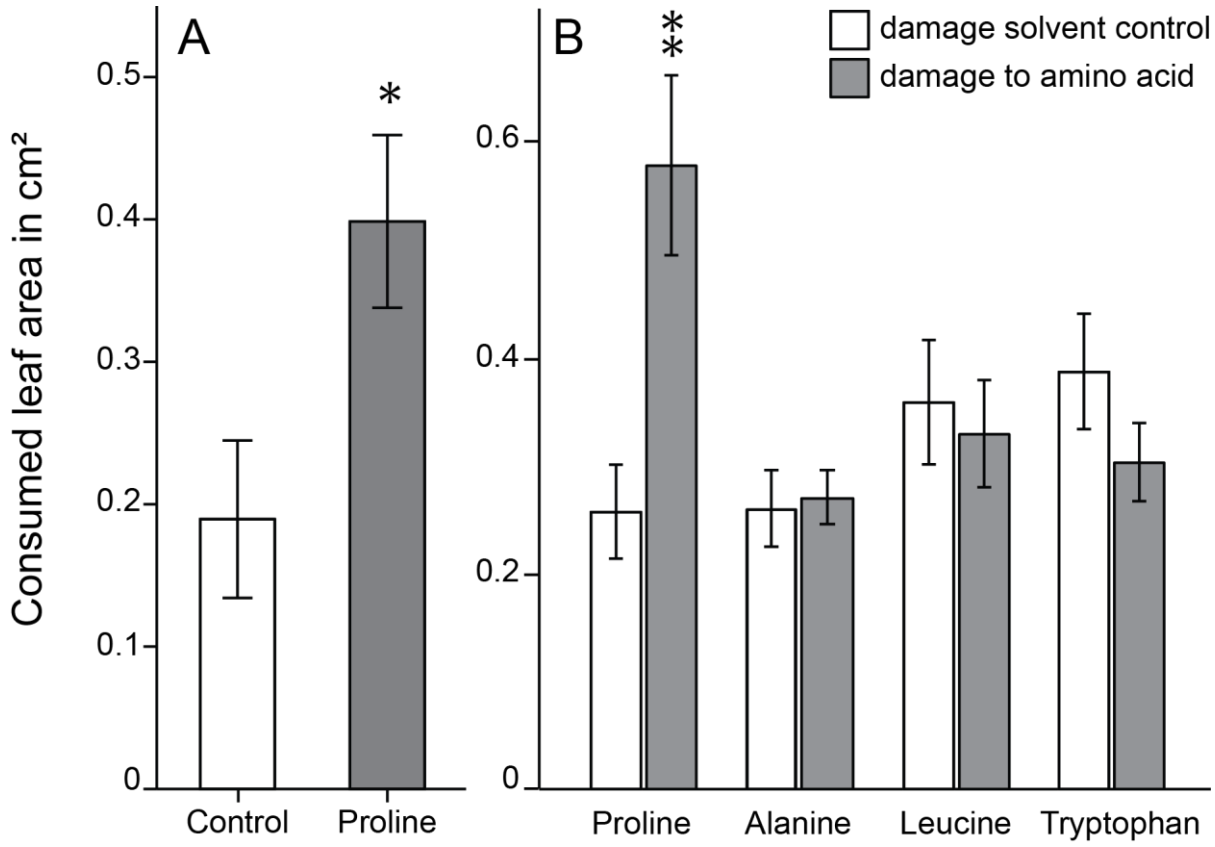

Figure 7: Feeding preference of *Lymantria dispar* caterpillars to leaf discs with enhanced proline levels (A) compared to control leaf discs **and to leaf discs supplemented with other amino acids (B)**. 20 µl of an amino acid dissolved in ethanol was pipetted onto leaf discs to increase amino acid concentration. Control leaf discs were treated with ethanol only. The leaf discs were allowed to dry before they were offered to the caterpillars. Asterisks indicate significant differences between treatments based on a related-samples Wilcoxon signed rank test for A (W-value = 3.053; p = 0.002) and paired t-test for B (proline: t = 3.618, p = 0.002; alanine: t = 0.27, p = 0.79; leucine: t = -0.449, p = 0.658; tryptophan: t = $^{-1}$.177, p = 0.255). Bars represent means ± SEM; n = 17 (A), n = 18-20 (B).

4. Manuscript II

Supplemental information

5. MANUSCRIPT III

Volatile mediated defense priming in black poplar.

Minor changes can cause major differences

Sandra Lackner, Thomas Fabisch, Heiko Vogel, Beate Rothe, Jonathan Gershenzon & Sybille B. Unsicker

In preparation for *Ecology Letters*.

5. Manuscript III

Title:

Volatile mediated defense priming in black poplar. Minor changes can cause major differences

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Abstract

In recent years evidence arose that herbivore-induced plant volatiles (HIPVs) can prime neighboring plants for increased defense upon possible future attack. However, the actual ecological consequences are not well studied for woody plants. The goal of this study was to elucidate whether volatile-mediated defense priming in black poplar would increase plant fitness. We analyzed phytohormones, transcriptional changes and multiple defense compounds in HIPV exposed poplar leaves. Additionally we conducted a food choice and a performance assay with gypsy moth caterpillars. HIPV exposure had no effect on phytohormones, transcriptional changes or protease inhibitors. Caterpillars avoided HIPV exposed leaves and showed an increased mortality and decreased performance when feeding on exposed leaves. We show that salicinoids are primed upon HIPV exposure and argue that the increase in salicinoids is responsible for reduced larval performance. Our results suggest that volatile mediated defense priming leads to increased fitness in black poplar.

5. Manuscript III

Introduction

Upon herbivore attack plants release herbivory-induced plant volatiles (HIPV) for direct and indirect defense (reviewed in Unsicker *et al*., 2009). Additionally those volatiles can function as signals between undamaged parts of the same plant or neighboring plants (Heil and Silva Bueno, 2007). The volatile receiving tissue or plant, which is now aware of a possible danger close-by, can prime their defense for a potential attack (Li *et al*., 2012). While early studies have received much skepticism in the past, numerous well-replicated, field and laboratory studies have proven that volatile communication leads to increased resistance against herbivory in multiple plant species (Karban *et al*., 2014).

By definition priming does not lead to phenotypical changes and will only become apparent after subsequent attack. But since plants do not possess a nervous system it should be possible to elucidate the underlying mechanism. Unfortunately so far only little is known about perception and transduction of the volatile signal. Only recently Nagashima *et al*. (2019) discovered that TOPLESS-like proteins can bind volatile compounds and function as transcriptional co-repressors in tobacco. But many open questions remain in regard to volatile-mediated signaling. Other kinds of priming like the systemic acquired resistance phenomenon are much better understood than volatile mediated defense priming. A wide array of possible mechanisms regarding a memory function and signal transduction have been described for pathogen defense like chromatin modifications, changes in cytosolic calcium, amino acids or sugars, gene activation and posttranslational modifications to only name a few (Conrath, 2011). Some evidence arose that very similar mechanism might play a role in volatile mediated defense priming as well (recently reviewed in Hilker & Schmülling, 2019).

A primed plant will show a faster, stronger or more sensitive response to a subsequent stress stimulus (Hilker *et al*. 2016). Therefore priming is believed to increase plant fitness since it would shorten the time where tissue is undefended, decrease the level of damage and additionally is argued to be less costly compared to induced defenses (van Hulten *et al*., 2006; Kost, 2006; Douma, 2017). Volatile-mediated priming can lead to an increase in terpenes (Engelberth *et al*., 2004), an increase in non-volatile plant defenses like protease inhibitors (PI) (Tscharntke *et al*., 2001) and/ or an upregulation of defense related genes, shown in most of the studies mentioned below.

This phenomenon has been described mainly for herbaceous plants like lima bean (Heil and Silva Bueno, 2007; Kost and Heil, 2006; Yi *et al*., 2009), Arabidopsis (Godard *et al*., 2008), maize (Engelberth *et al*., 2004; Ton *et al*., 2007; Erb *et al*., 2015), wheat (Ameye *et al*., 2015) and tobacco (Paschold *et al*., 2006). The literature for woody plants is sparser in general but there are some reports of priming in trees like alder (Tscharntke *et al*., 2001) and aspen (Li *et al*., 2012). It has been shown for birch that inter- and intra-plant signaling is possible even at

the same time (Girón-Calva *et al*., 2014). For perennial plants intra-plant priming might be of a greater ecological importance due to the short-lived nature of most volatile organic compounds under natural conditions and the fact that volatiles can move freely were there a restrictions in the vascular system (Karban *et al*., 2006; Frost *et al*., 2007 and 2008; Li and Blande, 2017).

Like mentioned above, most studies report an increase of defense related genes and/or a reduction of herbivore damage in plants upon HIPV exposure (Karban *et al*., 2014). Only a few studies focus on the consequences for the herbivore. Morrell and Kessler (2017) for example nicely showed that HIPV exposure of goldenrod increases herbivore movement and additionally decreases damage but the herbivore responded similar to directly damaged plants and plants that were exposed to HIPVs. Literature on woody plant species is sparse in general. Like mentioned above there are multiple reports of inter- and intra-plant volatile defense priming in several tree species. But little is known about direct consequences for feeding herbivores in trees. With the exception of a recent study that was conducted with willows. It was shown that larval performance was higher when feeding on control leaves compared to leaves that were exposed to HIPVs but this was only true for fast growing larvae. Volatile exposure had no effect on slow growing caterpillars (Hughes *et al*., 2015).

Many open questions remain regarding the priming phenomenon like which are the priming volatile compounds? What is the mechanism of the signal transduction and does priming have an actual consequence on feeding herbivores? Here we show that gypsy moth (*Lymantria dispar*) caterpillars actively avoid primed black popar (*Populus nigra*) leaves and show higher mortality and reduced performance when forced to feed on primed leaves. The exposure to HIPVs does not influence phytohormones, transcriptional changes, PIs, free sugars nor total protein content but primes salicinoids only. Furthermore we argue that the increase in salicinoids causes the reduction in larval performance.

Materials and Methods

Tree and insect rearing

Black poplar (*Populus nigra*) trees were grown monoclonal from stem cuttings of two genotypes growing in a common garden near Jena. All greenhouse experiments were carried out with the genotype “f65”, only the performance assay was carried out with a different genotype (f169). Stem cuttings were grown in 2 l pots filled with substrate mixture (55 % Klasmann-Tonsubstrat, 25 % Klasmann S1 [Klasmann-Deilmann GmbH, Geeste, Germany], 15 % sand and 5 % expanded shale) were grown and maintained in a greenhouse under summer conditions (24 °C, 60 % relative humidity, 16 h / 8 h light cycle) up to 1 – 1.20 m height until the start of the experiments.

Gypsy moth (*Lymantria dispar*) caterpillars were hatched from egg clutches and reared on artificial wheat germ diet (MP Biomedical, Eschwege, Germany) in a climate chamber (25 °C, 60 % humidity, 14:10 L:D period) until the start of the experiments.

Volatile collection of the emitters and exposure of receiver trees

The trees, both emitters and receivers, were cloaked in a PET bag (perimeter 62 cm, height according to tree height; Bratschlauch, Toppits, Minden, Germany). At 1.8 l min^{-1} air was pushed over an air purifying charcoal filter into the emitter bag (poplar infested with 10 4th instar *L. dispar* caterpillars). Via a diaphragm pump (Laboport, KNF Neuberger, Freiburg, Germany) the headspace was then transported from the bottom of one emitter to the top of three receivers using a three-way split at 0.5 l min^{-1} each. In total 1.5 l min^{-1} was pulled from the emitter resulting in a slight overpressure in the bags to avoid contamination from the outside. The PET bags and all hoses (Teflon tubing, 4mm in diameter) required for the air transport were fixed with cable ties. Receiving trees were exposed to emitter volatiles for 48 h. Control treatments were treated accordingly.

After the first 24 h of the 48 h of volatile transmission from emitting to receiving trees, volatile emissions from the emitter trees were collected for 2 h with Poropak traps (Alltech, Florida, USA). Following the collection the traps were eluted twice with 100 µl dichlormethane containing an internal standard (nonyl acetate, concentration = 10 ng $µl^{-1}$, Sigma Aldrich, Seelze, Germany). For identification and quantification of compounds we used GC-MS and GC-FID, for more detailed information see supplementary material.

Directly after the 48 h of HIPV exposure the air connection was removed and 10 4th instar *L. dispar* caterpillars were allowed to feed on the caterpillar and the HIPV exposed + caterpillar treatment **(Fig. 1)** for an additional 24h. Control and HIPV exposed treatments were resting for 24 h, resulting in total 72 h of experiment After 72 h the caterpillar food choice assay was carried out and 5 middle-aged leaves were harvested and stored for analyses.

Phenolic compound analysis

Phenolic compounds were extracted from 10 mg of freeze-dried *P. nigra* leaf material with 1 ml methanol containing an internal standard (0.8 mg ml^{-1} phenyl-β-glucopyranoside, Sigma Aldrich, Seelze, Germany). Extracts were diluted 1:2 with Milli-Q water before separation by an HPLC (1100 Series, Agilent Technologies, Santa Clara, CA, USA) equipped with a reversed phase column (EC 250/4.6 Nucleodur Sphinx, RP 5µm, Macherey-Nagel, Düren, GER). The mobile phases consisting of two solvents, solvent A (Milli-Q water) and solvent B (acetonitrile), were run with solvent B in gradient mode. The time/concentration (min/%) of the gradient was set to 0/14; 22.00/58; 22.10/100; 25.00/100; 25.10/14; 30.00/14 with a constant flow rate of 1 ml min^{-1}. The column oven temperature was set to 25 °C. The signal was detected with Photo Diode Array (PDA) and Evaporative Light Scatter (ELSD) detectors (Varian, USA). Concentrations were calculated on the basis of the peak areas as described in (Boeckler *et al*., 2013).

Other analyses

Phytohormones, phenylacetaldoxime and free sugars were extracted in parallel with the phenolic compounds and analyzed on HPLC-MS (for more information see Vadassery *et al*., 2012;Irmisch *et al*., 2013 and Eberl *et al*., submitted 2019; respectively). For more details on RNA extraction and transcriptome analysis see supplemental. Protease inhibitor concentration was determined *via* a radial diffusion assay as described in (Lackner *et al*., submitted 2019).

Caterpillar food choice assay

In a choice assay 19 2nd instar *L. dispar* caterpillars were offered leaf discs from either control or HIPV exposed plants to see whether they can distinguish between the treatments. Four leaf discs (16 mm Ø) of each treatment were stuck alternatingly on pins glued to a 90 mm petri dish equidistantly. A moist filter paper was placed at the bottom of each arena to avoid a drying of the leaf discs. One caterpillar per arena was then allowed to feed on the leaf discs for 24 h. Afterwards, the discs were photographed and herbivore damage was determined using Adobe Photoshop CS5 (Adobe, San Jose, CA, USA).

Caterpillar performance assay

To investigate whether HIPV exposure affects caterpillar development, a performance assay was conducted. Therefore, groups of 7 2nd instar *L. dispar* larvae were forced to feed on either HIPV exposed or control leaves, 12 groups each. Caterpillar groups were monitored and weighed over ten days. Dead larvae, if traceable, were removed before weighing. Each group was held in a 135 mm petri dish. Offered leaves were cut from pretreated trees and supplied with water. Leaves were removed from experiment, harvested for analysis and

exchanged with fresh leaves simultaneously according to larval feeding behavior, namely after 4, 6 and 9 days. Prior to the assay trees treatment were exposed to HIPVs for 48 h, as described above. The performance assay was then started directly after the 48 h of exposure. So at the time of leaf harvest, the exposure was respectively 4, 6 or 9 days ago. Additionally we conducted a caterpillar performance experiment on poplars with naturally varying salicinoid levels in a common garden (for more details see supplement).

Statistical analysis

All statistical analyses were done with IBM SPSS Statistics version 25 (SPSS, Chicago, IL, USA). All data were checked for statistical assumptions such as normal distribution, heterogeneity of variances and sphericity. In case of two group comparisons t-tests or related samples Wilcoxon rank tests were performed. ANOVA followed by Tuckey post hoc comparison was performed in case of normally distributed data with homogeneous variances. In case of non-parametric data, Kruskal-Wallis tests followed by Dunn´s post hoc tests were carried out. Data from the performance assay was analyzed using a repeated measurements ANOVA. To check whether phenolic compounds could explain the observed patterns of the performance assay, the analytes were one-by-one implemented as a co-variable into the repeated measurements model. Furthermore to compare survival of the caterpillars in the performance assay a Kaplan-Meier analysis was conducted.

Results

Emitter volatile emissions vastly increase by herbivore feeding

To evaluate the volatile signaling from emitter to receiver trees, volatile emissions from the emitting trees were collected. Altogether 27 volatile compounds could be identified (**Table S1**). Due to the herbivore treatment emissions of 15 volatile compounds increased significantly compared to constitutive emissions. Sesquiterpene emissions increased 4-fold, emission of aromatic compounds and monoterpenes increased about 8-fold, green leaf volatile emissions increased by 10-fold and *E*-DMNT emission increased by 65-fold. Nitrogenous compounds and isoamylacetate are exclusively emitted only after herbivore feeding.

Caterpillars performed worse when feeding on HIPV exposed leaves

A caterpillar choice assay was performed to elucidate whether *L. dispar* caterpillars can distinguish between leaf tissue that was exposed to HIPVs and control leaves. Second instar *L. dispar* caterpillars fed significantly more on control leaf discs than on leaf discs that were exposed to HIPVs (**Figure 3B**).

To test whether feeding on HIPV exposed leaves affects *L. dispar* caterpillars, a performance assay was conducted. Caterpillars that were forced to feed on HIPV exposed leaves grew significantly slower compared to caterpillars feeding on control leaves (**Figure 3A**). Additionally a higher mortality was observed for caterpillar groups feeding on HIPV exposed leaves. Of all caterpillars of the respective group 9.41 % died in control treatment whereas 23.81 % caterpillars died in groups that were forced to feed on HIPV exposed leaves. A log rank test was run to determine if there were differences in the survival distribution for caterpillar groups feeding on control or HIPV exposed leaves. The survival distributions were significantly different, $\chi^2(1) = 6.24$, $p = 0.012$. Furthermore occasional cases of cannibalism were noticed in groups that fed on HIPV exposed leaves (personal observation S.Lackner).

Receiver leaf analyses

To see whether HIPV signaling influences well-known plant hormones, salicylic acid, abscisic acid and jasmonic acid and its derivatives (referred to as jasmonates) were measured. All of the measured phytohormones showed a significant increase upon herbivore damage (**Figure S1**). The volatile signal, however, had no significant effect on any of the hormones.

We used next generation sequencing to elucidate whether HIPV signaling is regulated at the transcriptional level. No annotated sequences were significantly expressed differentially comparing control leaves to HIPV exposed leaves (for data and more information see supplementary data).

To check whether HIPV signaling has an effect on the nutritional value of a leaf, total protein content and free sugars were measured. The total protein content was not influenced by any treatment (**Table 2**). Similar the concentrations of sucrose, trisaccharides and tetrasaccharides were not significantly affected by any treatment (**Table S4**). In contrast glucose significantly increased after caterpillar feeding alone and fructose significantly increased upon herbivory (caterpillar treatment and HIPV exposed + caterpillar treatment) (**Table S4**). But, neither glucose nor fructose levels were influenced by HIPV signaling. Additionally we observed that the caterpillars dealt the same amount of damage to receiver trees that were exposed to constitutive black poplar emissions (caterpillar treatment: 7.49 ± 2.73 % leaf area loss) as to receivers that were exposed to HIPVs (HIPV exposed + caterpillar treatment: 8.36 ± 2.38 % leaf area loss; t-test showed no significant difference compared to caterpillar treatment).

Salicinoids increased after HIPV exposure and subsequent herbivore feeding

To test whether HIPV signaling influences the plants defensive metabolites we measured protease inhibitors, phenylacetaldoxime, catechin and salicinoid concentrations. The concentration of all trypsin inhibitors was determined *via* a radial diffusion assay. Trypsin inhibitor concentration increased significantly upon herbivory but there is no additional effect upon volatile signaling (**Table S2**). Herbivory significantly induced the leaf storage of phenylacetaldoxime but no additional volatile signaling effect was observed (**Table S3**).

None of the treatments had a significant effect on catechin (**Figure 2C**). However, there is a trend of lower catechin levels after exposure to HIPVs without subsequent feeding. To check whether the previously observed trend can be found again in a different black poplar genotype, catechin was measured in the leaves originating from the performance assay. At harvest time point 3 catechin levels are not influenced by the treatment. However, at the two later time points, catechin concentrations decrease significantly after HIPV exposure (**Figure 4C**).

To check whether HIPV signaling influences the salicinoids, salicin and salicortin concentrations were measured. Salicin significantly increases after exposure to HIPVs and subsequent caterpillar feeding (**Figure 2A**). There is also a trend of slightly higher salicin concentrations after HIPV exposure alone and caterpillar feeding alone. This trend is also apparent for salicortin. There are slighty higher concentrations after HIPV exposure and caterpillar feeding alone and in combination (**Figure 2B**). To monitor whether the previously observed patterns can be found again in a different black poplar genotype, salicin and salicortin were measured in the leaves originating from the performance assay. Salicin and salicortin both increase significantly after HIPV exposure and subsequent herbivore feeding at time point 5 (**Figure 4A+B**). Furthermore, there is a trend of an increase of salicin upon

HIPV exposure and herbivory at time points 3 and 8 (**Figure 4A**) and trend of an increase of salicortin at time point 3 (**Figure 4B**).

Field data suggests that there is a negative correlation between salicortin concentration and L. dispar larval weight

In a common garden setting *L. dispar* caterpillars were reared on nine black poplar genotypes, which vary naturally in salicinoid content, to monitor the effect of different levels of salicinoids on larval development. Therefore salicin, salicortin and catechin were measured after the caterpillars fed on the trees for 14 days and larval weight was recorded. The salicin levels varied from a minimum of 1.66 mg g^{-1} DW to a maximum of 6.50 mg g^{-1} DW and salicortin levels varied from a minimum of 32.74 mg g^{-1} DW to a maximum of 115 mg g^{-1} DW. We found a significant logarithmic connection between salicortin and average caterpillar weight ($y = 0.342 – 0.06 * \log(x)$, $F = 8.139$, $P < 0.05$, $R^2 = 0.546$, **Figure 5**). There was no connection between neither salicin nor catechin content and larval weight (Figure S3).

Discussion

For this study we wanted to investigate whether there is interplant volatile mediated signaling in poplar. Here we show that *L. dispar* caterpillars avoided feeding on primed leaves and performed worse when forced to feed on primed tissue. Furthermore, the salicinoids are primed and might serve as an explanation for the caterpillar behavior.

Black poplars herbivore-induced volatile blend has a huge signaling potential

The emitter volatile emissions of all major groups (green leaf volatiles, monoterpenes, sesquiterpenes, aromatic and nitrogenous compounds) highly increased after herbivory. Nitrogenous compounds were even completely absent on constitutive emissions (**Table S1**). It is well known that volatiles are inducible through herbivory. Green leaf volatiles are universally induced upon herbivore damage, there are numerous reports for herbaceous plants (Kigathi et al., 2009; Aharoni et al., 2003; Allmann and Baldwin, 2010) as well as for perennial species (Schmidt et al., 2011; Gossner et al., 2014; Arimura et al., 2004; Clavijo Mccormick et al., 2014; Frost *et al.*, 2007). Additionally several monoterpenes, sesquiterpenes, DMNT, aromatic and nitrogenous compounds are reported to be typically induced by herbivory (Arimura *et al.*, 2004; Clavijo Mccormick *et al.*, 2014; Danner *et al.*, 2011).

Priming leads to higher mortality and lower performance of L. dispar caterpillars

We conducted bioassays with *L. dispar* caterpillars to elucidate whether HIPV exposed leaves would influence the caterpillars' behavior. In a choice assay the caterpillars fed significantly more leaf area of the control leaves (**Figure 3 B**). Apart from test bites the larvae avoided leaves that were previously exposed to HIPVs. So it is very likely that they perceived a gustatory signal. Since the fed on HIPV exposed leaf disc resembles the HIPV exposed + caterpillar treatment we hypothesize that salicin, which was the only primed compound we could measure, might be responsible for the feeding decision as it has been reported to be repellent to caterpillars before (reviewed in Boeckler *et al*., 2011).

We also conducted a performance assay to check whether the observed patterns of the food choice assay has an impact on larval fitness. Significantly higher mortality was observed for caterpillars feeding on HIPV exposed leaves as well as significantly reduced larval weight gain (see results section and **Figure 3 A**). These findings lead to the conclusion that the poplar is better defended due to HIPV exposure. It has been reported that tobacco plants are more resistant to herbivores after receiving volatiles from a mechanically wounded sage bush (Karban *et al*., 2003). The increased mortality was most likely due to cannibalism. *L. dispar* is known to show cannibalism in populations with high density or when their nutrition is of poor quality (J. Mason *et al*., 2014). Since the density is constant during the performance assay it

is very likely that the nutritional quality of the leaves decreased due to HIPV exposure. However, we could not measure any changes in free sugar concentration or total protein content (**Table S2 + S4**). Therefore the change in food quality could very well arise from increased defensive compounds like the salicinoids. We saw a priming of salicin (**Figure 2**) but the increase is very marginal and the abundance of salicin is in general very low compared to the abundance of salicortin which was not affected by HIPV exposure.

The leafs chemistry is mostly unaffected by priming

All of the measured phytohormones, namely SA, ABA, JA and its conjugates (referred to as jasmonates), were induced upon herbivory (**Figure S1**) but no effect was observed when the trees were exposed to HIPVs. It is well known that jasmonates are inducible through herbivory (reviewed by Wasternack and Hause, 2013). Furthermore it has been shown that ABA increases after herbivory as it is an important regulator for herbivore induced resistance *via* JA dependent defenses (Vos *et al*., 2013). SA on the other hand is most often thought to play an important role in plant pathogen defense (Dempsey *et al*., 1999) but it has also been shown that SA is inducible upon sucking (Moran and Thompson, 2001) and chewing insects (Bi *et al*., 1997). However since none of the hormones reacted to HIPV exposure it is very likely that priming in black poplar is not regulated by SA, ABA or the jasmonates.

Another possible way of regulating priming is a modification on the transcriptome level therefore we analyzed our samples with next-generation sequencing. We hypothesized that transcriptional changes, if applicable, should be visible after HIPV exposure but before the subsequent herbivore attack, thereby functioning as the memory for the initial herbivore attack, the priming stimulus. Therefore we compared the RPKMs of control and HIPV exposed treatment for all sequences but no significant differential regulation was found for an annotated sequence (**supplementary material**). There was also no difference between caterpillar and HIPV exposed + caterpillar treatment (data not presented). However if there were a difference between said two treatments it would much rather hint at enhanced gene expression by chromatin modification which is very common in systemic acquired resistance against pathogens (van den Burg and Takken, 2009; reviewed in Conrath, 2011). Now one could make the argument that the low replicate number might be the reason that we cannot find a pattern but we are convinced the method worked because we can find statistically significant differences between control and caterpillar treatment for sequences that are related to wounding and/or defense *via* JA (**Figure S2**). Therefore we conclude that in black poplar priming is regulated on a different level. There are numerous possible signal transduction ways like changes in cytosolic calcium, tricarboxylic acids, reactive oxygen species, membrane depolarization, hormone conjugates, amino acids, sugars or post transcriptional modifications (Mauch-Mani *et al*., 2017).

In Addition we checked whether HIPV exposure would influence levels of free sugars or total protein content, since as mentioned before they are possible signal transduction ways (Mauch-Mani *et al*., 2017) and would maybe alter the nutritional quality of a leaf from an herbivores perspective. However the total protein content was not influenced by any of our treatments (**Table S2**). As for the free sugars, glucose and fructose significantly increases after caterpillar feeding but again there is no effect of HIPV exposure (**Table S4**). We therefore concluded that the nutritional value of the poplar leaf does not change due to volatile priming.

In addition to phytohormones and the transcripts, exposure to HIPVs did also not influence well-known defense compounds. We measured protease inhibitor concentrations since it has been proposed that PIs are affected by volatile defense priming (Farag *et al*., 2005; Kessler *et al*., 2006) but this is not the case in black poplar. We observed a significant induction of PIs after herbivory but there is no significant effect of HIPV exposure (**Table S2**).

We also measured phenylacetaldoxime, a semi volatile which accumulates in poplar leaves upon herbivory, that decreases the performance of *L. dispar* caterpillars (Irmisch *et al*., 2013). Furthermore it is a precursor of benzyl cyanide and other HIPVs (Irmisch *et al*., 2015). There was a significant induction of phenylacetaldoxime upon herbivory but again there is no significant influence of HIPV exposure (**Table S3**).

We measured catechin, a precursor on condensed tannin biosynthesis that is connected to salicinoid biosynthesis as well. It is known that an overexpression of the condensed tannins pathway leads to reduced concentrations of salicinoids (Mellway *et al*., 2009; Boeckler *et al*., 2014). Catechin and the salicinoids therefore are believed to behave antagonistically. We observed a trend of decreased catechin levels in HIPV exposed leaves but none of the treatments caused a significant change to catechin levels (**Figure 2**). Additionally we measured catechin in leaves originating from the performance assay to confirm our results from the initial experiment. Interestingly catechin levels decreased significantly after six and nine days post HIPV exposure (**Figure 4C**). Now this decrease could be explained by the previously mentioned antagonism of salicinoid and condensed tannins biosynthesis pathways but it is also possible that HIPV exposure leads to an increase substrate turnover of catechin and therefore an increase of condensed tannins. However the effects of condensed tannins on herbivores remain unclear (reviewed in Barbehenn and Peter Constable, 2011). It has been shown in poplar that neither catechin itself nor condensed tannins have a negative effect on *L. dispar* caterpillars (Boeckler *et al*., 2014).

HIPV exposure primes salicinoid levels

Another classic defense of the *Salicaceae* are the salicinoids. They are reported to be toxic and deterrent to herbivores (reviewed in Boeckler *et al*., 2011). We measured salicin and salicortin to see whether HIPV exposure would influence these compounds. There is no effect of any of the treatments on salicortin but there is a significant increase of salicin in leaves that were exposed to HIPVs and subsequently attack by caterpillars. The literature shows no universal pattern whether salicinoids are constitutive and/or inducible defenses (Boeckler *et al*., 2011), which makes the priming of salicin even more interesting.

We measured salicin and salicortin in the leftover fed upon leaves originating from the performance assay to elucidate whether the concentrations would change over a longer time period than the ones monitored during the initial experiment. There is a significant increase of salicin and salicortin in the leaves that were harvested six days after HIPV exposure (**Figure 4A+B**). Additionally there is a trend of increased salicortin and salicin four days after HIPV exposure and salicin is still increased at nine days post exposure. These results confirm our findings from the initial experiment. In black poplar salicin can be primed quite rapidly, one day after HIPV exposure, and shows increased levels until at least nine days post exposure. The effect of priming on a more complex salicinoid is visible only after couple of days after the priming event and will vanish quicker compared to salicin.

Marginal increase of salicortin can influence caterpillar performance

So far to our knowledge HIPV exposure in poplar leads to significant though marginal increase in salicin and salicortin and to a decrease of catechin. However we see strong effects on caterpillar behavior and performance. The decrease in catechin cannot serve as an explanation for the performance reduction. On the other hand there are multiple reports of salicinoids being toxic to *L. dispar* (Boeckler *et al*., 2011) and that feeding caterpillars a diet with artificially enhanced amounts of salicin and salicortin results in slower growth and pupation rates (Hemming and Lindroth, 1995; Kelly and Curry, 1991; Orians *et al*., 1997). Therefore we performed a repeated measurements ANCOVA to check whether salicin or salicortin can explain the pattern of the performance assay. Indeed as a co-variable salicin has a significant impact on the weight gain of the caterpillars (AIC of the whole model 486.04; F-value of the effect of the co-variable salicin = 6.61, p = 0.013). Additionally we observed a significant negative correlation between increasing in salicortin concentration and larval weight (**Figure 5**) during a performance experiment that was set up in a common garden setting with natural varying concentrations of salicinoids between genotypes. Alltogether, our findings strongly suggest that marginal increase of salicin and salicortin is indeed responsible for the decreased performance of *L. dispar* when feeding on primed leaves.

Conclusion

Though the actual mechanism of volatile-mediated defense priming still remains unknown our results suggest that exposure to HIPVs can prime salicinoids in black poplar trees for increased plant fitness upon subsequent herbivore attack. This supports that priming is an important mechanism for inter- and possibly intra-plant signaling (Frost *et al*., 2007; Li and Blande, 2017) in trees. Future studies will have to elucidate the actual signaling volatile compound and the underlying mechanism.

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Figures

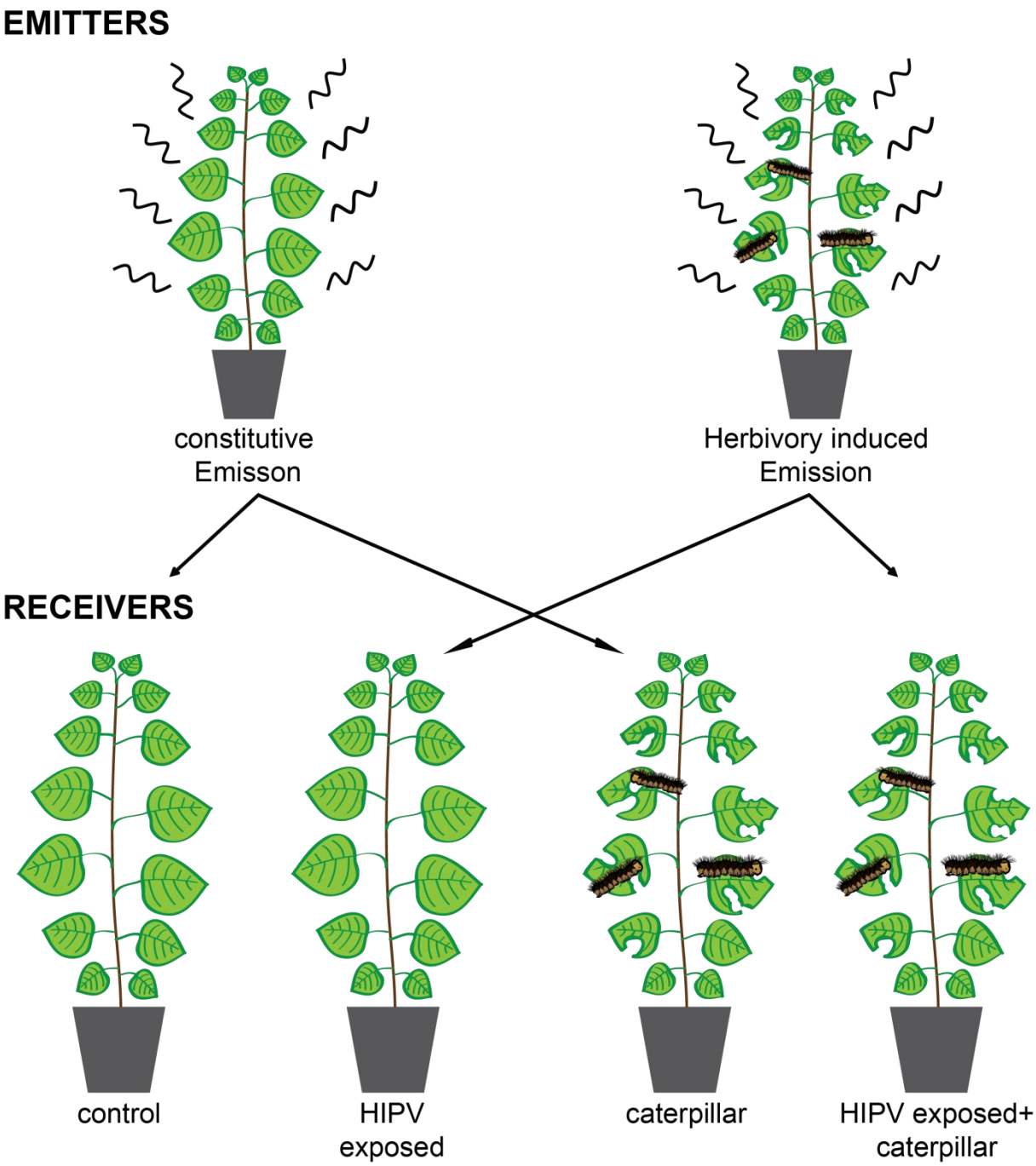

Figure 1: Experimental setup to prime the receiver trees. "Herbivory induced emission" emitter trees were infested with 10 4th instar *L. dispar* caterpillars. Directly after caterpillar onset, air connection was established for 48 h between emitter and receiver trees with airflow of 0.5 l min^{-1}. Afterwards air connection was removed and receiver trees of the caterpillar and the HIPV exposed + caterpillar treatment were infested with 10 4th instar *L.dispar* caterpillars, which were allowed to feed an additional 24 h. Control and HIPV exposed trees rested an additional 24 h, leading to a total experimental time of 72 h. Emitter volatile emissions were collected for 2 h after the first 24 h of established air connection.

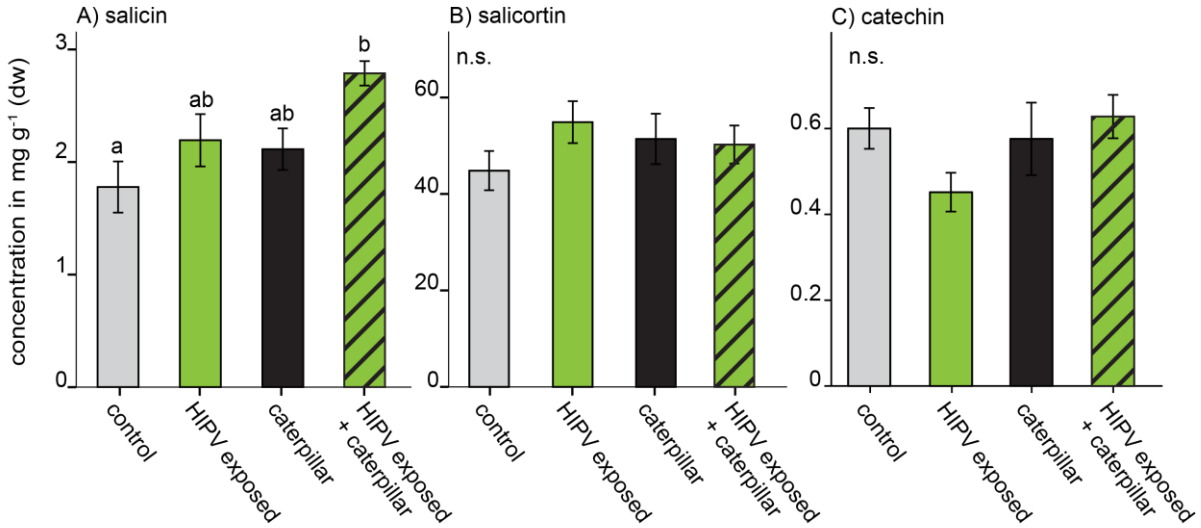

Figure 2: Salicin (A), Salicortin (B) and Catechin (C) concentrations of black poplar leaves after herbivore damage by gypsy moth caterpillars (caterpillar), previous exposure to HIPVs (HIPV exposed) and a combination of herbivore damaged and previously exposed to HIPVs (HIPV exposed + caterpillar) compared to control plants (control). Different letters indicate significant differences between treatments based on an ANOVA with Tukey´post hoc (A: F = 4.782; p = 0.012; C: n.s.) or a Kruskal Wallis test (B: n.s.). Bars represent means ± SE; n = 5-6.

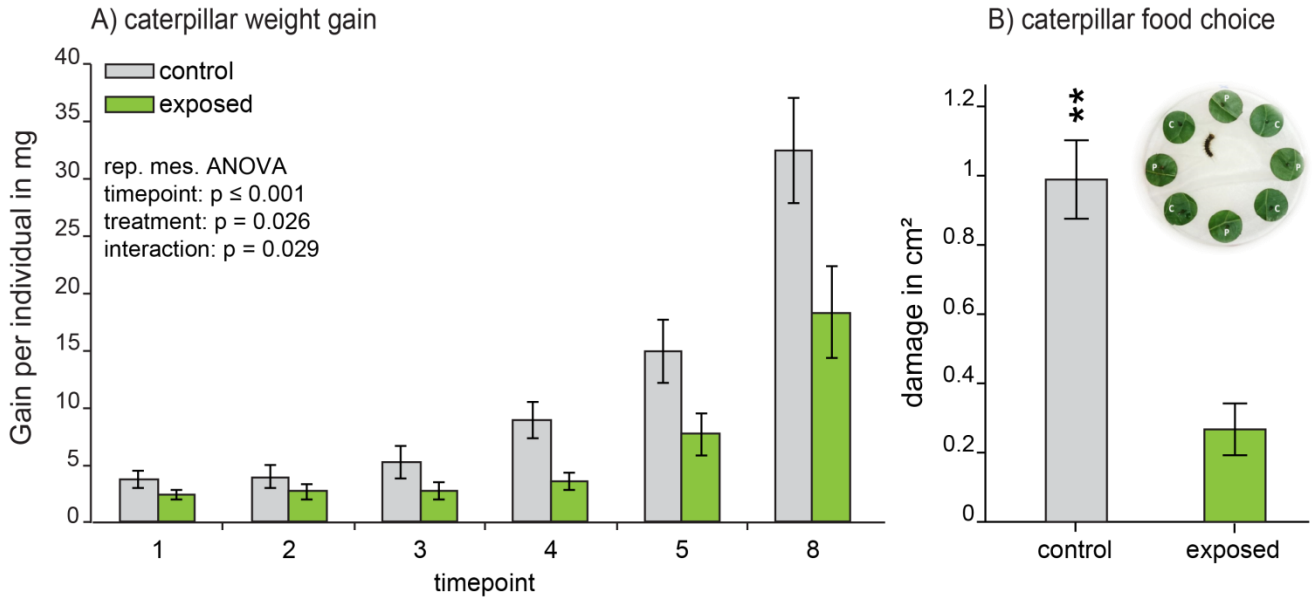

Figure 3 A: Caterpillar performance. Groups of 7 second instar *L. dispar* larvae were kept in separate petri dishes and forced to feed on either control or HIPV exposed leaves and weighed for 10 days. Dead larvae, if traceable, were removed before weighing. Offered leaves were exchanged twice, see method section for details. Bars represent means + SE. Given p-values result from a repeated measures ANOVA, n = 12. **B: Caterpillar food choice.** Leaf discs of either control or HIPV exposed trees were offered to one second instar *L. dispar* larva per petri dish arena. Caterpillars were allowed to feed for 24 h. Afterwards feeding damage was analyzed using Photoshop. Bars represent means +SE. Asterisks indicate significant difference based on a related samples Wilcoxon signed rank test: W= -3.179, p = 0.001, n = 19.

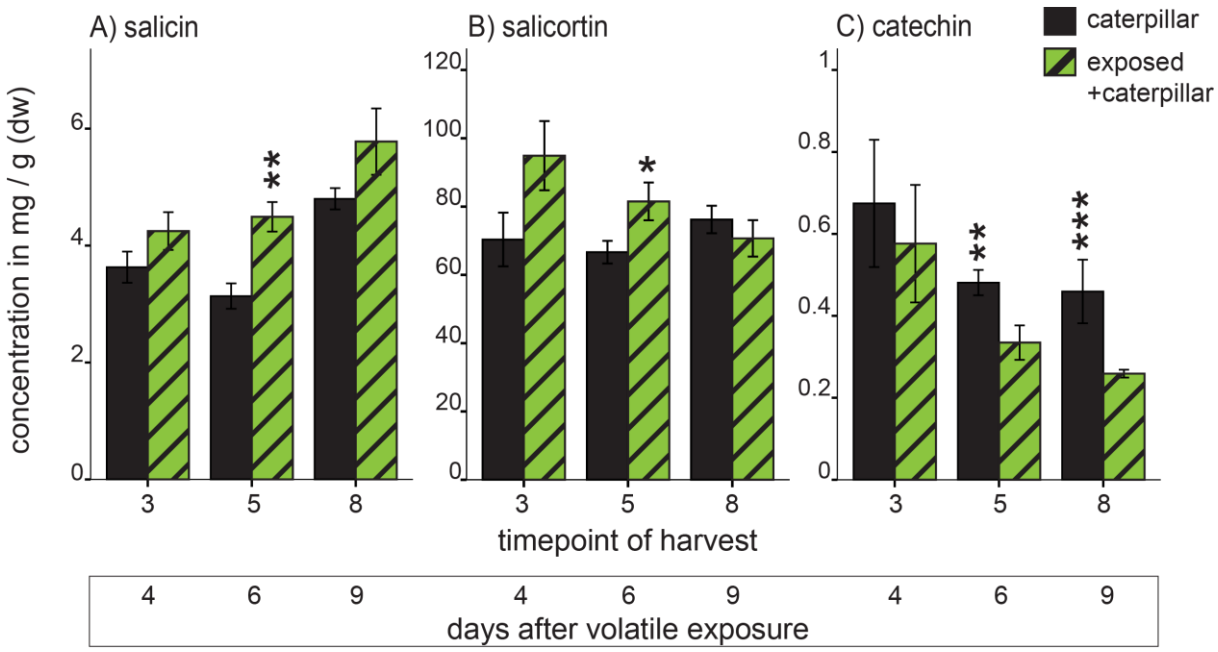

Figure 4: Salicin (A), Salicortin (B) and Catechin (C) concentrations of black poplar leaves analyzed after being fed on by caterpillars of the performance assay (Fig. 3A). Black bars represent leaves that were exposed to constitutive volatiles and afterwards offered to *L. dispar* caterpillars. Striped green bars represent leaves that were exposed to HIPVs and afterwards offered to *L. dispar* caterpillars. Displayed time points mirror time points of the performance assay **(Fig. 3A)**. To compare differences between treatments within one time point a t-test was performed (A 5: $F = 18$, $p = 0.001$; B 5: $F = 37$, $p = 0.045$; C 5: $F = 23$, $p = 0.004$; C 8: $F = 10$, $p < 0.001$; all other comparisons were not significant) Asterisks indicate significance level based on the p-value. Bars represent means ± SE, n = 12.

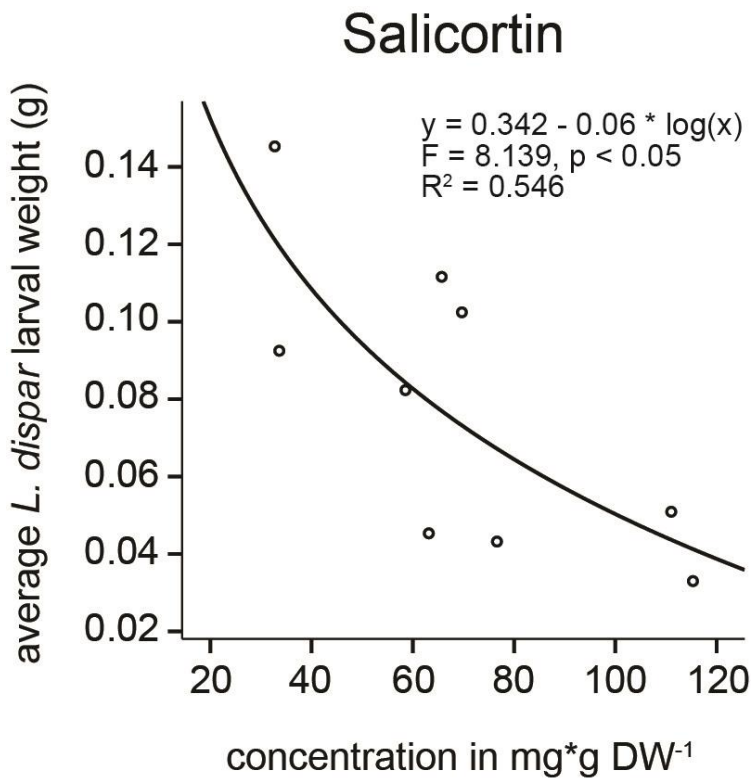

Figure 5: Average *L. dispar* larval weight in dependence of salicortin levels in black poplar trees of nine genotypes. The data derives from a common garden experiment performed on poplar trees in July 2016. Twenty 2nd instar *L. dispar* caterpillars were allowed to feed on a leaf pool consisting of eight leaves for 14 days. Afterwards caterpillars were weighed (dead larvae excluded from analysis), leaves were harvested and analyzed for salicortin content. Shown are means, n = 5-10.

5. Manuscript III

Supplemental information

6. DISCUSSION

Plants face a constant risk of being attacked by insect herbivores aboveground (AG) as well as belowground (BG). Therefore, they have evolved an arsenal of defensive mechanisms to resist herbivore pressure. Many studies investigating plant defenses have focused on attack of a single herbivore. But, in nature multiple insect species can feed simultaneously on a plant or different parts of the plant or one attack may follow others. Plant response to multiple attackers might differ from that to a single herbivore, and later arriving herbivores may find more or less favorable conditions on a previously attacked plant. Experiments with multiple attackers therefore present us with a more realistic picture of plant defense responses, especially for large species like trees that can participate in a multitude of biotic interactions. In recent years a number of researchers have started to investigate multiple herbivore interactions in herbaceous plants, but our knowledge on such interactions in trees is still scarce

6.1. Black poplar roots release monoterpenes upon root damage

Upon herbivore attack, many plants change their volatile emission profile as part of direct and indirect defense measures and for intra-plant communication. For AG tissues this has been documented countless times in many plant species (reviewed in Unsicker *et al*. 2009) including black poplar (**manuscript II**; **supplementary material for manuscript III**). However, the emission of VOCs from roots into the soil is poorly understood and underrepresented in the literature (Penuelas *et al*. 2014). It has been reported that root herbivory induces volatile emissions in both herbaceous and woody plant species (Rasmann *et al*. 2005; Weissteiner *et al*. 2012; Abraham *et al*. 2015) but the role of the emitted compounds is not fully understood.

In recent years, the main focus of studies investigating root volatiles has been the attraction of herbivore enemies. For example, damaged roots of maize and citrus trees can attract entomopathogenic nematodes with the help of sesquiterpenes (Rasmann *et al*. 2005; Degenhardt *et al*. 2009; Ali *et al*. 2010). Whether root volatiles can also act as direct defenses, as reported for AG systems (L. Bernasconi *et al*. 1998; von Mérey *et al*. 2013; De Moraes *et al*. 2001; Rostás & Hilker 2002), remains unknown. Bioassays conducted with the

common cockchafer (*Melolontha melolontha*) larvae, an important agricultural pest feeding on the roots of crops and fruit trees, have shown that the larvae, known as grubs, can be both repelled and attracted by certain pure volatile compounds (Eilers 2012). These findings hint at the possibility of root VOCs acting as direct defenses, but they should be verified in a more natural system. Other studies investigating the direct effects of volatiles on BG herbivores came to the conclusion that herbivores exploit the volatile cues to find their hosts in the soil. For example, it has been shown that the western corn root worm and the forest cockchafer are attracted to volatiles emitted from maize and oak roots respectively that have been damaged by conspecifics (Robert *et al*. 2012; Weissteiner *et al*. 2012).

We investigated the formation and emission of volatiles from black (*P. nigra*) and western-balsam (*P. trichocarpa*) poplar roots upon herbivore damage by grubs of the common cockchafer. We could show that both poplar species emit monoterpenes from roots, among them α- and β-pinene, camphene and 1,8-cineole (**manuscript I**). Most of the measured VOCs were emitted constitutively but some compounds are inducible by herbivory. For example, 1,8-cineole significantly increased after damage to *P. trichocarpa* roots, whereas in *P. nigra* roots camphene and an unidentified monoterpene were emitted in higher amounts. Furthermore, there was a trend of increased β-pinene emission with herbivory in both species. We were able to identify and characterize three terpene synthases that produce the emitted compounds and could show that the emission pattern of these volatiles is most likely regulated *via* upregulation of the corresponding genes (**manuscript I**). Our results therefore suggest that the mechanism of BG monoterpene emission in roots is very similar to that from AG tissues, which is very well understood (reviewed in Degenhardt *et al*. 2009b).

Additionally, we were able to show that 1,8-cineole and β-pinene significantly reduce the growth of *Phytophthora cactorum* (**manuscript I**). An oomycete, *P. cactorum* is a plant pathogen with a broad host spectrum and causes root and crown rots in over 200 species of ornamentals, trees and fruit crops like strawberries and apple trees (Davison 1998). Infection with this pathogen is accomplished *via* mobile zoospores that can enter the plant through wounds (Seemuller 1998). Damaged tissues BG as well as AG are in general thought to be sites where pathogens can easily enter their hosts (Savatin *et al*. 2014). The two monoterpenes that showed anti-microbial activity, 1,8-cineole and β-pinene, were induced *via* root damage caused by herbivory. Our data suggest that the emission of these compounds could act as a defense against a subsequent pathogen attack. Terpenes and especially 1,8-cineole have been reported as antibiotics against bacteria and fungi (Kalemba *et al*. 2002; Hammer *et al*. 2003; Quintana-Rodriguez *et al*. 2018). The other compounds tested, 2-phenylethanol and limonene, have demonstrated antimicrobial activity in other systems (Liu *et al*. 2014; Hammer *et al*. 2003) but had no effect on *P. cactorum* (**manuscript I)**. Defense against microorganisms *via* VOCs therefore seems to be target species-specific. As 2-

phenyethanol and limonene were not emitted from the roots but are well-known leaf volatiles, it is plausible that they do not affect a soil-borne pathogen. Of course the emission of volatiles upon root damage could also act as a defense against the feeding herbivore or as attractants for herbivore enemies. 1,8-Cineole and limonene have been reported to be toxic to the American wheat weevil and the red flour beetle (Prates *et al*. 1998). In addition, nematodes attracted to root volatiles, as mentioned earlier, reduce the performance of *M. melolontha* larvae (Erbaş *et al*. 2014). It is therefore possible that poplar root volatiles are emitted as direct and indirect defenses as well as anti-microbial agents. Future studies are needed to answer these questions.

6.2. Root herbivory causes water stress-like symptoms in poplar

The root is one of the three basic organs of all seed plants. Its primary functions are the absorption of water and minerals from the soil and anchoring the plant to the ground. Thus when an herbivore feeds on the roots of a plant it will not only affect the damaged root but also have an effect on AG tissues. The consequences of root herbivory are mediated in part by the reduction of the plant´s capability to take up water and nutrients. BG herbivory negatively affects plant growth (Tsunoda *et al*. 2014) and the nutritional value of the leaves (Blossey and Hunt-Joshi 2003). However, AG plant defenses can also be affected by BG herbivory, mediated through intra-plant communication *via* phytohormones. In general it has been observed that root herbivory benefits piercing insects like aphids or spider mites (reviewed by Johnson *et al*. 2012; Kammerhofer *et al*. 2015; Hoysted *et al*. 2017), but hinders leaf chewers like caterpillars (van Dam *et al*. 2005; Erb *et al*. 2011; Bakhtiari *et al*. 2018). Most of the studies investigating BG-AG interactions have focused on annual herbaceous plants (Papadopoulou & Dam 2016) and very little is known about how trees are affected by BG herbivory (Zvereva & Kozlov 2012).

In **manuscript II** we therefore investigated how BG and AG herbivory affect the phytochemistry of black poplar leaves and the consequences for the leaf herbivore *L. dispar*. The phytohormone SA was neither affected by root nor shoot herbivory, consistent with what has been described in literature previously (Erb *et al*. 2009[a]; Pierre *et al*. 2012; Pieterse *et al*. 2012). However, JA and its derivatives increased drastically in leaves upon AG herbivory (**manuscript II & III**), which is well-known for herbaceous as well as woody plant species (reviewed in Wasternack & Hause 2013; Clavijo Mccormick *et al*. 2014b; Eberl *et al*. 2017). In contrast, root herbivory had no significant impact on leaf jasmonate levels consistent to what was previously described for maize plants under BG herbivore attack (Erb *et al*. 2009[a]; Erb *et*

al. 2011). Leaf ABA levels, on the other hand, did not significantly increase after leaf herbivory, but increased upon root damage and increased even more during combined BG and AG attack (**manuscript II**). ABA is known to act as a systemic signal from roots to shoots (Jackson 1997) and it has been reported as a key player in plant response to both water loss and wounding (Aleda *et al*. 2005; Erb *et al*. 2009[a]; Nguyen *et al*. 2016). Therefore, it seems likely that ABA mediates BG-AG intra-plant communication upon root attack. Further, increased levels of ABA in leaves potentially interfere with established JA-dependent defense mechanisms against chewing insects. It has been proposed that JA-ABA interactions regulate growth-defense tradeoffs as they are known to co-regulate plant growth under healthy conditions as well (Erb *et al*. 2011; Lackman *et al*. 2011).

In order to see whether root herbivory influences AG defense mechanisms of black poplar, volatiles, protease inhibitors (PI) and salicinoid levels, which are well-known anti-herbivore defenses in poplars (Major & Constable 2008; Boeckler *et al*. 2011; Clavijo McCormick *et al*. 2014b), were measured. Upon leaf herbivory by *L. dispar* caterpillars, the emission of GLVs, monoterpenes and *E*-DMNT increased significantly as well as the emission of aromatic and nitrogenous compounds (**manuscript II & III**). This is consistent with studies investigating herbaceous (Arimura *et al*. 2004; Kigathi *et al*. 2009; Allmann & Baldwin 2010) as well as woody plant species (Schmidt *et al*. 2011; Gossner *et al*. 2014) including poplars (Frost *et al*. 2007; Danner *et al*. 2011; Clavijo McCormick *et al*. 2014b). BG herbivory had no significant impact on AG volatile emissions, neither by itself nor in combination with an AG attack (**manuscript II**). Therefore, volatile emissions that are direct and indirect defenses of black poplar foliage are most likely not affected by BG herbivory. Previous studies have reported the induction as well as no change in AG volatile emissions upon BG herbivory (reviewed by Papadopoulou & Dam 2016).

Other well-known direct defenses of poplar are PIs and salicinoids. PIs irreversibly inhibit proteases in the insect gut and are therefore considered very efficient direct defenses against feeding herbivores (Howe & Jander 2008; Saadati & Bandani 2011; Zhu-Salzman & Zeng 2015). In hybrid poplar (*Populus trichocarpa* × *P. deltoides*) it has been shown that genes related to PIs are amongst the most upregulated upon mechanical wounding and insect feeding (Major & Constable 2006). Also in *P. nigra* leaves trypsin inhibitor activity increases significantly after leaf herbivory (**manuscript II & III**) and combined BG-AG attack (**manuscript II**). Additionally upon BG herbivory alone there is a trend of higher PI levels in the leaves (**manuscript II**), similar to maize and tomato, where a systemic induction of PIs through root damage has been reported (Erb *et al*. 2011; Arce *et al*. 2017). In general, PIs are thought to be induced *via* the JA-dependent pathway (Dinh *et al*. 2013; Vos *et al*. 2013). In *P. nigra*, however, jasmonate levels were not induced after BG herbivory, whereas ABA significantly increased (**manuscript II**). The systemic induction of PIs therefore hints at the

co-regulation of PIs *via* JA and ABA, which was previously hypothesized by Nguyen *et al*. (2016).

Salicinoids, the direct defense compounds exclusive to the Salicaceae family, are not influenced by leaf herbivory (**manuscript II**), which is consistent with the results of other studies conducted with black poplar (Boeckler *et al*. 2013; **manuscript III**). Of the four salicinoids monitored, only homaloside D was affected by the applied treatments. It significantly increased only after combined BG and AG attack (**manuscript II**). Additionally, upon root herbivory alone there is a non-significant trend of increased foliar levels of salicin, homaloside D and 6'-O-benzoylsalicortin, a salicinoid which is first described in **manuscript II.** Other groups of phenolic defense compounds have been reported to be inducible in AG tissues through BG herbivory. Coumaroylquinic acids, for example, increase in leaves of *Brassica* plants under root attack (Jansen *et al*. 2008) and maize plants induce chlorogenic acid in leaves upon root feeding (Erb *et al*. 2009[a] & 2009[b]). The induction of AG phenolic defenses upon root herbivory, therefore, might be a general defense strategy that occurs across species. However, future studies are needed to provide more evidence on this hypothesis.

Taken together, this means that BG herbivory only partially influenced AG direct defenses of black poplar. The levels of PIs increased significantly in the leaves of BG-damaged trees and there is a trend of higher salicinoid levels as well (**manuscript II**). These increased levels of direct defenses might indicate that the leaves of root-damaged poplars are better defended than those of undamaged trees. Interestingly, in contrast to that assumption, we observed that *L. dispar* caterpillars showed a preference for leaves of root-damaged plants over leaves of control plants (**manuscript II**). Additionally, caterpillars fed on less leaf area during combined BG and AG attack compared to single AG attack (**manuscript II supplement**).

As the feeding preference of the caterpillars could not be fully explained by the levels of defense compounds, we further analyzed the leaf material for potential changes in nutritional quality. We observed a non-significant increase of tri-, tetra- and pentasaccharide concentrations and a significant increase of the amino acid proline after BG herbivory (**manuscript II**). Proline has already been reported to have phagostimulatory properties (Behmer & Joern 1994; Meyer *et al*. 2006; Ximénez-Embún *et al*. 2016). Therefore, an additional food choice assay was conducted with leaf discs that were supplemented with proline or alanine, leucine or tryptophan. Indeed *L. dispar* caterpillars preferred to feed on leaf discs with a proline supplement over control discs (**manuscript II**), while none of the other tested amino acids influenced food choice.

Due to the loss of root tissue, BG herbivory is often associated with water stress (Blossey & Hunt-Joshi 2003; Erb *et al*. 2011). A well-known indicator for drought stress in leaves is the increase of ABA (Seki *et al*. 2007; Nguyen *et al*. 2016) and proline among other free sugars

and amino acids (Masters *et al*. 1993; Yamada *et al*. 2005; reviewed by Kavi Kishor & Sreenivasulu 2014). Plants are known to mobilize free sugars and amino acids from proteins and carbohydrates as osmotic adjustments to cope with drought stress (Rosa *et al*. 2009; Hummel *et al*. 2010; Bowne *et al*. 2012). Unfortunately for the plant, this strategy increases the available nitrogen for leaf feeding insects, which ultimately leads to a higher performance for shoot herbivores (Brown & Gange 1990; Poveda *et al*. 2003). The increase of proline in combination with enhanced levels of ABA upon BG feeding in our experiment, indicate that the trees might have suffered from water stress due to root damage. We therefore conclude that BG herbivory in poplar can lead to water stress, which will result in the accumulation of ABA and proline in the leaves and ultimately leads to an increased preference of gypsy moth caterpillars for BG infested trees. Whether or not this will also result in a fitness benefit for the caterpillars should be tested in future studies.

6.3. Caterpillars perform worse on primed black poplar leaves

In addition to internal plant signaling *via* vascularly transported phytohormones, plants can use airborne signals to communicate from one part of the plant to another or even to neighboring plants. One aspect of airborne communication that has gained increasing attention in recent years is volatile-mediated defense priming. When plant parts or a neighboring plant perceive herbivore-induced plant volatiles (HIPV) emitted from plant parts under herbivore attack, they can prime their defenses for a possible future attack (Li *et al*. 2012). During such a future attack, primed plant tissues will then show a faster or greater defense response compared to a non-primed plant. However, how plants perceive volatiles and what signals a primed state is largely unknown. It has been shown that volatile defense priming can increase a plant´s resistance to herbivores for many herbaceous and woody plant species (reviewed in Karban *et al*. 2014), including poplar (Frost *et al*. 2007 & 2008; Li *et al*. 2012). Yet only a few studies have tried to investigate the influence of plant priming on the feeding herbivore (Hughes *et al*. 2015; Morrell & Kessler 2017) and so far there is no clear evidence that priming causes a more detrimental response for insect herbivores than regular induced plant defenses.

In **manuscript III** we investigated whether the exposure of black poplar saplings to HIPVs from adjacent saplings leads to an induction or priming of defenses and how this influences herbivore behavior and performance. To gain insight into the regulatory mechanisms of induction and priming, we analyzed defense related hormones in herbivore-treated and HIPV-exposed poplar leaves. As expected, herbivory alone induced the levels of JA and its

conjugates (**manuscript III supplement**; **manuscript II**) as well as SA and ABA concentrations (**manuscript III supplement**). The induction of ABA might be expected considering its role as a co-regulator for JA-dependent defenses (Vos *et al*. 2013). Even though SA is mostly connected with defense against biotrophic pathogens or phloem-sucking insects (Dempsey *et al*. 1999; Moran & Thompson 2001), it has been reported to increase after the attack of leaf chewing insects as well (Bi *et al*. 1997). However, we did not find an induction of SA or ABA upon leaf herbivory in **manuscript II**, most likely due to the fact that different *Populus nigra* genotypes were used in the two experiments. Poplars of the same species are known to differ greatly in their leaf chemistry based on natural genotypic variations (Donaldson & Lindroth 2007; Rubert Nason *et al*. 2015) and our results indicate that even regulatory mechanisms, i.e. defense hormone signaling, might be subject to such intraspecific variation. The exposure to HIPVs on the other hand, had no significant effect on any of the measured plant hormones (**manuscript III**). Thus it can be concluded that neither SA, ABA nor JA are responsible for the regulation of the priming stimulus in black poplar.

Most studies investigating defense priming report an upregulation of defense-related genes upon exposure to HIPVs and /or following herbivore attack (Tscharntke *et al*. 2001; Frost *et al*. 2008; Yi *et al*. 2009; Li *et al*. 2012; Ameye *et al*. 2015). We therefore searched for differences in the transcriptome of HIPV-exposed leaves compared to control leaves as transcriptional changes upon exposure might function as a memory of the initial priming stimulus (Hilker & Schmülling 2019). We did not find any significant differential expression of annotated sequences in HIPV-exposed leaves (**manuscript III supplement**). However, there are other possible mechanisms for storing the perception of the primary stimulus apart from transcriptional modifications, for example changes in the concentration of cytosolic calcium, tricarboxylic acids, reactive oxygen species, membrane depolarization, hormone conjugates, amino acids, sugars or chromatin and post-transcriptional modifications (van den Burg & Takken 2009; Conrath 2011; Mauch-Mani *et al*., 2017).

Since priming has been reported to mainly affect the inducibility of defenses, we analyzed known defense compounds of black poplar in HIPV-exposed leaves. For example PIs, which inhibit the digestive function of the herbivore´s gut, increase significantly in black poplar leaves upon herbivory (**manuscript II & III supplement**), and have been previously reported to be affected by volatile priming (Tscharntke *et al*. 2001; Farag *et al*. 2005; Kessler *et al*. 2006). However, there was no significant effect of HIPV exposure on PI concentration in black poplar (**manuscript III supplemental**). Additionally, we measured the semi-volatile, nitrogenous compound phenylacetaldoxime, which is known to increase in black poplar leaves after herbivore feeding (Irmisch *et al*. 2013). Phenylacetaldoxime is a precursor of the toxic compound benzyl cyanide and has been reported to reduce larval performance of gypsy moths (Irmisch *et al*. 2013; Irmisch *et al*. 2014). Similar to PIs, we found increased levels of

phenylacetaldoxime in leaves attacked by herbivores, but HIPV exposure did not affect the concentration of this aldoxime, either alone or when followed by an herbivore attack (**manuscript III supplement**).

Although HIPV exposure to black poplar leaves had no effect on defense hormones, the transcriptome or the levels of inducible defense compounds, gypsy moth caterpillars were strongly affected by the exposed leaves. When given a choice caterpillars avoided feeding on leaf discs that were previously exposed to HIPVs (**manuscript III**). Furthermore, when forced to feed on exposed leaves, caterpillars showed higher mortality and lower performance compared to caterpillars that were reared on control leaves (**manuscript III**). We therefore measured the total protein content of the leaves and the concentrations of free sugars to determine whether the nutritional value of the leaves was altered by HIPV exposure. Free sugars were also measured since they can have signaling functions and may transduce the priming stimulus (Mauch-Mani *et al*. 2017). Yet none of the treatments had an effect on the total protein content (**manuscript III supplement**), consistent with the findings of **manuscript II** (**supplement**), nor on the levels of free sugars (**manuscript III supplement**).

There is no universal pattern regarding herbivore inducibility of salicinoids, a group of poplar phenolic defense compounds (reviewed in Boeckler *et al*. 2011). Consistent with my previous findings, there was no induction of total salicinoids due to leaf feeding (**manuscript III; manuscript II**). Yet there was a minor though significant increase of the salicinoid salicin in leaves that were exposed to HIPVs and subsequently attacked by *L. dispar* caterpillars (**manuscript III**). Furthermore, in the caterpillar performance experiment, HIPV exposure and herbivory led to a significant increase in salicin and salicortin (two main components of the salicinoid pool) even at four to nine days post volatile exposure (**manuscript III**). This indicates that there is volatile defense priming in black poplar for salicinoids.

There are multiple reports that salicinoids are toxic to *L. dispar* (reviewed in Boeckler et al 2011). Furthermore it has been shown that the addition of salicin and salicortin to artificial diets leads to slower growth and pupation rates of *L. dispar* larvae (Hemming & Lindroth, 1995; Kelly & Curry, 1991; Orians et al., 1997). Of herbivore defensive traits measured in black polar leaves, salicin and salicortin were the only compounds that responded to the volatile signal. However, the increase is rather small compared to the high constitutive levels at which these compounds occur naturally. It is therefore questionable whether this salicinoid increase can serve as an explanation for the observed caterpillar performance and behavior. A statistical evaluation indicates that salicin significantly affects the weight gain of the larvae monitored in the performance assay (**manuscript III**). Additionally, a performance experiment conducted in a common garden with black poplar trees that vary naturally in levels of salicinoids revealed a significant negative correlation between increasing salicortin levels and caterpillar weight (**manuscript III**). Taken together, these results do indicate that the increase

of salicinoids in black poplar leaves can reduce the performance of feeding *L. dispar* caterpillars. Therefore, volatile-mediated defense priming can be considered an important mode of inter-plant communication (and perhaps between parts of the same plant as well) resulting in increased tree resistance to a subsequent herbivore attack.

6.4. Conclusion and Outlook

The results of my thesis shed light on the complex interactions and modes of intra- and inter-plant communication in trees. To my knowledge, this is the first study to investigate belowground-aboveground interactions in a woody plant species. I showed that root herbivory induces the emission of monoterpenes from the roots and that these compounds can act as a defense against subsequently attacking microbial pathogens. Furthermore, belowground feeding causes water stress-like symptoms in aboveground tissues, notably through the accumulation of drought stress-related signal molecules in the leaves. This result indicates that, like herbaceous species, woody plants rely on vascular intra-plant communication *via* phytohormones from roots to shoots. Moreover, I showed that leaf-feeding herbivores prefer plants that have suffered root herbivory, most likely due to the osmotic changes leaves undergo as a response to water stress. This implies that belowground attackers can influence aboveground feeders *via* phytochemical changes in their common host. Seen from an ecological point of view, this means that trees can connect spatially distant food webs, such as soil-dwelling root-feeders, decomposers and root-colonizing microbes, on the one side, and aboveground leaf-feeders and their parasitoids and predators on the other side.

Additionally, I confirmed that there is aboveground volatile-mediated defense priming between tree saplings of the same genotype, a form of inter-plant communication that presumably also occurs within branches of the same tree. Exposure to herbivore-induced volatiles had strong detrimental effects on the behavior and performance of a generalist herbivore feeding on the exposed leaves. My thesis is one of the first studies to show that plant volatile defense priming has severe consequences for herbivores. Thus, this kind of plant-plant communication should be considered in future studies investigating plant-herbivore interactions. In mature trees, where vascular connections between branches are distant, intra-plant priming might have greater ecological relevance than inter-plant priming.

Many aspects of tree communication related to herbivory remain unknown. For example, root volatiles such as monoterpenes might not only inhibit pathogens but also influence mutualistic mycorrhizal fungi. Additionally, it would be interesting to investigate whether the chemistry of root tissues changes upon root herbivory. I investigated the effects of belowground herbivory

on aboveground tissues, which seem very likely to be affected as most vascular transport and signaling is believed to be directed upward. However, it has been argued that aboveground herbivory can reciprocally influence belowground plant defenses and the performance of root feeders as well (reviewed in Erb *et al*. 2008). More research clearly needs to be carried out with respect to belowground-aboveground interactions, especially in trees. Their size and lifespan enables trees to establish many more biotic interactions both below- and aboveground compared to herbaceous species. In addition, although our knowledge of volatile-mediated defense priming is substantial, very little is known about the perception of volatiles and the downstream signaling events.

There are also other directions for future research on within- and between-tree communication triggered by herbivory. While I performed experiments with young trees in controlled greenhouse and laboratory conditions, these can never completely mimic the complexity of a natural system. All findings need to be validated under field conditions with naturally growing young and mature trees, which might react differently to biotic stresses than a young tree growing in a greenhouse. Furthermore, there is a huge imbalance between the abundance of literature investigating plant-herbivore and plant-plant interactions in herbaceous, especially crop species, compared to trees and other perennial woody species. Woody species structure many ecosystems, with forests in particular containing 80 % of our planet´s plant biomass. Our knowledge about interactions in these systems is limited and needs to be expanded.

7. SUMMARY

Plants have evolved a wide arsenal of defenses against the manifold herbivore species that feed on both belowground as well as aboveground tissues. In the process, they utilize internal communication channels to signal the presence of an herbivore to distal tissues and consequently trigger the induction of defenses. These signals can travel through the vascular system of the plant e.g. from roots to shoots. However, many plants also rely on airborne signals for communication. Neighboring plants, even from another species, can eavesdrop on these signals. For both belowground – aboveground interactions and volatile mediated signaling, research has focused mainly on herbaceous species, and little is known about these phenomena in trees.

In my thesis I studied the volatile emissions of black poplar (*Populus nigra*) roots that were damaged by larvae of the common maybug (*Melolontha melolontha*). Furthermore, I investigated how belowground herbivory by this insect influences defenses against a generalist herbivore, the gypsy moth caterpillar (*Lymantria dispar*). Additionally, I examined whether exposure to herbivore-induced plant volatiles changes *P. nigra´*s leaf chemistry and how this might affect feeding gypsy moth caterpillars.

Root herbivory induced the emission of monoterpenes from roots. These compounds are biosynthesized *de novo* in root tissue by terpene synthases similar to the biosynthesis of monoterpenes aboveground. The compounds 1,8-cineole and β-pinene, which increased upon root herbivory, showed antimicrobial activity against an oomycete, *Phytophthora cactorum* that causes root rots in many fruit crop species. Furthermore, belowground herbivory caused water stress-like symptoms in the leaves resulting in the increase of the phytohormone abscisic acid and the amino acid proline. *L. dispar* caterpillars showed a strong preference for leaves influenced by root herbivory, which is most likely due to the fact that proline acts as a feeding stimulant.

Upon leaf herbivory by *L. dispar*, black poplar leaves alter their volatile bouquet both quantitatively and qualitatively. If another poplar tree perceives these herbivore-induced volatile emissions no measurable phenotypic changes occur. However, upon subsequent herbivore attack the salicinoid compounds increase significantly. Given a choice, *L. dispar* caterpillars avoid feeding on volatile-exposed leaves and, if forced to, show a higher mortality and lower performance compared to feeding on plants not exposed to HIPVs. This strongly suggests that black poplar is capable of inter- and intra-plant volatile-mediated defense priming, and that priming leads to increased plant resistance.

7. Summary

This thesis is the first study to investigate belowground-aboveground interactions in a woody plant species (Figure 1; left). It was shown that root damage can influence the behavior and putatively the performance of aboveground herbivores. Therefore, trees can mediate a connection between otherwise distant food webs. This highlights the need for more research in the field of belowground-aboveground interactions, especially in woody plants, to gain a more comprehensive understanding of ecosystems. Furthermore, this thesis confirmed the existence of volatile defense priming in the genus *Populus* (Figure 1; right). Defenses in primed leaves of black poplar saplings had more severe consequences for feeding herbivores than defenses in unprimed leaves. Yet further work on volatile priming is necessary as the mechanism of priming still remains unknown. In mature trees, volatile signaling might even be of greater ecological importance than in saplings as airborne signals can overcome the vascular constrains in a large tree crown. Further study of volatile-mediated signaling will undoubtedly help to understand more about the complex responses of trees to the many organisms associated with them.

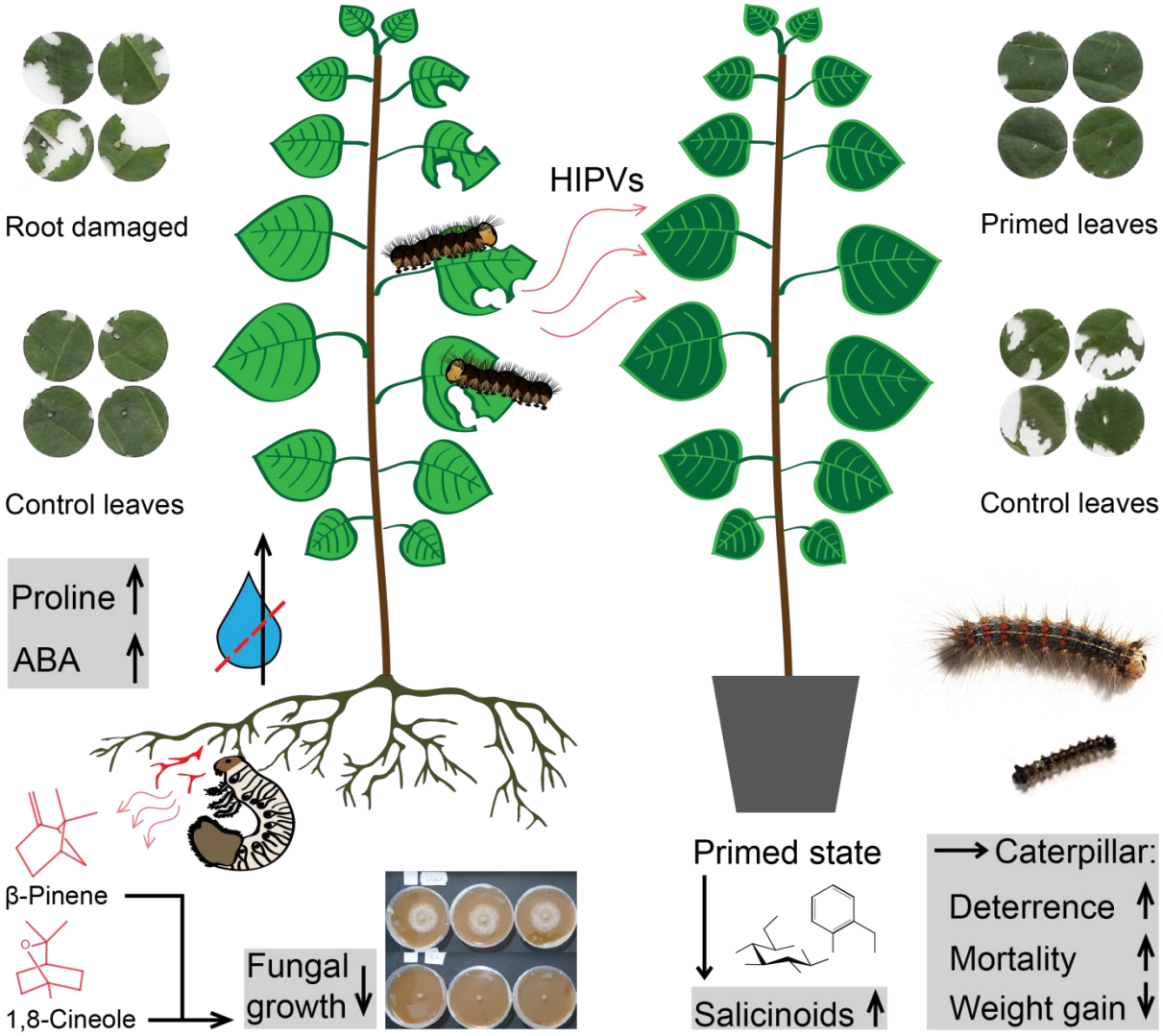

Figure 1. Graphical summary for the consequences of intra- (left) and inter-plant (right) signaling in black poplar (*Populus nigra*) on the pathogenic oomycete *Phytophtora cactorum* and the leaf herbivore *Lymantria dispar*. Root herbivory by *Melolontha melolontha* grubs induced root monoterpene emission. Two of these monoterpenes, β-pinene and 1,8-cineole, inhibited the growth of *P. cactorum*. Moreover, the intra-plant signal molecules proline and abscisic acid (ABA) increased in aboveground tissue of root-damaged trees, which strongly indicates that, the trees suffered from water stress (left center). Furthermore, *L. dispar* caterpillars showed a strong preference for leaves influenced by root herbivory, which might be due to the phagostimulatory properties of proline (upper left). The inter-plant communication *via* herbivore-induced plant volatiles (HIPVs) primed the salicinoid levels of the receiving plant (bottom right). Caterpillars avoided to feed on primed leaves (top right) and showed higher mortality and lower weight gain, when being forced to feed on primed leaves (right).

8. ZUSAMMENFASSUNG

Pflanzen haben im Laufe der Evolution ein großes Arsenal an Verteidigungsmechanismen entwickelt, um sich gegen Fressfeinde zu wehren, die sich von unterirdischen wie überirdischen Pflanzenorganen ernähren. Dabei nutzen sie Signale über interne Kommunikationswege vom befressenen (lokalen) zum unbefressenen (systemischen) Gewebe, um ganzheitlich die Verteidigung zu induzieren. Solche Signale können durch das gesamte vaskuläre System transportiert werden, zum Beispiel von der Wurzel bis in die Blätter. Zusätzlich nutzen viele Pflanzen auch volatile, durch die Luft übertragene Signale, sogenannte Pflanzenduftstoffe. Benachbarte und selbst artfremde Pflanzen können diese volatilen Signale „belauschen“ und daraufhin ihre Verteidigungen in Alarmbereitschaft versetzten, das sogenannte „Priming“, um im Falle eines Befalls durch Herbivoren besser reagieren zu können. Die meisten wissenschaftlichen Studien, die sich mit unter- und oberirdischen Interaktionen oder der Kommunikation mittels Duftstoffe beschäftigen, werden an krautigen Pflanzenarten durchgeführt. Daher ist über diese beiden Kommunikationswege bei Bäumen bisher nur wenig bekannt.

In meiner Dissertation habe ich die Duftstoffemissionen der Wurzeln von Schwarzpappeln (*Populus nigra*) untersucht, welche von Maikäferlarven (*Melolontha melolontha*) befressen wurden. Des Weiteren habe ich den Einfluss solcher Wurzelherbivorie auf die oberirdischen Verteidigungsmechanismen der Schwarzpappel erforscht, die sich gegen die Raupen des Schwammspinners (*Lymantria dispar*) richten. Darüber hinaus, habe ich die Phytochemie von Schwarzpappeln untersucht, die fraß-induzierten Pflanzenduftstoffen ausgesetzt waren und inwieweit Schwammspinner-Raupen durch diese Behandlung ihrer Wirtspflanzen beeinflusst werden.

Wurzelherbivorie induzierte die unterirdische Emission von Monoterpenen. Diese Stoffe werden *de novo* im Wurzelgewebe von Terpensynthasen gebildet, ähnlich zur überirdischen Monoterpenbiosynthese. Die Stoffe 1,8-Cineol und β-Pinen, deren Emission durch Wurzelherbivorie anstieg, zeigten antimikrobielle Wirkung gegen den Oomycet, *Phytophthora cactorum*, der in vielen Obstkulturen Wurzelfäule verursacht. Weiterhin führte Wurzelherbivorie zu Veränderungen in den Blättern, die Symptomen von Trockenstress ähnlich waren. Diese äußerten sich in erhöhten Konzentrationen des Phytohormons Abscisinsäure und der Aminosäure Prolin. Die Schwammspinner-Raupen präferierten diese von Wurzelherbivorie beeinflussten Blätter, was zumindest anteilig, auf die fraßstimulierende Wirkung von Prolin zurückzuführen ist.

Sobald Schwammspinner-Raupen an den Blättern der Schwarzpappel fressen, ändern diese ihre Duftstoffemissionen sowohl qualitativ als auch quantitativ. Ist eine benachbarte Schwarzpappel diesen Duftstoffen ausgesetzt ist, treten zunächst keine phänotypisch messbaren Veränderungen auf. Bei einem nachfolgenden Befall durch Herbivoren, steigen jedoch die Konzentrationen der Salicinoide signifikant an. In Wahlfraßexperimenten vermeiden es die Schwammspinner-Raupen, an Blättern zu fressen, die den Pflanzenduftstoffen ausgesetzt waren. Darüber hinaus weisen Raupen, die an Duftstoff-exponierten Blättern fressen, eine erhöhte Mortalität und eine verlangsamte Entwicklung auf, verglichen mit Raupen, die an nicht-exponierten Blättern gefressen haben. Diese Ergebnisse weisen darauf hin, dass Schwarzpappeln in der Lage sind ihre Verteidigungen mit Hilfe von Duftstoff-vermittelter Signalübertragung zu „primen“. Dies führt letztlich, zu einer erhöhten Resistenz der Pflanze gegenüber Schädlingen.

Diese Dissertation ist die erste Studie, welche die Interaktion von unter- und oberirdischer Herbivorie in Gehölzpflanzen untersucht (Abbildung 1; links). Aus den Ergebnissen geht hervor, dass Wurzelherbivorie das Verhalten und möglicherweise auch die Fitness von blattfressenden Insekten beeinflussen kann. Dadurch wird der Baum zu einem Vermittler zwischen ober- und unterirdischen Nahrungsnetzen, die räumlich voneinander getrennt sind und andernfalls kaum miteinander interagieren können. Die Erforschung solcher komplexen Zusammenhänge ist unumgänglich, um ein vollständiges Verständnis über natürliche Ökosysteme zu erhalten. Des Weiteren bestätigte diese Dissertation, dass duftstoffvermitteltes „Priming“ von Pflanzenverteidigungen im Genus *Populus* existiert (Abbildung 1; rechts). Es wurde gezeigt, dass „geprimte“ Verteidigungen schwerwiegendere Konsequenzen für Herbivoren haben als nicht-„geprimte“ Verteidigungen. Es ist essentiell, dass duftstoffvermitteltes „Priming“ und dessen Konsequenzen für Antagonisten der Pflanzen ausführlicher untersucht werden, da der zugrundeliegende molekulare Mechanismus weiterhin nicht bekannt ist. Duftstoffvermittelte Kommunikation könnte in ausgewachsenen Bäumen eine größere ökologische Bedeutsamkeit haben, da Signale, die über die Luft übertragen werden, Grenzen des vaskulären Systems innerhalb einer Baumkrone überwinden können. Die Erforschung von duftstoffvermitteltem „Priming“ wird zweifelsohne hilfreich sein, um die Signalübertragung innerhalb eines Baumes, sowie die komplexen Interaktionen zwischen Bäumen und ihren vielen assoziierten Organismen, zu verstehen.

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11. EIGENSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät 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 in der Auflistung der Manuskripte (Kapitel 2, *Overview of Manuscripts*) 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.

Sandra Lackner Jena, den 24.06.2019

12. Curriculum Vitae

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Work experience

May 2015 – present	PhD thesis. Max Planck Institute (MPI) for Chemical Ecology, Department of Biochemistry (Prof. J. Gershenzon), Jena; Friedrich-Schiller Universität, Institut für Biodiversität (Nicole van Dam), Jena. "Consequences of intra- and inter-plant communication for tree-herbivore interactions."
Jan 2014 – Apr 2015	Master thesis. MPI for Chemical Ecology, Department of Biochemistry (Dr. S.B. Unsicker), Jena. "Volatile mediated communication in black poplar trees."
Jun – Sep 2012	Bachelor thesis. MPI for Chemical Ecology, Department of Biochemistry (Dr. S.B. Unsicker), Jena. " Herbivor-induzierte Emission von Duftstoffen bei der Schwarzpappel (*Populus nigra*) unter Freilandbedingungen "

Education

May 2015 – present	Doctorate. Member of IMPRS graduate school.
Oct 2012 – Apr 2015	M.Sc. in Evolution, Ecology and Systematics (1.5). Friedrich-Schiller Universität, Jena.
Oct 2009 – Sep 2012	B.Sc. in Biology (2.3). Friedrich-Schiller Universität, Jena.
Aug 2001 – Jul 2009	Abitur (2.4). Goethe Gymnasium, Auerbach (Vogtland).

List of Publications

Lackus, N., Lackner, S., Gershenzon, J., Unsicker, S., Köllner, T. G. (2018). The occurrence and formation of monoterpenes in herbivore-damaged poplar roots. Scientific Reports, 8: 17936. doi:10.1038/s41598-018-36302-6.

List of Conference contributions

Oral presentations

Lackner S. (2018). Belowground herbivory increases palatability of black poplar leaves. Talk presented at 10th Scandinavian Plant Physiology Society PhD Student Conference, Copenhagen, DK

Lackner S. (2017). How belowground herbivory influences aboveground defense mechanisms of black poplar. Talk presented at 16th IMPRS Symposium, International Max Planck Research School, Dornburg, DE

Lackner S. (2016). Can herbivore-induced plant volatiles prime defenses in Black poplar. Talk presented at Plant Volatiles: Diversity of Targets, Effects and Applications of Plant Volatiles, Gordon Research Conferences, Ventura, CA, US

Poster presentations

Lackus N., Lackner S., Gershenzon J., Unsicker S., Köllner T. (2018). The odour of roots: Biochemical basis of terpene biosynthesis in poplar roots. Poster presented at Gordon Research Conference - Plant Volatiles: The Role of Plant Volatiles in Communication, Barga, IT

Lackner S., Lackus N., Köllner T., Unsicker S. (2018). Below- and aboveground defenses of black poplar. Poster presented at 34th ISCE Meeting, International Society of Chemical Ecology, Budapest, HU

Lackner S., Unsicker S. (2018). Belowground defenses in black poplar. Poster presented at 17th IMPRS Symposium, International Max Planck Research School, Dornburg, DE

Lackus N., Lackner S., Gershenzon J., Unsicker S., Köllner T. (2018). The odour of roots: Biochemical basis of terpene biosynthesis in poplar roots. Poster presented at 17th IMPRS Symposium, International Max Planck Research School, Dornburg, DE

Eberl F., Lackner S., Fabisch T., Unsicker S., Gershenzon J. (2016). Pop(u)lar Science. Poster presented at SAB Meeting 2016, MPI for Chemical Ecology, Jena, DE

Lackner S., Gershenzon J., Unsicker S. (2016). Can herbivore-induced plant volatiles prime defenses in black poplar? Poster presented at 15th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

Lackner S., Gershenzon J., Unsicker S. (2016). Can herbivore-induced plant volatiles prime defenses in black poplar? Poster presented at Plant Volatiles: Diversity of Targets, Effects and Applications of Plant Volatiles, Gordon Research Conferences, Ventura, CA, US

Eberl F., Lackner S., Fabisch T., Gershenzon J., Unsicker S. (2015). Pop(u)lar Science. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE

Lackner S., Wright L., Gershenzon J., Unsicker S. (2014). Volatile mediated communication in black poplar trees. Poster presented at GFÖ 44th Annual Conference, Hildesheim, DE

Awards

Sep 2018	Best talk on 10th Scandinavian Plant Physiology Society PhD Student Conference
Mar 2016	IMPRS travel award for best poster at the 17th IMPRS Symposium

Scientific activities

Jul – Oct 2018	Supervision of master student for internship.
Nov 2017	Demonstration at “Long Night of Science”.
Jul – Aug 2016	Supervision of master student for internship.
Oct 2015	Supervision of bachelor student for internship.

Language Skills

German (mother tongue), English (fluent)

12. Curriculum Vitae

13. Supplementary Data

13.1. Manuscript I – Supplementary data

Supplementary material:

The occurrence and formation of monoterpenes in herbivore-damaged poplar roots

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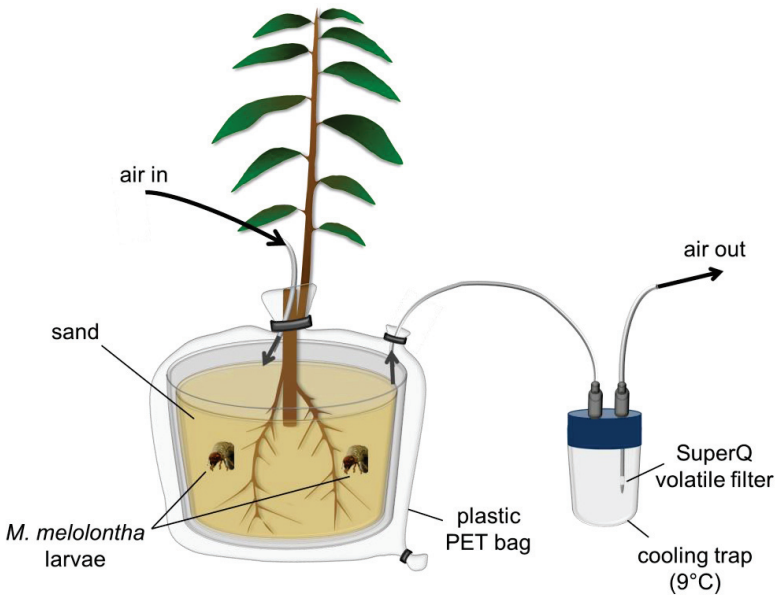
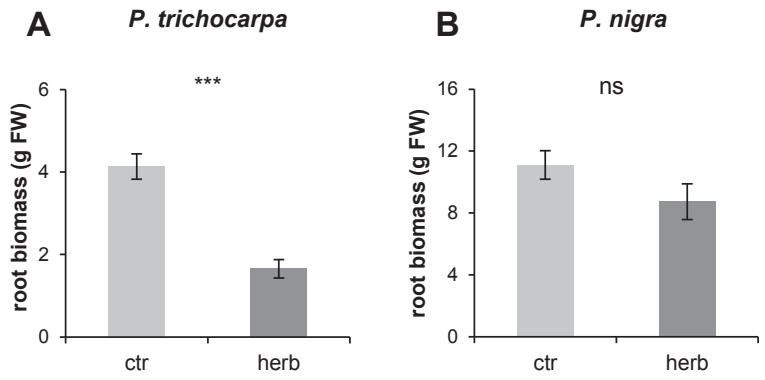
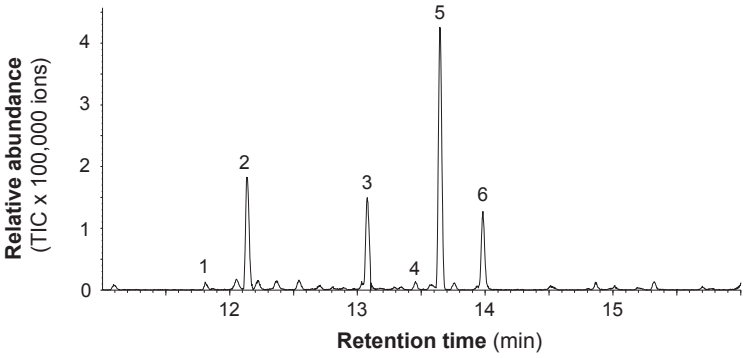


Supplementary Figure S1: Root volatile collection system. Volatiles were sampled for 68 h with a dynamic push-pull system and a trap packed with Poropak adsorbent. Trees were grown in sand and the pots were bagged in PET foil for volatile collection.



Supplementary Figure S2: Effect of herbivory on root mass of *Populus trichocarpa* (A) *and P. nigra* (B) trees. Root masses are displayed for undamaged roots (ctr) and roots damaged by cockchafer (*Melolontha melolontha*) larvae (herb) and shown as means ± SE in g (n = 8). Asterisks indicate statistical significance in Student's t-tests. *P. trichocarpa* ($P \leq 0.001$, t = 6.973); *P. nigra* ($P = 0.108$, t = 1.717).

PnTPS4



Supplementary Figure S3: Sesquiterpene synthase activity of PnTPS4. The gene was heterologously expressed in *E. coli* and partially purified protein was incubated with (*E,E*)-FPP as substrate. Enzyme products were analyzed using GC-MS. 1, (*E*)-α-bergamotene; 2, (*E*)-β-farnesene; 3, (*E,E*)-α-farnesene; 4, sesquiphellandrene; 5, (*Z*)-α-bisabolene; 6, nerolidol. Compounds were tentatively identified by database comparisons.

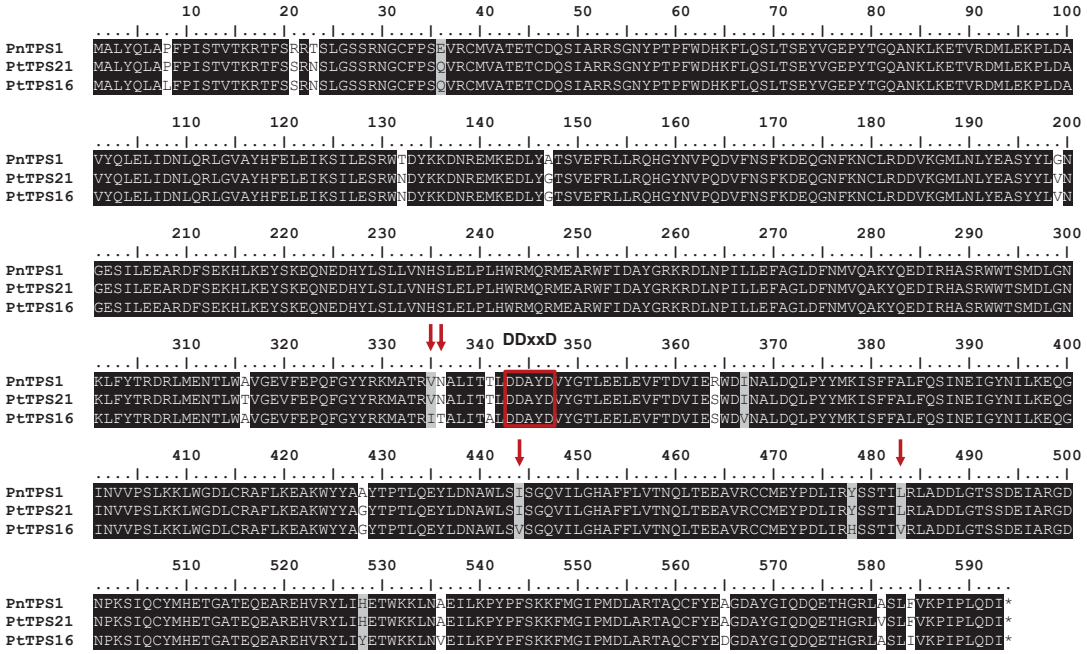
A

```
### chlorop v1.1 prediction results ##########################
Number of query sequences: 3

Name                    Length     Score    cTP    CS-      cTP-
                                                  score    length
-----------------------------------------------------------------
PnTPS4_full_length       589       0.568    Yes    11.435      40
PtTPS16_full_length      593       0.569    Yes    11.435      38
PtTPS21_full_length      593       0.570    Yes    11.435      38
-----------------------------------------------------------------
```

B

```
### targetp v1.1 prediction results ##################################
Number of query sequences:  3
Cleavage site predictions not included.
Using PLANT networks.

Name                   Len     cTP    mTP     SP  other  Loc  RC
-------------------------------------------------------------------------
PnTPS4_full_length     589   0.913  0.108  0.083  0.040   C    1
PtTPS16_full_length    593   0.826  0.486  0.029  0.016   C    4
PtTPS21_full_length    593   0.894  0.352  0.028  0.025   C    3
-------------------------------------------------------------------------
cutoff                       0.000  0.000  0.000  0.000
```

Supplementary Figure S4: Signal peptide prediction of PnTPS4, PtTPS16, and PtTPS21. Signal peptide (cTP) prediction was done using the web-based prediction programs chlorop v1.1 (http://www.cbs.dtu.dk/services/ChloroP/) (A) and targetp v1.1 (http://www.cbs.dtu.dk/services/TargetP/) (B).



Supplementary Figure S5: Amino acid sequence comparison of PtTPS16 and PtTPS21 from *Populus trichocarpa* and PnTPS1 from *P. nigra*. Amino acids with identical side chains are marked by black boxes and amino acids with similar side chains are marked by gray boxes. Red arrows highlight different amino acid residues in the active site. The conserved DDxxD motif is marked in red.

Supplementary Table S1. Emission of aromatic volatile compounds from undamaged (ctr) and *Melolontha melolontha*-damaged (herb) roots of *Populus trichocarpa* and *P. nigra.* Volatiles were analyzed using GC-MS/FID and emission levels are displayed as means ± SE in pg g^{-1} h^{-1} fresh weight (n = 8). *P*-values are based on the results from Student's t-tests or from Mann-Whitney Rank Sum Tests between control and herbivore treatments.

	P. trichocarpa				***P. nigra***			
	ctr	**herb**	***P*-value**	**t-value**	**ctr**	**herb**	***P*-value**	**t-value/ *T-value***
benzaldehyde	123 ± 44	297 ± 98	0.063	-2.023	69 ± 15	75 ± 34	0.442	*76.00*
benzyl alcohol	244 ± 63	269 ± 61	0.765	-0.305	284 ± 68	364 ± 131	0.931	0.0887
salicylaldehyde	749 ± 401	1403 ± 631	0.236	-1.239	1467 ± 520	1002 ± 324	0.376	0.914

Supplementary Table S2. Accumulation of aromatic volatile compounds and camphene in undamaged (ctr) and *Melolontha melolontha*-damaged (herb) roots of *Populus trichocarpa* and *P. nigra.* Root material was extracted with hexane and analyzed using GC-MS/FID. Accumulation levels are displayed as means ± SE in µg g^{-1} fresh weight (n = 8). *P*-values are based on the results from Student's t-tests or from Mann-Whitney Rank Sum Tests between control and herbivore treatments.

	P. trichocarpa				***P. nigra***			
	ctr	**herb**	***P*-value**	**t-value/ *T-value***	**ctr**	**herb**	***P*-value**	**t-value/ T-value**
camphene	NA	NA	NA	NA	0.0 ± 0.0	0.3 ± 0.1	0.002	*40.00*
benzaldehyde	7.9 ± 4.4	48.9 ± 6.6	≤0.001	*37.00*	0.1 ± 0.05	0.2 ± 0.03	0.373	-0.920
benzyl alcohol	11.9 ± 1.4	1.9 ± 1.9	≤0.001	5.298	NA	NA	NA	NA
salicylaldehyde	550.1 ± 135.4	1948.6 ± 209.5	≤0.001	-7.408	33.4 ± 4.3	73.4 ± 8.9	≤0.001	-4.538

Supplementary Table S3: Oligonucleotides used for isolation, qRT-PCR analysis and site-directed mutagenesis of *TPS* genes.

Name	Sequence	Usage
PtTPS16-PtTPS21-fwd	CACCATGGTAGCGACCGAAACTTG	cloning of *PtTPS16/21*
PtTPS16-rev	CTATATATCTTGGAGAGGAATGG	cloning of *PtTPS16*
PtTPS21-rev	GATTTATCAAGAAAACTTAAC	cloning of *PtTPS21*
PnTPS4-fwd	ATGGTAGGTCTCAGCGCGTTGCCACCGAAGCTGCTGGT	cloning of *PnTPS4*
PnTPS4-rev	ATGGTAGGTCTCATATCATAAAGGCTTAATAAGTAAGGATTTC AC	cloning of *PnTPS4*
PnTPS1-PtTPS21-qRT-PCR-fwd	TATCGAAAAATGGCGACCAGGG	qRT-PCR
PnTPS1-PtTPS21-qRT-PCR-rev	GCAGTTGGTCCAATGCATTGAT	qRT-PCR
PtTPS16-qRT-PCR-fwd	TATCGAAAAATGGCGACCAGGA	qRT-PCR
PtTPS16-qRT-PCR-rev	GCAGTTGGTCCAATGCATTGAC	qRT-PCR
PnTPS4-qRT-PCR-fwd	CATATACAGCGCGAATCGAAAAGT	qRT-PCR
PnTPS4-qRT-PCR-rev	AGATAACCCAAGTCTTTGCAAGGC	qRT-PCR
PtTPS16-I335V-fwd	ATGGCGACCAGGGTTACTGCTCTAATAACAGCATTAGAT	site-directed mutagenesis
PtTPS16-I335V-rev	ATCTAATGCTGTTATTAGAGCAGTAACCCTGGTCGCCAT	site-directed mutagenesis
PtTPS16-T336N-fwd	ATGGCGACCAGGATTAATGCTCTAATAACAGCATTAGAT	site-directed mutagenesis
PtTPS16-T336N-rev	ATCTAATGCTGTTATTAGAGCATTAATCCTGGTCGCCAT	site-directed mutagenesis
PtTPS16-V444I-fwd	GATAATGCTTGGTTGTCAATTTCCGGACAAGTCATACTA	site-directed mutagenesis
PtTPS16-V444I-rev	TAGTATGACTTGTCCGGAAATTGACAACCAAGCATTATC	site-directed mutagenesis
PtTPS16-V483L-fwd	CGTCACTCGTCAACGATTTTGCGACTCGCAGATGACCTA	site-directed mutagenesis
PtTPS16-V483L-rev	TAGGTCATCTGCGAGTCGCAAAATCGTTGACGAGTGACG	site-directed mutagenesis
PtTPS16-I335V-T336N-fwd	ATGGCGACCAGGGTTAATGCTCTAATAACAGCATTAGAT	site-directed mutagenesis
PtTPS16-I335V-T336N-rev	ATCTAATGCTGTTATTAGAGCATTAACCCTGGTCGCCAT	site-directed mutagenesis

13.2. Manuscript II – Supplementary data

Table S1: Volatile organic compounds (VOCs) released from black poplar leaves after belowground damage by one *Melolontha melolontha* larva (grub), aboveground damage by *Lymantria dispar* caterpillars (caterpillar), and a combination of both herbivores (grub + caterpillar) as compared to non-damaged control plants (control) in ng*g^{-1} (FW). Shown is the mean ± SEM, n = 13-15. Bold numbers indicate the total emission within the groups of VOCs.

	Control	Grub	Caterpillar	Grub + Caterpillar
Green leaf volatiles	**70.52 ± 17.54**	**43.09 ± 5.31**	**319.49 ± 69.54**	**229.59 ± 25.74**
(*E*)-2-Hexenal	2.90 ± 0.97	2.46 ± 0.88	46.66 ± 7.71	67.24 ± 31.31
(*Z*)-3-Hexenol	51.80 ± 13.62	30.84 ± 3.95	219.37 ± 54.80	271.76 ± 122.09
(*Z*)-3-Hexenylacetate	15.83 ± 3.86	9.79 ± 1.74	53.46 ± 10.82	47.77 ± 8.18
Monoterpenes	**2644.39 ± 529.26**	**3753.08 ± 856.20**	**5166.63 ± 538.94**	**5684.18 ± 986.40**
α-Pinene	191.54 ± 42.49	146.06 ± 27.15	227.79 ± 34.14	161.45 ± 22.99
Camphene	230.67 ± 54.80	174.61 ± 30.88	290.91 ± 47.36	186.61 ± 26.11
α-Thujene	22.32 ± 4.81	17.71 ± 3.76	25.61 ± 3.52	19.58 ± 2.44
Sabinene	155.52 ± 33.47	127.33 ± 27.53	200.67 ± 27.34	145.47 ± 19.98
β-Pinene	184.34 ± 40.18	144.83 ± 27.54	214.37 ± 32.78	151.51 ± 20.79
β-Myrcene	49.44 ± 10.46	44.73 ± 9.86	63.78 ± 7.63	57.76 ± 8.30
Limonene	90.16 ± 19.25	72.40 ± 15.44	107.35 ± 14.86	79.30 ± 10.67
1,8-Cineole	637.03 ± 99.64	594.71 ± 101.21	625.54 ± 90.34	569.55 ± 77.24
(*Z*)-Ocimene	49.16 ± 14.38	96.86 ± 31.88	131.55 ± 19.23	188.90 ± 40.45
(*E*)-β-Ocimene	817.54 ± 301.03	2065.01 ± 767.86	2886.58 ± 437.20	4307.87 ± 938.60
γ-Terpinene	13.81 ± 3.43	15.52 ± 3.33	16.59 ± 1.51	17.79 ± 2.11
(*Z*)-Linalool oxide	8.11 ± 2.60	6.69 ± 2.46	41.04 ± 8.23	36.19 ± 8.77
α-Terpinolene	43.16 ± 9.13	39.27 ± 7.58	132.97 ± 32.85	132.47 ± 33.57
Linalool	17.52 ± 3.17	18.38 ± 3.01	19.77 ± 1.88	43.63 ± 9.68
Camphor	90.24 ± 22.40	145.16 ± 22.57	118.69 ± 21.42	125.33 ± 24.07
Borneol	16.43 ± 5.27	15.48 ± 4.18	25.97 ± 4.41	18.48 ± 3.90
α-Terpineol	27.41 ± 5.35	28.32 ± 4.73	37.46 ± 6.65	35.86 ± 6.64
Sesquiterpenes	**894.16 ± 122.65**	**750.32 ± 88.98**	**1461.65 ± 235.60**	**1200.11 ± 131.14**
α-Copaene	5.00 ± 0.84	4.66 ± 0.90	13.68 ± 2.46	10.34 ± 1.43
β-Cubebene	1.64 ± 0.59	4.66 ± 1.22	30.88 ± 6.02	20.67 ± 3.25
(*E*)-β-Caryophyllene	510.92 ± 70.96	393.83 ± 52.93	555.21 ± 88.25	460.24 ± 53.18
α-Guaiene	12.60 ± 2.07	9.79 ± 1.50	11.02 ± 2.45	10.48 ± 1.62
α-Humulene	56.75 ± 8.02	46.99 ± 6.58	94.74 ± 16.10	73.87 ± 9.27
Germacrene D	28.66 ± 5.18	44.39 ± 9.78	357.15 ± 72.97	223.36 ± 36.46
(*Z*,*E*)-α-Farnesene	7.44 ± 1.86	7.57 ± 2.18	14.03 ± 2.25	18.50 ± 4.02
(*E*,*E*)-α-Farnesene	89.48 ± 19.29	88.07 ± 27.59	190.64 ± 40.34	209.91 ± 63.55
γ-Cadinene	15.14 ± 2.51	12.46 ± 2.57	28.93 ± 4.31	23.02 ± 5.29
δ-Cadinene	25.57 ± 3.88	23.24 ± 3.41	49.64 ± 8.25	42.14 ± 6.20
Caryophyllene oxide	42.46 ± 7.08	36.12 ± 4.79	38.65 ± 7.63	38.20 ± 5.55

13. Supplementary Data

Guaiol	98.50 ± 20.80	78.55 ± 14.33	77.07 ± 19.53	80.14 ± 17.19
Aromatic compounds	**31.48 ± 5.83**	**38.07 ± 6.01**	**89.98 ± 13.74**	**119.64 ± 37.24**
Benzaldehyde	13.31 ± 1.91	18.21 ± 2.43	24.99 ± 2.80	31.56 ± 5.19
Salicylaldehyde	4.43 ± 2.02	4.48 ± 2.44	58.19 ± 12.23	63.93 ± 12.59
Methyl salicylate	13.74 ± 4.25	15.37 ± 3.63	6.80 ± 2.18	35.17 ± 24.24
Nitrogenous compounds	**18.44 ± 4.63**	**36.56 ± 10.85**	**511.48 ± 82.41**	**509.51 ± 92.95**
(*Z*)-2-Methylbutyraldoxime	6.20 ± 2.14	16.39 ± 5.62	189.06 ± 27.46	243.02 ± 62.79
(*E*)-2-Methylbutyraldoxime	2.14 ± 0.55	3.72 ± 1.17	48.41 ± 7.98	90.13 ± 41.23
(*E*)-3-Methylbutyraldoxime	0.96 ± 0.70	1.64 ± 0.78	25.05 ± 6.68	23.44 ± 8.49
Benzyl cyanide	9.14 ± 1.88	14.80 ± 4.68	248.95 ± 47.50	268.23 ± 48.90
(*E*)-DMNT	**170.95 ± 43.34**	**177.62 ± 45.97**	**1847.46 ± 311.47**	**1186.10 ± 130.26**

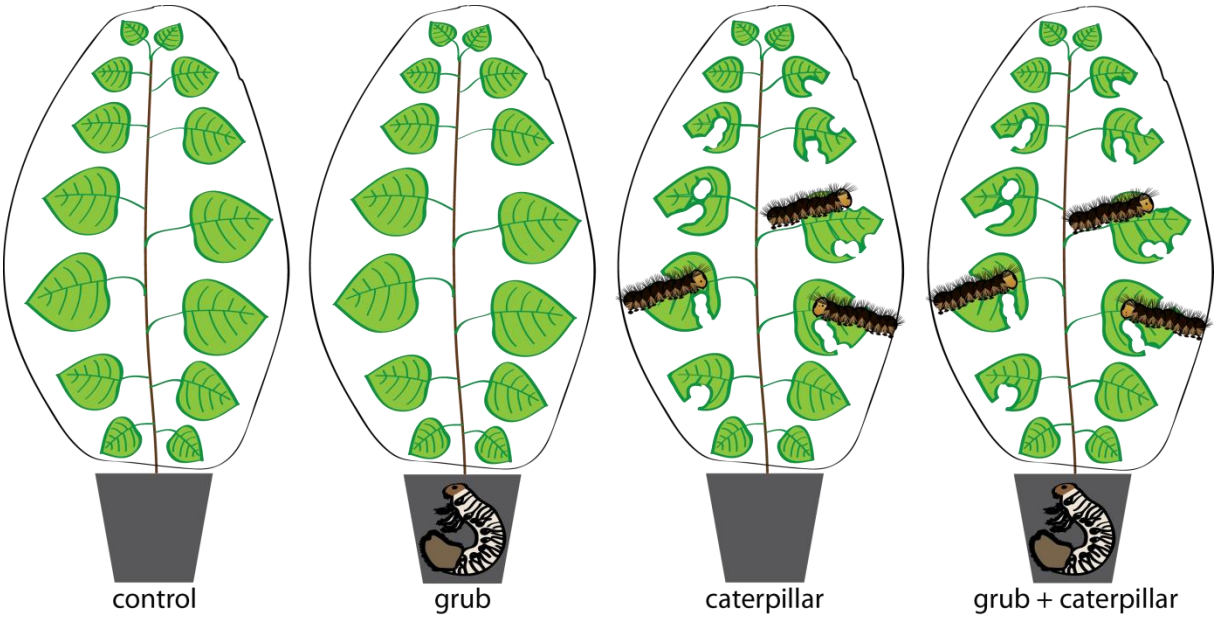


Figure S1: Schematic view of the experimental treatments. From left to right: control (non-treated control tree), grub (tree infested with one *Melolontha melolontha* larva for 6 days), caterpillar (tree infested with six 4th instar *Lymantria dispar* caterpillars for 40 hours), and grub + caterpillar (tree infested with one grub for 6 days and six 4th instar caterpillars for 40 hours).

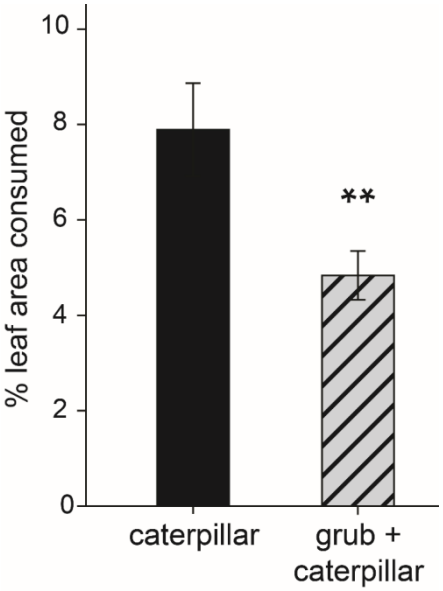


Figure S2: Leaf area loss (%) caused by *Lymantria dispar* caterpillars in the treatment with no belowground herbivory (caterpillar) and the treatment with additional belowground herbivory by one *Melolontha melolontha* larva (grub + caterpillar). Asterisks indicate the significant difference between treatments based on a Mann-Whitney U-test ($U = -2.573$; $p < 0.01$). Bars represent means ± SEM; n = 14-15.

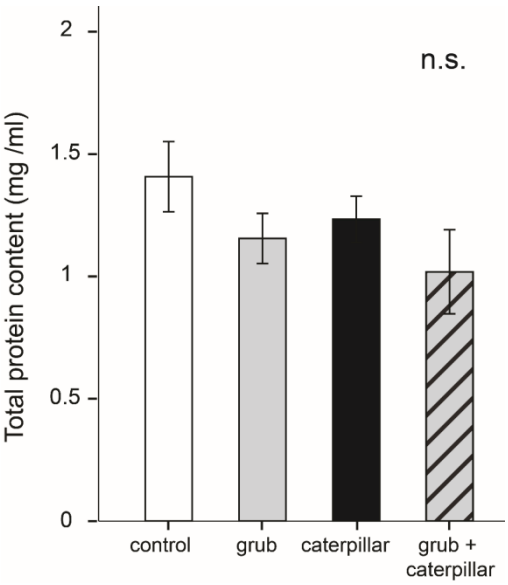


Figure S3: Total protein content of black poplar leaves after belowground damage by one *Melolontha melolontha* larva (grub), aboveground damage by *Lymantria dispar* caterpillars (caterpillar), and a combination of both herbivores (grub + caterpillar) as compared to non-damaged control plants (control). n. s. = non-significant (Kruskal Wallis test; $H = 5.836$; $p = 0.12$). Bars represent means ± SEM; n = 13-15.

13. Supplementary Data

Table S2: Amino acid concentrations after belowground damage by one *Melolontha melolontha* larva (grub), aboveground damage by aboveground damage by *Lymantria dispar* caterpillars (caterpillar), and a combination of both herbivores (grub + caterpillar) compared to non-damaged control plants in nmol g^{-1} DW. Bold p values indicate significant differences based on an ANOVA or Welch-ANOVA. Superscript letters indicate the results of Games-Howell post hoc tests. Bars represent means ± SEM; n = 12-15.

	Control	Grub	Caterpillar	Grub + Caterpillar	F	Welch F	p-value
Alanine	4622.89 ± 188.33	4802.23 ± 413.56	4393.32 ± 377.52	4826.39 ± 336.47	0.37		0.774
Serine*‡	5004.61 ± 428.80^{a}	4612.25 ± 504.31^{ab}	3853.54 ± 635.72^{b}	3661.99 ± 409.67^{b}	3.17		**0.032**
Proline	3273.84 ± 75.84^{a}	3709.57 ± 98.85^{b}	2703.41 ± 177.59^{c}	4924.21 ± 166.96^{d}		27.18	**<0.001**
Valine†	544.77 ± 47.51	730.02 ± 128.14	634.57 ± 74.58	698.70 ± 69.70	0.65		0.589
Threonine†	1655.17 ± 130.35^{a}	1562.14 ± 142.00^{ab}	1139.17 ± 118.72^{b}	1230.01 ± 57.32^{b}	5.91		**0.002**
Isoleucine	461.04 ± 45.48	647.20 ± 139.29	442.47 ± 57.07	505.72 ± 45.99	0.46		0.713
Leucine	268.74 ± 24.07	519.25 ± 194.58	341.58 ± 42.61	435.83 ± 61.53	1.93		0.137
Aspartic acid	3692.44 ± 339.54	3208.31 ± 258.01	3028.49 ± 353.50	3422.69 ± 321.48	0.82		0.487
Glutamic acid	26854.92 ± 2011.18	26641.88 ± 2756.79	22399.27 ± 2486.04	24346.02 ± 2296.55	0.92		0.436
Methionine	43.74 ± 3.33	41.22 ± 4.74	47.98 ± 4.87	46.94 ± 4.24	0.90		0.448
Histidine	581.86 ± 41.49	773.23 ± 183.38	500.75 ± 29.63	635.88 ± 73.71	0.94		0.428
Phenylalanine	270.59 ± 29.65	260.70 ± 44.61	229.86 ± 47.94	213.33 ± 29.44	1.69		0.181

Arginine‡	398.56 ± 144.82	471.28 ± 296.43	119.14 ± 22.35	212.44 ± 57.94	1.95	0.144
Tyrosine	293.05 ± 32.04	266.31 ± 37.21	253.44 ± 39.38	231.58 ± 26.41	1.05	0.388
Tryptophan‡§	156.91 ± 6.88[a]	209.73 ± 29.72[ab]	274.41 ± 30.61[b]	329.12 ± 52.85[b]	13.21	**<0.001**
Asparagine	454.92 ± 87.82	427.81 ± 64.38	331.76 ± 73.50	383.64 ± 53.73	0.45	0.718
Glutamine‡	6924.97 ± 635.18[a]	7199.47 ± 670.29[a]	6578.29 ± 542.58[a]	8673.09 ± 599.76[b]	4.97	**0.007**
Lysin	27.52 ± 3.55	32.29 ± 3.45	29.07 ± 3.53	24.87 ± 3.79	1.15	0.337

*Games-Howell post hoc test p-value = 0.068 and 0.079.

† data was $\log_{10}$ transformed; ‡ data was $\log_{10}$ transformed twice

§One outlier in the grub treatment was removed from the dataset

Table S3: Amino acid concentrations *in planta, in the experimental coating, and the coated leaf discs used in the preference assays†.** The coating concentration is based on the calculated concentration difference *in planta*. Concentrations are given in in nmol g^{-1} DW.

	Proline	**Alanine**	**Leucine**	**Tryptophan**
In planta* concentrations				
Control treatment	3275.54	4457.08	250.07	154.7
Grub treatment	3686.44	5138.42	310.98	175.54
Difference	410.9	681.34	60.91	20.84
Coating concentration	400	700	60	20
Concentration in coated leaf discs†				
Ethanol treatment	94.3	4723.8	122.2	36.5
Amino acid + ethanol treatment	320.2	5313.9	172.1	44.3
Difference	225.9	590.1	49.9	7.8

*Values represent medians (n = 12-15; for means ± SEM see Table S2).
†Values represent concentrations in bulk sample consisting of 5 leaf discs.

13. Supplementary Data

Structure elucidation of 6'-*O*-benzoylsalicortin. The structure of the previously undescribed compound with the retention time rt = 18.5 min, 6'-*O*-benzoylsalicortin, was elucidated by means of nuclear magnetic resonance (NMR) spectroscopy. As depicted in Fig. S4**,** the ^{1}H-NMR spectrum showed only little overlap. The elucidation was hence mainly based on ^{1}H-^{1}H correlations. Numbering in the following text is in accordance to the structure given in Fig. S8**.** Two spin systems in the low field range, an AMX system attributable to a phenyl ring (d_H 8.02 (H-2"/H-6"), 7.63 (H-4") and 7.49 (H-3"/H-5")) and an AA'XX' system (δ_H 7.25 (H-3), 7.14 (H-6), 7.08 (H-5) and 6.97 (H-4)) belonging to a 1,2-disubstituted aromatic ring, were identified. The AMX system was further characterized as a benzoate moiety by a long-range ^{1}H-^{13}C correlation from H-2"/H-6" to a carbonyl function at δ_C 167.8 (C-7", Fig. S7). In addition, the latter position showed a long-range ^{1}H-^{13}C correlation to the methylene signal of H-6', which eventually revealed the presence of a 6-benzoylated glucopyranosyl moiety. The identity of the latter was deduced from ^{1}H-^{1}H COSY correlations (Fig. S5**)** between a seven-membered spin system. The doublet signal of the anomeric position H-1' at δ_H 4.95 (J=7.7 Hz) was coupling to the overlapping H-2'/H-3' at δ_H 3.53, and the latter showed a coupling to H-4' (δ_H 3.47, dd, J=9.5/9.5 Hz). Another ^{1}H-^{1}H correlation of H-4' to H-5' (δ_H 3.47, ddd, J=2.2/7.3/9.5 Hz) and that of H-5' to the methylene signals of H-6' (δ_H 4.70, dd, J=11.7/2.2 Hz and δ_H 4.40, dd, J=11.7/7.3 Hz) completed the identification. The large coupling constants were characteristic for the presence of a b-glucopyranosyl element. Corresponding ^{13}C chemical shifts were extracted from ^{1}H-^{13}C heteronuclear single quantum correlations (HSQC, Fig. S6). The order of chemical shifts in the above mentioned AA'XX' system was established from ^{1}H-^{1}H COSY correlations. A long-range ^{1}H-^{13}C correlation from H-3 (δ_H 7.25) to C-7 (δ_C 64.6) led to the identification of a methylene extension of the 1,2-disubstituted aromatic ring at position 2. No further ^{1}H-^{1}H couplings from H-7 (δ_H 5.36 and 5.24, both (d, J=12.3 Hz)) were observed. Another long-range ^{1}H-^{13}C correlation from H-1'' to C-1 (δ_C 156.9) led eventually to the conclusion that a salicinyl substructure was present. The remaining part of the structure was identified based on a long-range ^{1}H-^{13}C correlation from H-7 to the carboxy function C-8 (δ_C 171.6). C-8 was furthermore showing a long-range ^{1}H-^{13}C correlation to H-10 (δ_H 5.72, ddd, J=1.8/1.8/9.8Hz). As evident from ^{1}H-^{1}H COSY correlations, H-10 was part of a 6-membered spin system, and the order of chemical shifts were established based on ^{1}H-^{1}H couplings together with HSQC data. Finally, the presence of a long-range ^{1}H-^{13}C correlation from H-10 to the carbonyl C-14 (δ_C 207.5) and from H-11 (δ_H 6.10, ddd, J=4.0/4.0/9.8Hz) to C-9 (δ_C 79.4), identified the substructure as a hydroxyl-cyclohexenonoate (HCH). Hence, a salicortin with a 6-benzoylated glucopyranosyl moiety was present. The NMR data showed uniform stereochemistry for this diastereomeric structure. Since the chemical shifts were in accordance with previously identified salicortin derivatives (Feistel et al. 2015), we consequently concluded (*S*)-configuration at C-9.

Figure S4: ^{1}H-NMR spectrum of 6'-*O*-benzoylsalicortin.

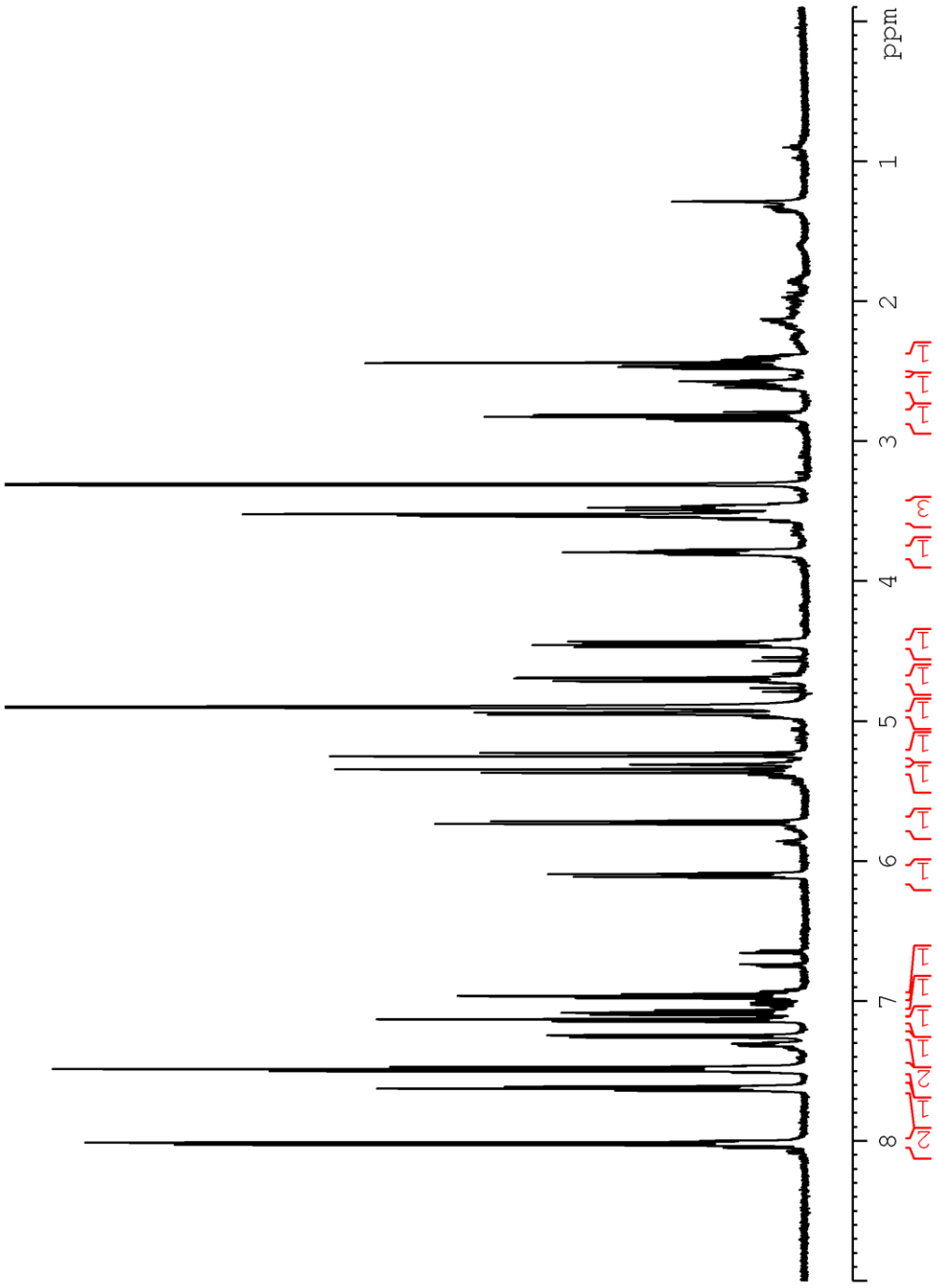


Figure S5: ^{1}H-^{1}H COSY NMR spectrum of 6'-*O*-benzoylsalicortin.

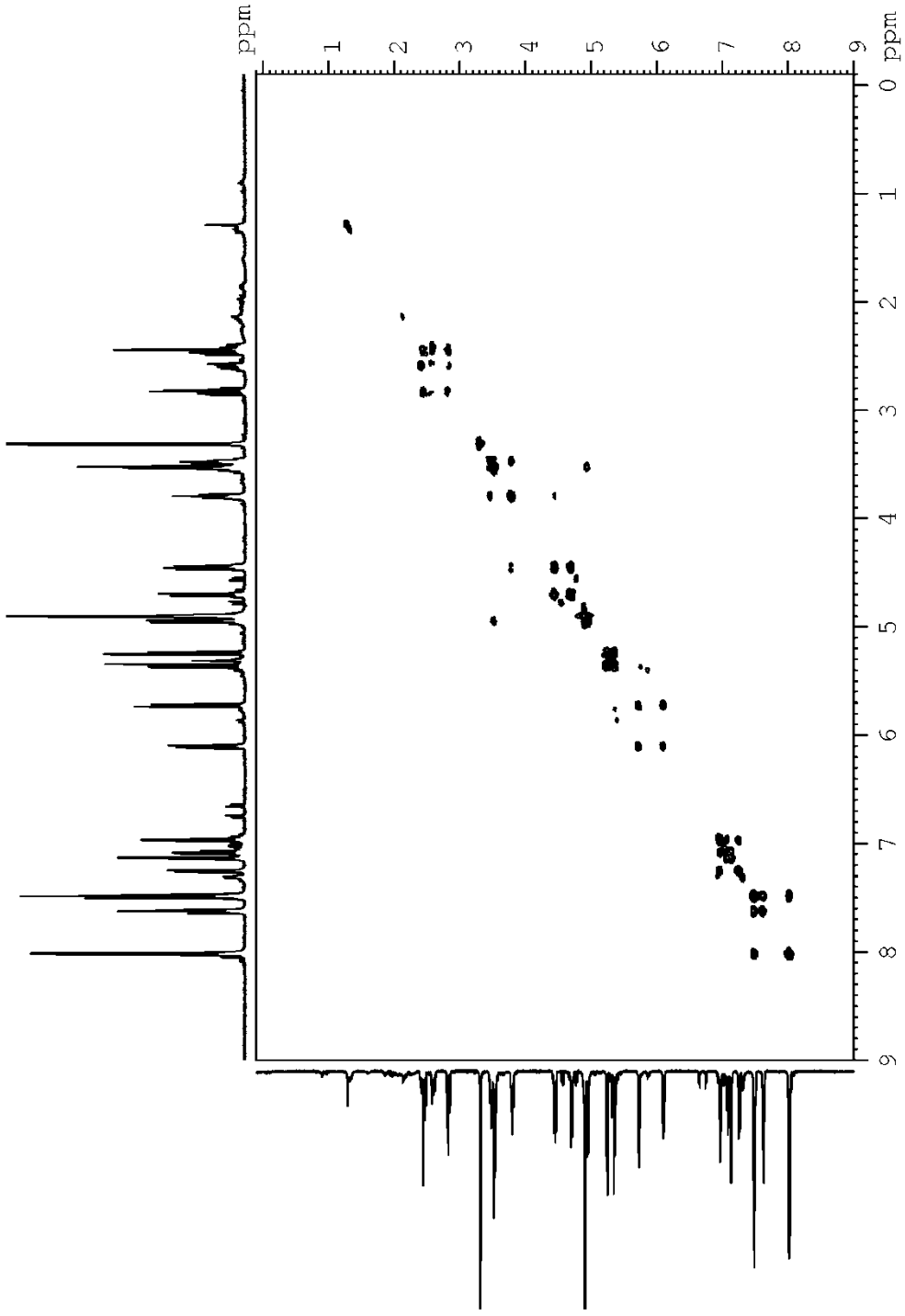


Figure S6: ^{1}H-^{13}C HSQC NMR spectrum of 6'-*O*-benzoylsalicortin.

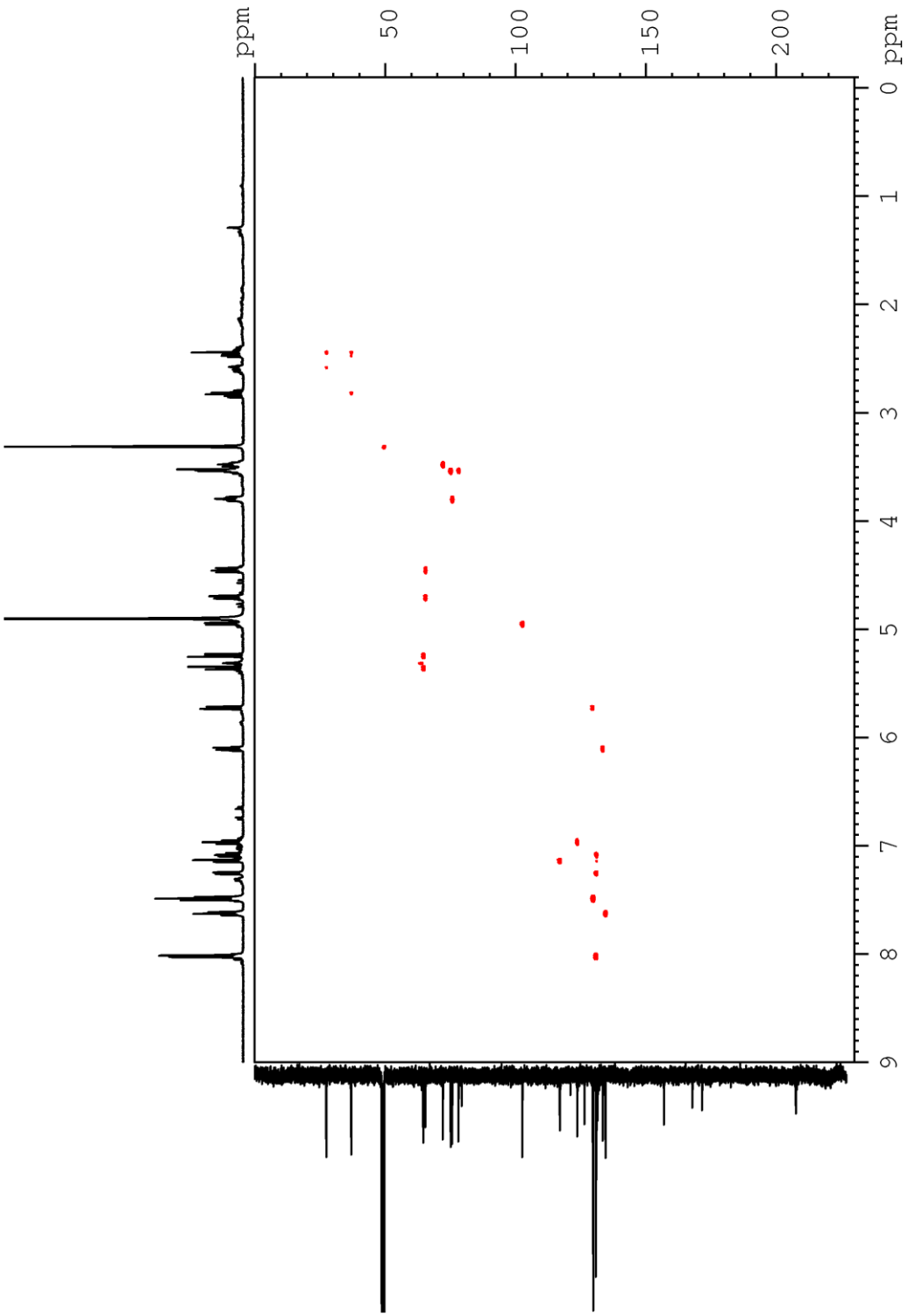


Figure S7: ^{1}H-^{13}C HMBC NMR spectrum of 6'-*O*-benzoylsalicortin.

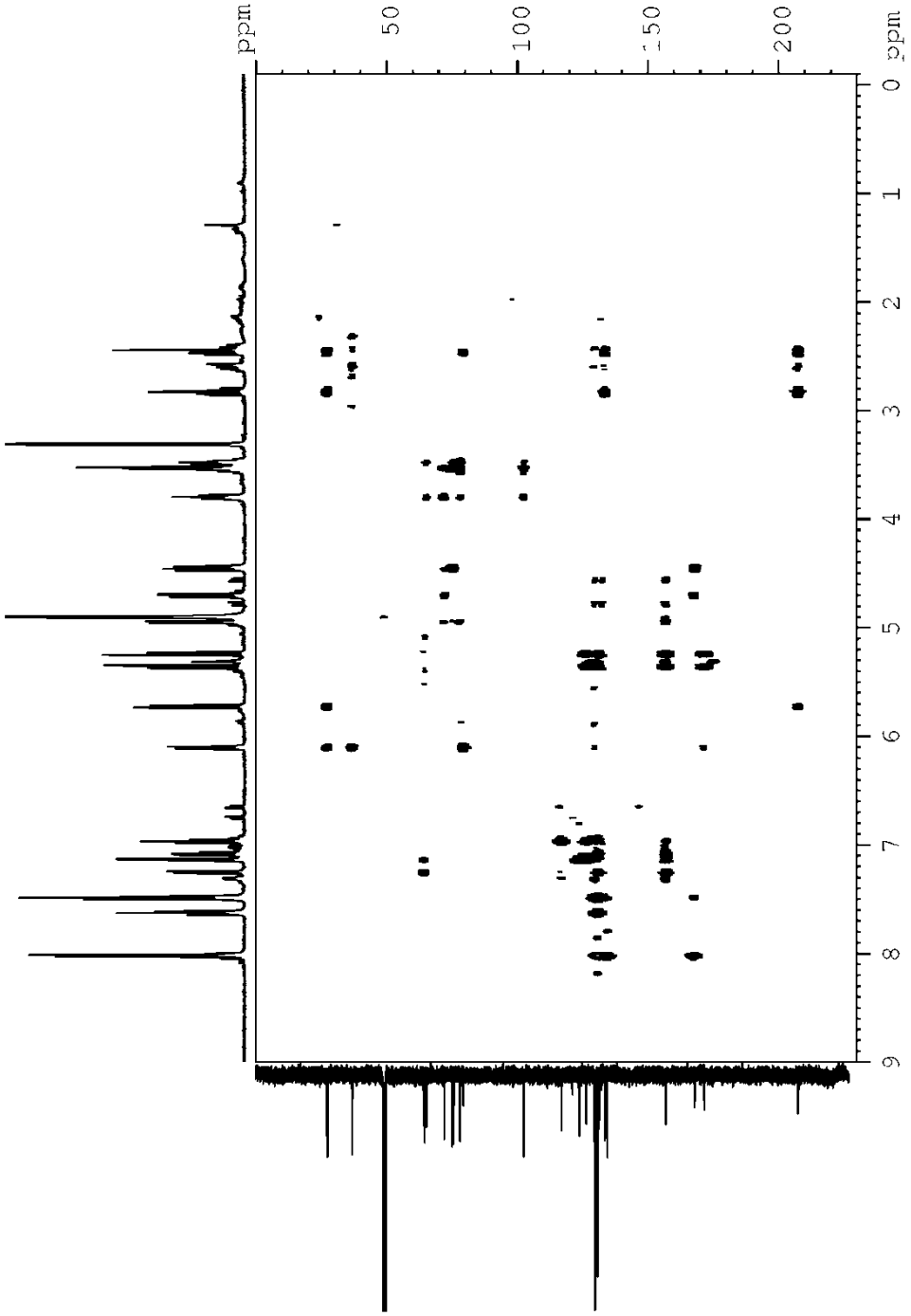
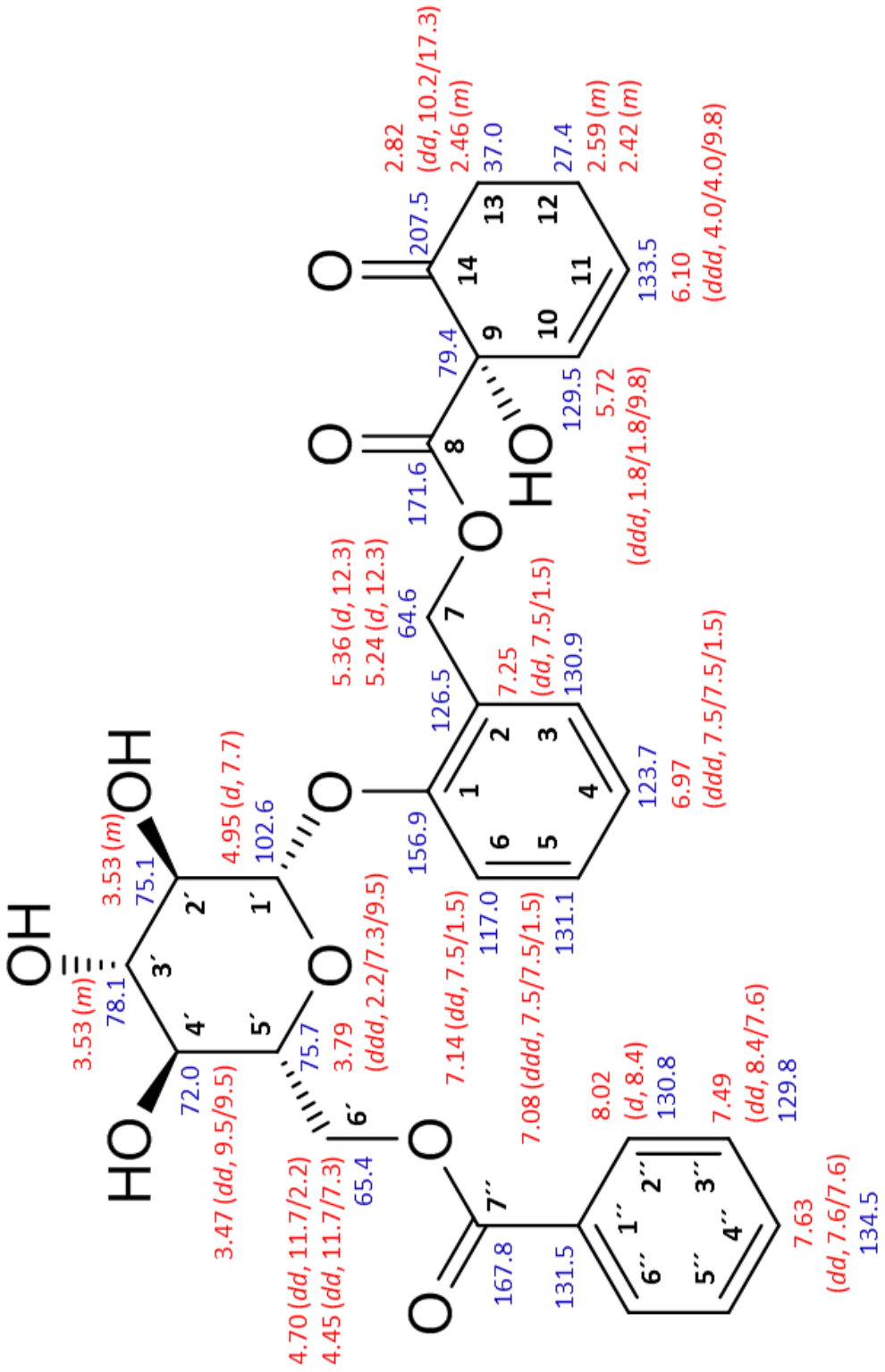


Figure S8: Structure of 6'-*O*-benzoylsalicortin with chemical shifts.



References

Feistel F, Paetz C, Lorenz S, Schneider B. (2015) The absolute configuration of salicortin, HCH-salicortin and tremulacin from *Populus trichocarpa* × *deltoides* Beaupré. *Molecules* 20, 5566.

13. Supplementary Data

13.3. Manuscript III – Supplementary data

Qualification and quantification of volatile organic compounds

2 µl of the eluate was injected splitless into a gas chromatograph (6890 series, Hewlett-Packard, Agilent Technologies, Santa Clara, CA, USA) equipped with a 30 m × 250 µm × 0.25 µm DB5-MS column (WicomGmbH, Heppenheim, Germany) coupled to a quadrupole mass spectrometer (5973 series, Hewlett-Packard, Agilent Technologies, Santa Clara, CA, USA) (in short GC/MS). The injector was held at 230 °C with helium used as carrier gas at 1 ml/min. The oven temperature of the GC/MS was held at 50 °C for 3 minutes after injection and then heated up to 95 °C at a rate of 4 °C/min. Afterwards, the oven temperature was increased to 145 °C with a 15 °C/min gradient and then to 180 °C with a 10 °C/min gradient. Finally, the oven temperature was kept stable for 3 min at 300 °C. Mass spectra were recorded (transfer line temperature: 230 °C, source temperature: 230 °C, quadrupole temperature: 150 °C, ionization energy: 70 eV, mass range: 40-500 m/z). Compounds were identified by comparing their mass spectra to authentic standards and three libraries (Wiley275, NIST, ADAMS). For quantification, the samples were separated with the same GC method as described above with hydrogen as the carrier gas. Afterwards the samples were analyzed with a flame ionization detector (FID, 9200 Hydrogen detector, Packard, Agilent Technologies, Santa Clara, CA, USA) operating at 300 °C. Absolute amounts of all compounds were calculated based on the relation of their FID peak area and the area of the internal standard according to the “effective carbon number (ECN) concept” (Scanlon & Willis 1985).

13. Supplementary Data

Table S1: Means ± Standard Error (SE) of constitutive or herbivore induced volatile emissions from black poplar emitter trees (*n* = 4) in ng g^{-1} (fw) h^{-1}.

	constitutive		Herbivory induced		U-test	
	Mean	±SE	Mean	±SE	U	p
GLV	**96.14**	**32.12**	**1228.35**	**532.50**	**2.309**	**0.021**
Z-3 hexenol	3.65	1.32	266.56	129.92	**2.309**	**0.021**
Z-3-hexenylacetate	92.50	31.08	961.79	457.22	2.309	0.021
MT	**355.96**	**129.92**	**2951.04**	**1607.43**	**2.309**	**0.021**
α-pinene	24.93	7.25	55.45	24.14	ns	ns
camphene	39.42	11.95	76.55	33.39	ns	ns
sabinene	30.28	10.44	59.88	31.20	ns	ns
β-pinene	11.95	9.77	6.00	3.76	ns	ns
myrcene	24.99	6.73	59.22	33.97	ns	ns
limonene	72.10	43.35	150.37	127.81	ns	ns
Z-ocimene	18.94	8.34	209.92	115.72	2.309	0.021
E-β-ocimene	100.75	37.58	2246.59	1200.03	2.309	0.021
camphor	20.62	10.67	64.46	29.30	ns	ns
borneol	11.98	5.28	22.61	11.94	ns	ns
***E*-DMNT**	**18.27**	**7.30**	**1379.98**	**678.91**	**2.309**	**0.021**
ST	**157.02**	**88.81**	**616.77**	**355.08**	**ns**	**ns**
β-caryophyllene	13.73	8.91	248.72	140.95	2.309	0.021
α-humulene	9.37	6.14	50.28	31.84	1.732	0.083
β-cubebene	7.80	4.73	133.07	76.76	2.323	0.02
E,*E*-α-farnesene	120.39	67.49	170.98	97.06	ns	ns
δ-cadinene	5.73	2.53	13.72	8.53	ns	ns
Aromatics	**31.94**	**16.53**	**248.80**	**114.11**	**1.732**	**0.083**
benzaldehyde	8.22	2.19	17.52	6.77	ns	ns
salicylaldehyde	0.00	0.00	199.13	97.14	2.460	0.014
eugenol	23.72	14.56	24.05	12.51	ns	ns
Nitrogenous	**0.00**	**0.00**	**433.81**	**185.99**	**2.460**	**0.014**
E-2-methylbutyraldoxime	0.00	0.00	223.59	97.50	1.984	0.047
Z-2-methylbutyraldoxime	0.00	0.00	63.47	23.47	2.460	0.014
(*E*/*Z*)-3-methylbutyraldoxime	0.00	0.00	63.22	20.79	2.460	0.014
benzyl alcohol	0.00	0.00	8.09	2.72	1.984	0.047
benzyl cyanid	0.00	0.00	83.53	54.59	1.984	0.047
isoamylacetate	**0.00**	**0.00**	**93.48**	**46.27**	**2.460**	**0.014**

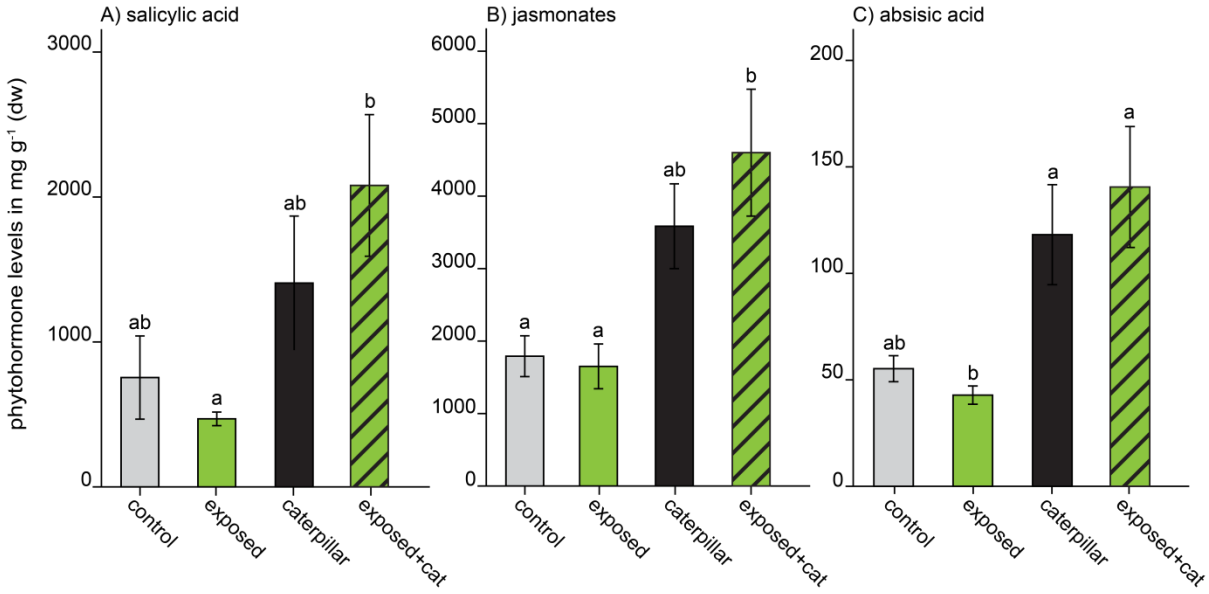


Figure S1: Salicylic acid (A), jasmonates (B) and absisic acid (C) concentrations of black poplar leaves after herbivore damage by gypsy moth caterpillars (caterpillar), previous exposure to HIPVs (HIPV exposed) and a combination of herbivore damaged and previously exposed to HIPVs (HIPV exposed + caterpillar) compared to control plants (control). The group jasmonates represents the sum of jasmonic acid and its derivatives (JA-Ile, OH-JA, OH-JA-Ile, COOH-JA-Ile and cisOPDA). Different letters indicate significant differences between treatments based on a Kruskal Wallis test with Dunns post hoc (A: H = 11.894; p = 0.008; B: H = 15.123; p = 0.002; C: H = 15.663; p = 0.001). Bars represent means ± SE; n = 5-6.

13. Supplementary Data

RNA isolation, RNA-Seq, de novo assembly and differential gene expression analysis

RNA was isolated from frozen, ground leaf material using the InviTrap Spin Plant Mini Kit (Stratec Biomedical AG) according to the manufacturer's manual. Additionally, a DNA digestion was included (DNase set; Qiagen). RNA concentration and purity were tested with a NanoDrop2000c spectrophotometer (Peqlab Biotechnology AG).

Sequencing of the poly(A)+ mRNA enriched samples was done at the Max Planck-Genome-Centre (Köln, Germany) on a HighSeq3000 instrument (Illumina, San Diego, California, USA) generating appr. 15 Mio paired-end reads (2 x 150 bp) per sample. Quality control measures, including the filtering of high-quality reads based on fastq file scores, the removal of reads containing primer/adapter sequences, and trimming of the read length, were carried out using CLC Genomics Workbench v11 (http://www.clcbio.com). The same software was used for *de novo* transcriptome assembly using a total of 185 Mio sequence reads, combining three replicates of each RNA-Seq treatment group, and selecting the presumed optimal consensus transcriptome as previously described (Vogel *et al.* 2014). The final *de novo* reference transcriptome assembly (backbone) of *Populus nigra* contained 65,866 contigs. Minimum contig size was 300 bp with an N50 contig size of 1430 bp. The transcriptome was annotated using BLAST, Gene Ontology (GO) and InterPro terms (InterProScan, EBI), enzyme classification (EC) codes, and metabolic pathways (Kyoto Encyclopedia of Genes and Genomes, KEGG) as implemented in BLAST2GO v5.1 (http://www.blast2go.de). To assess transcriptome completeness, we performed a BUSCO (Benchmarking Universal Single-Copy Orthologs; http://busco.ezlab.org) analysis by comparing our assembled transcript set against a set of highly conserved single-copy orthologs. This was accomplished using the BUSCO v3 pipeline (Waterhouse *et al.* 2017) compared to the predefined set of 303 Eukaryota single-copy orthologs from the OrthoDB v9.1 database. The assembled *P. nigra* transcriptome was determined to be 82.2% complete and 6.2% of the BUSCO genes were missing. The Illumina data have been deposited in the EBI short read archive (SRA) with the following sample accession numbers: ERS 3356354- ERS 3356361. The complete study can also be accessed directly using the following URL: http://www.ebi.ac.uk/ena/data/view/ PRJEB32064.

Digital gene expression analysis was carried out using CLC Genomics Workbench v11 to generate BAM (mapping) files, and QSeq Software (DNAStar Inc., Madison, WI, USA) was then used to estimate expression levels. The $\log_2$ (RPKM) values (normalized mapped read values; geometric means of the biological replicate samples) were subsequently used to calculate fold-change values. To identify differentially expressed genes, we used the Student´s t-test (as implemented in Qseq) and corrected for multiple testing using the Benjamini–Hochberg procedure to check the false discovery rate (FDR). In addition to the method implemented in Qseq, we used an alternative method for normalization and

differential gene expression analysis. Mapped reads were log2-transformed and normalized using the quantile method and statistical analysis of the normalized data was carried out using the “empirical analysis of digital gene expression” (EDGE) tool, implemented in CLC Genomics Workbench v8.1. For both methods, a gene was considered significantly differentially expressed with a minimum two-fold change and if the FDR-corrected p-value was less than 0.05.

Transcriptomic data of black poplar leaves exposed to HIPVs compared to control leaves. See Excel file on attached CD.

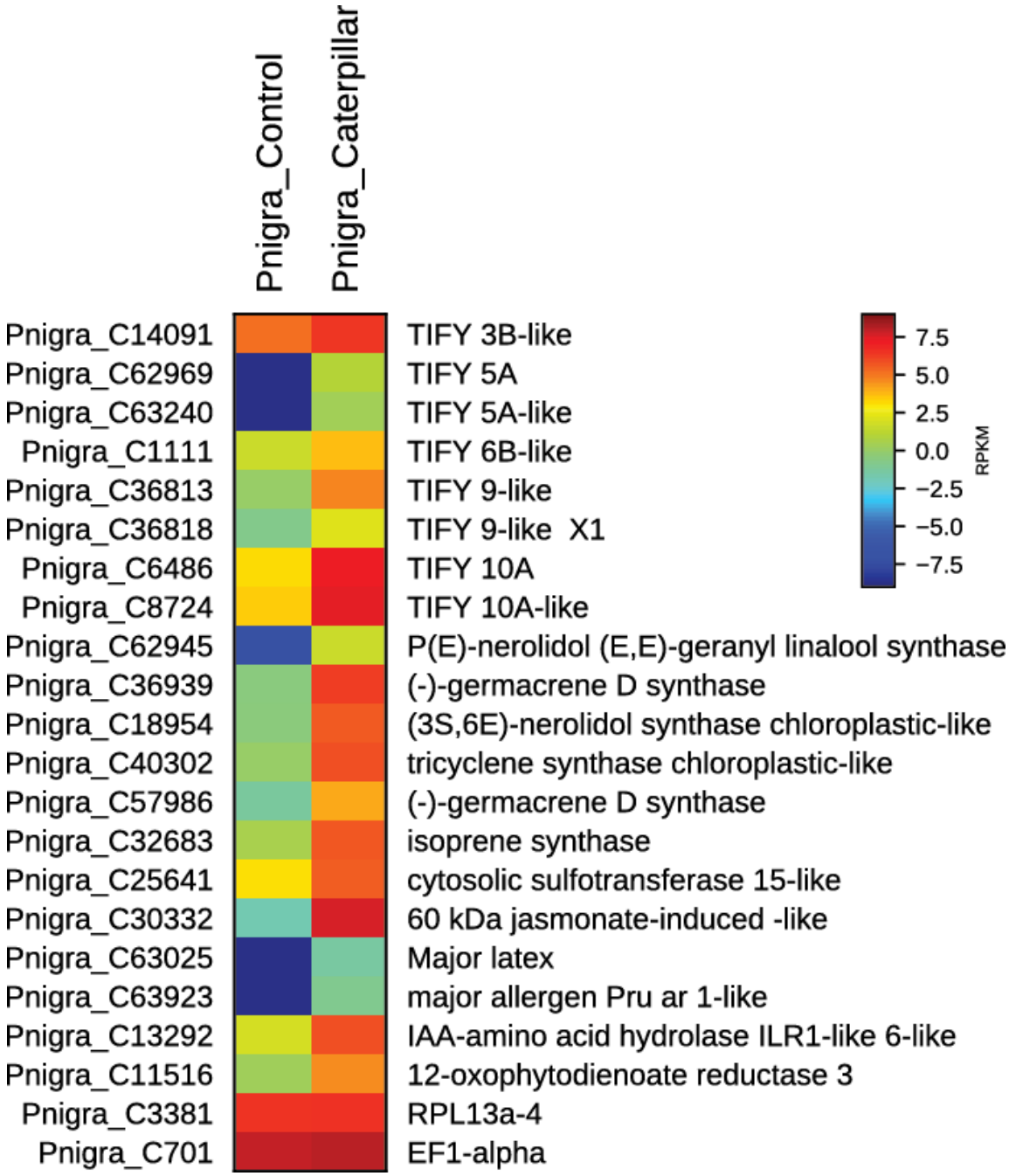


Figure S2: Heat map of 20 exemplary wounding or JA-mediated defense related genes identified in the *P. nigra* transcriptome, for leaves that were damaged by gypsy moth caterpillars (Pnigra_caterpillar) compared to controls (Pnigra_control). The map is based on log2-transformed RPKM values (blue represents low-expressed genes, and red represents highly-expressed genes). RPL13a-4 and EF1-alpha were included as control genes.

Table S2: Protease inhibitor levels of black poplar leaves after herbivore damage by gypsy moth caterpillars (caterpillar), previous exposure to HIPVs (HIPV exposed) and a combination of herbivore damaged and previously exposed to HIPVs (HIPV exposed + caterpillar) compared to control plants (control). Different letters indicate significant differences between treatments based on a Kruskal Wallis test with Dunns post hoc (protein content: n.s.; trypsin inhibitor: H = 11.045, p = 0.011; n = 4-6).
Shown are means ± SE; n = 4-6.

	control		HIPV exposed		caterpillar		HIPV exposed + caterpillar	
	Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE
total protein content [mg ml^{-1}]	1.09	0.10	0.81	0.15	0.59	0.09	0.84	0.13
trypsin inhibitor content [µg mg^{-1} protein]	7.71^{a}	0.85	13.88^{ab}	4.36	19.09^{b}	1.87	25.25^{b}	5.73

Table S3: phenylacetaldoxime content in black poplar leaves after herbivore damage by gypsy moth caterpillars (caterpillar), previous exposure to HIPVs (HIPV exposed) and a combination of herbivore damaged and previously exposed to HIPVs (HIPV exposed + caterpillar) compared to control plants (control). Different letters indicate significant differences between treatments based on a Kruskal Wallis test with Dunns post hoc (H = 12.445, p = 0.006; n = 5-6). Shown are means ± SE; n = 5-6.

	control		HIPV exposed		caterpillar		HIPV exposed + caterpillar	
	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE
phenylacetaldoxime [µg g^{-1} DW]	0.03^{a}	0.01	0.03^{a}	0.02	0.77^{ab}	0.39	0.68^{b}	0.21

Table S4: Levels of free sugars in black poplar leaves after herbivore damage by gypsy moth caterpillars (caterpillar), previous exposure to HIPVs (HIPV exposed) and a combination of herbivore damaged and previously exposed to HIPVs (HIPV exposed + caterpillar) compared to control plants (control). Different letters indicate significant differences between treatments based on an ANOVA with Tukey´post hoc (sucrose and trisaccharide n.s.; fructose: F = 5.389; p = 0.007) or a Kruskal Wallis test with Dunns post hoc (glucose: H = 10.5; p = 0.033; tetrasaccharide n.s.). Shown are means ± SE; n = 5-6.

	control		HIPV exposed		caterpillar		HIPV exposed+caterpillar	
	Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE
Glucose	1.27[a]	0.17	1.47[ab]	0.18	3.79[b]	0.66	2.71[ab]	0.80
Fructose	3.71[a]	0.35	3.48[a]	0.38	5.71[b]	0.67	5.45[b]	0.57
Sucrose	29.81	1.57	28.39	1.00	30.11	1.02	30.26	1.03
Trisaccharide	0.29	0.03	0.26	0.02	0.27	0.04	0.28	0.02
Tetrasaccharide	0.04	0.01	0.02	0.00	0.03	0.01	0.03	0.01

Common garden performance experiment

The effect of salicinoid concentration on the performance of young *L. dispar* larvae was studied in a common garden experiment under natural conditions in June 2016. The trees derived from monoclonal stem cuttings of a natural black poplar population located in a floodplain forest along the Oder River of northeastern Germany (52°34'1" N, 14°38'3" E). Nine trees of different genotypes, which vary naturally in salicinoid concentration, were selected for the experiment. On each tree one branch was selected. Starting from the youngest fully developed leaf and counting in basal direction 8 young leaves were enclosed with a net bag, fixed on both ends with cable binders. Subsequently, twenty 2nd instar *L. dispar* larvae were released into the leaf pool. The larvae were allowed to feed for 14 days. After 14 days caterpillars were weighed. Dead larvae were not considered in the analysis. Afterwards all leaves were harvested and shock-frozen in liquid nitrogen. In the lab all leaf material was lyophilized (ALPHA 1-4 LDplus, Christ, Germany) and stored at -20 °C until further analysis.

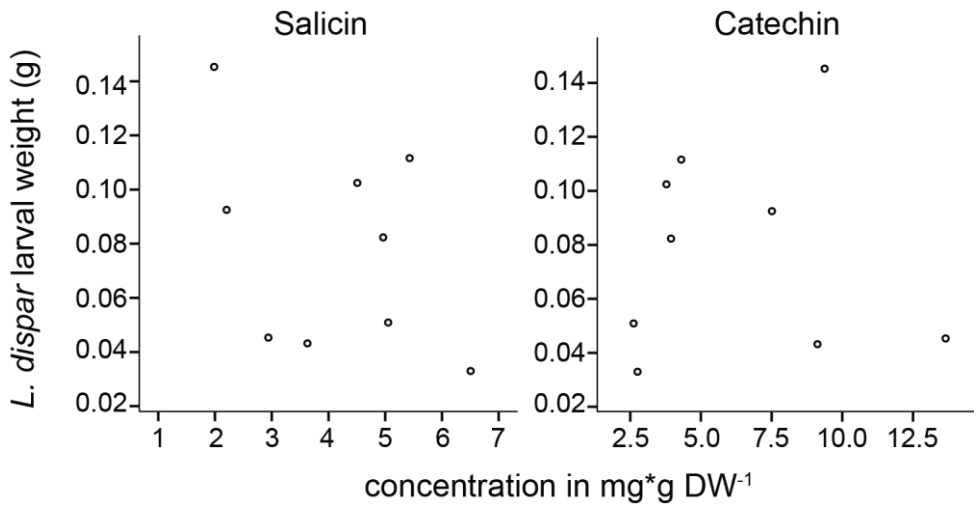


Figure S3 Average *L. dispar* larval weight in dependence of salicin (A) and catechin (B) levels in black poplar trees of nine genotypes. Shown are means, n = 5-10.