Tripartite relationships:
The chemical ecology of tree-herbivore-pathogen interactions

DISSERTATION

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1. INTRODUCTION

One third of the landmass of our planet is covered with forest (FAO 2015), and the immense ecological importance of the trees growing in these forests is almost impossible to describe. Beside their atmospheric impact by carbon fixation, oxygen production and emission of volatile organic compounds, trees shape the biotic environment in these ecosystems. Each tree harbors a huge diversity of microbial as well as vertebrate and invertebrate species, which inhabit below- and aboveground tissues. Microbes, especially bacteria and fungi, might have beneficial or detrimental effects on their hosts as symbionts or pathogens, respectively. At the same time, plants are continuously exposed to herbivores, especially insects, which feed on their leaves, roots and other tissues. Trees play a pivotal role in forest ecosystems as their longevity and large size offers numerous possibilities for interactions with other organisms, as compared to short-lived and small-sized herbs. This makes woody plants ideal study organisms to elucidate complex ecological interactions. Nevertheless, studies on plant-insect or plant-microbe interactions have been carried out predominantly with herbs and crop plants. The characterization of trees and their biotic environment, however, will open up new perspectives and provide knowledge to elucidate the chemical interactions between multiple organisms in complex ecosystems.

1.1. Fungal colonization and anti-fungal defenses in plants

Prevalence and importance of leaf pathogens

Microbial species occur in all ecosystems of the world, and are often associated with other organisms which they colonize internally or externally, such as plants and animals. Plant-microbe associations have been described for a wide range of microbial species including bacteria, protists, archaea and fungi (Berg *et al.* 2016; Hardham 2007). Fungi can colonize every organ of a plant, i.e. leaves, roots, stems, flowers and, in case of trees, also bark and wood (Baldrian 2017). They are hence universally abundant, and under natural conditions every plant is associated with fungi. However, the role of fungi in these associations may take different forms, ranging from mutualistic (e. g. mycorrhizae), commensal (the microbe benefits without negative effects on its plant host) to pathogenic (as causing agent of diseases). Other fungi, the saprophytes, only

colonize dead plant tissue and therefore do not influence plant physiology actively but play an important role in nutrient cycling (Azcón-Aguilar & Barea 2015).

In this thesis I focus on leaf pathogens, disease-causing fungi that inhabit the foliage of plants. Leaf pathogens can be classified according to their life style into biotrophic or necrotrophic pathogens (Glazebrook 2005). Biotrophic pathogens require living plant cells from which they withdraw nutrients, often by specialized hyphae called haustoria (Vögele & Mendgen 2003). Necrotrophic pathogens, on the other hand, kill host cells to deprive nutrients from dead or dying tissue. Some pathogens, the hemibiotrophs, are able to switch from one life style to the other, usually passing through a biotrophic phase first before turning into necrotrophs (Ferreira *et al.* 2006). Pathogens that infect crop plants and thereby affect economic yields have been studied most intensively. Well known examples are molds such as the necrotrophic *Botrytis cinerea* infecting grapes and wine, biotrophic rusts such as *Puccinia graminis* infecting wheat, and the hemibiotrophic *Colletotrichum spp*. which infect a wide range of vegetables (Dean *et al*. 2012). Plants infected by necrotrophic pathogens suffer from the loss of photosynthesizing tissue, while plants infected by biotrophic pathogens are deprived of nutrients. Both infection modes reduce carbon fixation in their host plants, lower levels of stored reserves and result in less investment into growth and reproduction. To protect plants, especially crops, from pathogen-related damage great efforts have been made to breed resistant varieties. However, in order to select for resistant genotypes it is necessary to have a detailed understanding of pathogen-recognition and anti-pathogen defense in plants.

Recognition and defense against fungal pathogens in plants

Pathogens can enter the plant matrix through natural openings, such as stomata, or by wounds, insect vectors (Kluth *et al*. 2002) or pegs and appressoria, specialized fungal structures that penetrate the plant epidermis (Dean *et al.* 2012). Once inside the host, the pathogen is recognized by the plant through pathogen-specific molecules (Dodds & Rathjen 2010). Non-specific recognition occurs by detection of “pathogen-associated molecular patterns” (PAMPs; sometimes referred to as “microbial-associated molecular patters”, MAMPs) (Chisholm *et al.* 2006), such as β-glucan or chitin which are constituents of the fungal cell wall (Newman *et al* 2013). The perception of PAMPs in the plant triggers a signaling cascade leading to “pathogen-triggered immunity” (PTI) (Newman *et al.* 2013). PTI induces various defense mechanisms that directly or

indirectly target the pathogen. Reactive oxygen species and nitric oxide are involved in plant-signaling to enhance the response, but can also have direct toxic effects on the pathogen or induce oxidative cross-linking of polymers (Newman *et al.* 2013). These polymers result in cell wall fortification, as does the deposition of callose (Halim *et al*. 2007) or lignification (Bhuiyan *et al.* 2009). Furthermore, antimicrobial compounds can be synthesized which act as a direct chemical defense (Ahuja *et al.* 2012). Additionally, a whole set of so called "pathogenesis-related proteins" (PR proteins) is produced, such as glucanases, chitinases or proteinase inhibitors (van Loon *et al.* 2006; Ferreira *et al*. 2007). However, pathogens have evolved various strategies to evade plant recognition and suppress the defense reaction (Jones & Dangl 2006), for example by inhibiting the signaling pathway downstream of the PAMP receptors (He *et al*. 2006). The suppression of recognition and defense is usually achieved by the secretion of effector molecules by the pathogen (Dodds & Rathjen 2010). However, plants can recognize such pathogen-derived effectors and evolved specialized "resistance proteins" (R proteins) that can bind the effectors to activate a defense response known as "effector-triggered immunity" (ETI) (Jones & Dangl 2006). The ETI is the second, more specific strategy of plants to recognize and fight the pathogenic invader. ETI arose due to co-evolution of plants and their pathogens and is an extension of the "gene-for-gene theory" (Flor 1971) since a specific effector protein of the pathogen is recognized by a specific R protein of the plant. The ultimate consequence of ETI is usually similar to that of the PTI, but faster and stronger, leading to the hypersensitive response, a form of programmed cell death at the site of infection that isolates the pathogen in an island of dead cells (Ferreira *et al.* 2006).

Most of the defense responses to pathogens described above are activated by signaling cascades mediated by defense hormones in the infected plant. In order to differentially defend against distinct types of pathogens (biotrophic and necrotrophic), plants have evolved specific signaling cascades to target each type of attacker. Usually, biotrophic pathogens induce a defense response activated by salicylic acid (SA), whereas necrotrophic pathogens induce defense responses mediated by jasmonic acid (JA) (Glazebrook 2005). The latter is also induced by chewing herbivores and will be discussed in the following section in more detail. SA triggers local signaling cascades, but also acts as systemic signal which can result in "systemic acquired resistance" (SAR) (Park *et al.* 2007), conferring long-lasting and far-ranging resistance against future pathogen attacks. On a molecular level, SA activates the key regulator "non-repressor of PR genes 1" (NPR1), which translocates into the nucleus where it induces the expression of SA-

responsive genes (Mur *et al*. 2013). Most important among those SA-responsive genes are the PR genes mentioned above. However, SA also activates other transcription factors such as members of the WRKY family which induce expression of defensive genes (Eulgem & Somssich 2007).
Anti-pathogen defense mechanisms in plants have been almost exclusively studied in herbaceous species. In contrast, much less is known about woody plants, which are thought to have a more complex response, as they have to evaluate the trade-off between defense *versus* future growth (Herms & Mattson 1992) more carefully to have sufficient resources for the next growth season. Additionally, woody plants, due to their large seize, might have to constantly fight multiple infections at different sites within the canopy. Some aspects of the defense signaling cascades of woody plants have already been shown to be different from those in herbs. For example, the role of SA is still not clear in woody plants, as contradictory results have been reported (Germain & Seguin 2011; Naidoo *et al.* 2013). Also NPR1, which is described as a key regulator in SA signaling in herbs, was not found to be induced by SA accumulation in poplar (Xue *et al*. 2013). However, there are also many parallels between the anti-pathogen defenses of trees and herbs, such as the involvement of WRKY transcription factors in disease resistance (Jiang *et al*. 2017), the expression of PR genes (Naidoo *et al*. 2013) and the biosynthesis of phytoalexins (Gottstein & Gross 1991).

1.2. Plant-herbivore interactions

In natural environments plants are not only colonized by microbes, but also face attacks by a plethora of herbivores, animal species that feed on plants. Arthropods are certainly the biggest and evolutionarily oldest group among the herbivores. Fossils depicting arthropod herbivory date back to the early Devonian, more than 400 million years ago (Labandeira 2007). Within the arthropods, insects account for the largest group (Stork 2018) and at least half of all insect species feed on plants (Price et al. 2011). This long co-evolution between plants and insects (Ehrlich & Raven 1964) has shaped a dynamic interaction between them starting with the origin of plant defenses against insects, followed by insect circumvention of the defense and plant development of novel defense mechanisms in repeating cycles.
Insect herbivores can feed on different tissues of a plant, such as roots, stems, wood or leaves. Apart from that, the feeding guild of the herbivores has an important effect on the host plant. Herbivores might bite whole chunks from plant tissue (“chewing”) or pierce into the tissue to feed

from phloem sap ("piercing-sucking"). Both types of feeding provoke entirely different responses in the host (Walling 2000), which correspond to the distinct signaling cascades upon the infection with biotrophic and necrotrophic pathogens (chapter 1.1.). Furthermore, some insects produce galls ("galling"), feed inside the leaf or stem ("mining") or remove the tissue between the leaf veins ("skeletonizing"). Herbivorous insects differ not only in feeding guilds but also in the range of their host plants. Whereas some insects accept only closely related plant species of the same family or genus ("specialists" or "oligophages"), others can feed on a broad range of plants across different families ("generalists" or "polyphages") (Ali & Agrawal 2012).

Nutritional value of plant tissue for insect herbivores

Food quality is a key component of insect performance and fitness as it influences growth, survival, immune response and fecundity (Awmack & Leather 2002; Bukovinszky *et al.* 2009). For herbivores, plant tissue is a matrix of water and nutrients on the one hand, and anti-digestive or toxic compounds on the other hand. When feeding, insects have to satisfy their demand for nutrition and at the same time circumvent the defense strategies of the host. Even though the general requirement for water, carbon (carbohydrates, lipids, sterols), nitrogen (protein, amino acids), vitamins, essential nutrients and minerals are common to all insect species, the specific nutritional target, i.e. the optimal composition of the diet, might differ among insect species (Behmer 2009). In order to meet their nutrient target insects are able to self-select their optimal diet, by changing or mixing food sources within one plant (for example young *vs.* old leaves) or from different plants (Behmer 2009; Waldbauer & Friedman 1991). Beyond the challenge of taking up the ideal amounts of macro- and micronutrients, insects also have to deal with various defense compounds which they encounter in the plant tissue. The detoxification of those defense compounds involves an additional metabolic cost (Gatehouse 2002) and constrains the feeding choice of the herbivore.

Anti-herbivore defenses in plants

As plants are sessile organisms, they have to adapt to external stresses by mechanisms other than escape. Against insect herbivores, plants have evolved a broad spectrum of defense mechanisms. The diversity of these defense mechanisms can be categorized by different features: temporal abundance (constitutive *vs*. inducible), effect on the attacker (direct *vs.* indirect) or function

(chemical, biochemical, physiological, morphological or ecological). Constitutive or "preformed" defenses are present at all times, whereas inducible defenses are only formed upon attack (Eyles 2010; Fürstenberg-Hägg *et al.* 2013). Both, constitutive and inducible defenses can have direct or indirect effects on the attacking herbivore. Direct effects might be physical barriers e.g. thorns or lignification (morphological defense), reduction of digestibility by proteinase inhibitors or chitinases (biochemical defense), or deterrence and intoxication by specific compounds (chemical defense) (Fürstenberg-Hägg *et al.* 2013; Chen *et al.* 2005; De Moraes *et al.* 2001). In the past, researchers intensively explored chemical defenses comprising many different chemical compound classes, such as cyanogenic glycosides, phenolic compounds, alkaloids and terpenoids (Mithöfer & Boland 2012). Some compounds within these and other classes are volatile and play an important role in the indirect defense. Indirect defenses involve additional players, such as parasitoids and predatory insects that will be attracted by plant volatiles and attack the feeding herbivore (Arimura *et al.* 2005; Pare & Tumlingson 1999). Upon herbivory, plants induce the emission of herbivore-induced plant volatiles (HIPVs), which are usually composed of green leaf volatiles (C_6-alcohols and -aldehydes), aromatics, nitrogenous compounds and terpenoids. Terpenoids, the main component of HIPV blends, represent the most diverse group of natural products with ca. 25,000 reported and > 30,000 estimated structures (Gershenzon & Dudareva 2007; Mithöfer & Boland 2012). They are formed from isoprenoid units (C_5) and involve enzymes called "terpene synthases", which are transcriptionally upregulated after herbivory (Irmisch *et al.* 2014a).

Before the transcription of terpene synthases and other defense-related genes are upregulated, an intracellular signaling cascade is triggered after herbivore perception. Analogous to the recognition of pathogens by PAMPs, herbivores are recognized by herbivore-associated molecular patterns (HAMPs), chemical substances from the oral secretions or oviposition fluids from insects (Mithöfer & Boland 2008), as well as the temporal and spatial patterns of feeding (Mithöfer *et al.* 2005). Early signaling events after herbivore recognition in the plant include changes in the membrane potential, calcium ion influx, formation of reactive oxygen species, and the activation of several protein kinases (Fürstenberg-Hägg *et al.* 2013; Wu & Baldwin 2010). The most important signal – locally and systemically – after these early signaling events is the accumulation of jasmonic acid (JA) and its active form, the isoleucine conjugate jasmonoyl-isoleucine (JA-Ile). Through complex interactions with specific proteins, JA-Ile ultimately

activates transcription factors which in turn induce the expression of JA-responsive genes (Wasternack & Hause 2013), such as proteinase inhibitors (Farmer & Ryan 1990) or terpene synthases (Clavijo McCormick *et al.* 2014).

Although our knowledge of anti-herbivore defenses in plants derives from model organisms like arabidopsis or tomato, there is good evidence for similar mechanisms in woody plants. Many examples validate the defense-related role of JA in deciduous trees and conifers (Boeckler *et al.* 2013; Arnerup *et al*. 2013; Semiz *et al.* 2012; Lawrence *et al*. 2006; Martin *et al.* 2003; Kozlowski *et al.* 1999). Also some anti-herbivore defenses known from herbaceous plants are described in woody species (Eyles *et al*. 2010), for example, HIPVs (Miller *et al*. 2005; Clavijo McCormick *et al.* 2014), proteinase inhibitors (Major & Constabel 2008) and polyphenoloxidases (Ruuhola *et al*. 2008).

1.3. Complex tripartite interactions

Plants regularly face a multitude of different stresses in their natural environment, which includes abiotic factors such as drought or high temperature, as well as biotic actors such as viruses, pathogens and herbivores. Despite the fact that these stressors typically co-occur in nature, the interaction between them and their additive effects on plants are rarely studied under controlled conditions. As a consequence, our knowledge on complex interactions between more than two species or stress factors is still at a very early stage.

Simultaneously occurring plant antagonists, including herbivores and pathogens, can affect not only their common host plant but also each other. The effects of one plant antagonist on another can be either direct or indirect. Examples for direct interactions are the dispersion of plant pathogens by insect vectors (Kluth *et al.* 2002), the exploitation of fungal enzymes by insect herbivores (Kukor *et al.* 1988) and the ingestion of microbial tissue or microbe-derived compounds by herbivores (Sumarah & Miller 2009; Mondy *et al.* 2000; Martin 1979). Indirect effects, on the other hand, are conveyed by the host plant that the antagonists share and are therefore also termed “plant-mediated effects”. For example, infestation with whiteflies reduces the emission of HIPVs in cotton, which act as a defense against simultaneously feeding caterpillars (Rodriguez-Saona *et al.* 2003). Similarly, a belowground pathogen infection in oak increases the performance of caterpillars feeding aboveground by chemical changes in the leaves (Milanovic *et al.* 2015). The molecular mechanisms behind these observed effects are still not

entirely understood, but the crosstalk of phytohormones, which are induced simultaneously or subsequently, certainly plays a central role. JA, induced by chewing herbivores and necrotrophic pathogens, and SA, induced by piercing-sucking herbivores and biotrophic pathogens, are assumed to be the key players in this interaction. Their parallel activation might lead to synergistic, antagonistic or no effects (Derksen *et al.* 2013). However, among defense-activated signaling pathways there is a clear trend towards antagonism, usually caused by SA-signaling repressing JA-induced responses (Caarls *et al.* 2015; Spoel *et al.* 2007; Preston *et al.* 1999; Thaler *et al.* 1999). Apart from these two dominant defense hormones, other phytohormones such as ethylene, abscisic acid or gibberellin might also play a role in the signaling crosstalk upon multiple stresses (Robert-Seilaniantz *et al.* 2011).

However, studies investigating such complex interactions and especially the underlying mechanisms in woody plants are rare. The outcome of tripartite interactions seems to be dependent on several independent factors, such as host tree and attacker identity (Busby *et al.* 2015; Ahlholm *et al.* 2002) or the fertilization level in the soil (Eyles *et al.* 2007), making it difficult to draw general conclusions. More importantly, the field of complex interactions in trees lacks studies that combine ecological observations with a detailed analysis of molecular and chemical changes. This kind of comprehensive investigations is needed to understand and predict the outcomes of multiple attack scenarios in trees.

1.4. The study organisms: black poplar, poplar leaf rust and gypsy moth

In order to investigate the chemical ecology of complex tripartite interactions I chose three European species as study organisms: black poplar as the host tree, poplar leaf rust as the fungal plant pathogen, and gypsy moth as the insect herbivore.

Black poplar (*Populus nigra*; Figure 1 A) is a deciduous European tree species belonging to the Salicaceae family, which comprises, among others, members of the genera *Populus* (poplars) and *Salix* (willows) and is widely distributed throughout the northern hemisphere (Isebrands & Richardson 2014). Black poplar trees and other Salicaceae species inhabit riverbanks and floodplain forests. Even though regular disturbances by flooding and poor soil conditions impede the development of plant seedlings here, species of the Salicaceae are well adapted to this environment (Karrenberg *et al.* 2002). Poplar and willow trees therefore play an important role in shaping these ecosystems, by being a food source, habitat or shelter for a multitude of other

organisms. For poplar, more than 500 insect and mite species are reported to be found on trees of this genus (Mattson *et al.* 2001). Pathogens are also numerous on poplar trees, such as rusts (*Melampsora* spp.), stem canker (*Septoria* sp.), leaf and shoot blight (*Venturia* spp.) and leaf spot disease (*Marssonia* sp.), to mention only the most prevalent ones (Newcombe *et al.* 2001).

To defend against herbivores and pathogens, poplar trees possess various direct and indirect defense mechanisms. For example, feeding-damaged black poplar leaves emit a diverse bouquet of volatile organic compounds consisting of more than 80 different compounds, dominated by terpenoids (pers. observation). Certain compounds within this volatile blend are able to attract parasitoids for indirect defense (Clavijo McCormick *et al*. 2014) or to repel herbivores directly (Irmisch *et al.* 2014a). Constitutively, black poplar trees contain high amounts of phenolic compounds, especially condensed tannins and phenolic glucosides, which are termed "salicinoids" due to their occurrence in the Salicaceae and possession of salicin as a chemical core structure (Boeckler *et al.* 2011). Both condensed tannins and salicinoids are believed to be important in the direct defense against mammalian and insect herbivores (Boeckler *et al*. 2014; Barbehenn & Constabel 2011; Hemming & Lindroth 2000). Additionally, phenolic compounds are known to be crucial in the anti-microbial defense of poplars, as recently demonstrated for flavan-3-ols, which includes monomers such as catechin and polymers such as condensed tannins (Ullah *et al*. 2017). Due to black poplar's chemical defenses, ecological relevance and ease in cultivation (fast growth, clonal reproduction), this species is an ideal model organism for chemical-ecological studies. For similar reasons – fast growth, clonal reproduction and the ability to regrow from stumps – hybrids of this species are grown in plantations for economic use. The wood harvested from short-rotation coppices of poplars is processed to paper, pulp and biofuel (Karp & Shield 2008; Rytter 2006).

However, plantations as well as natural populations of poplar trees are regularly impacted by severe infections of rust fungi of the genus *Melampsora*, which are among the most damaging diseases of poplar (Stochlova *et al*. 2016; Pei & Shang 2005). Infection with these biotrophic fungi cause dramatic losses in biomass production from plantations (Wan *et al*. 2013; Gerard *et al*. 2006), premature defoliation and even mortality of young trees (Polle *et al*. 2013; Benetka *et al.* 2011). Rust fungi are as widespread as their host trees, and spores easily travel long distances by wind and water to other individuals and populations for further distribution (Nagarajan & Singh 1990). Additionally, the production of asexual uredospores, which can infect poplar over

prolonged periods, does not require the full life cycle which includes a second host *Larix spp.* and requires one year to complete (Hacquard *et al.* 2011). Instead, the rust fungus undergoes a vegetative life cycle in summer and autumn in which it repetitively produces massive amounts of the orange-colored uredospores within one week (Hacquard *et al.* 2011), resulting in dissemination to other poplar trees and reinfection of the same individual host. The rust infection process starts with germ tubes, which develop from the spores and penetrate through the stomata on the abaxial side of the leaf (Mlodzianowski *et al*. 1978; Duplessis *et al*. 2011). Inside the mesophyll cells of the leaf specialized structures differentiate from fungal infection hyphae to haustoria (Laurans & Pilate 1999). Haustoria are responsible for the nutrient uptake from the plant cell (Garnica *et al* 2014; Voegele & Mendgen 2003) and also play an important role in the release of effectors suppressing the host's defense response (Duplessis *et al.* 2009; Voegele *et al*. 2009). In a successful infection, the leaf mesophyll is densely colonized by fungal hyphae and haustoria and finally uredinia, spore-bearing structures, are formed for dispersal of the fungus (Hacquard *et al.* 2011). The economic losses caused by rust infections combined with the high prevalence of rust fungi in ecosystems have triggered increasing interest in studying poplar-rust interactions. *Melampsora larici-populina* (family: Melampsoraceae, order: Uredinales; Figure 1 B), the species responsible for most of the economic losses in European poplar plantations (Frey *et al*. 2005), has a sequenced genome (Duplessis *et al*. 2011) and is currently established as a model pathosystem in woody plant research.

Another threat for black poplar trees are herbivorous insects that feed on roots, wood, bark and leaves. Folivorous insects that consume leaf material are able to defoliate whole branches or even complete small trees and so limit the possibility for the host tree to assimilate carbon by photosynthesis. One species among the Lepidoptera, the gypsy moth (*Lymantria dispar,* family: Erebidae, order: Lepidoptera; Figure 1 C), is one of the most devastating tree defoliators in North America (Davidson *et al*. 1999). This insect, which is endemic to Europe, is an invasive species in the United States and Canada, where it is spreading (USDA 2017) since its first introduction into Massachusetts in 1869 (Hoover 2000). One reason for the success of this pest species is its broad host range. Even though larvae of gypsy moth prefer deciduous tree species such as oak, poplar or birch, they can feed on a broad range of broadleaf and coniferous trees of more than 40 different plant families (Robinson *et al*. 2010). The larvae not only cause damage to trees, but are also – apart from human-induced transportation – responsible for the spread of the species. Since female

moths are unable to fly (Wilson, 2018), the larvae represent the most mobile stage. Dispersal occurs by active movement along the branches as well as passively when young larvae hang down from silk threads and get transported by wind (Lance & Barbosa 1981). The gypsy moth is usually univoltine, producing one generation per year. However, under favorable environmental conditions a second generation can arise within the same year, causing a second wave of leaf damage on the host.

The three species used in this thesis, black poplar, poplar leaf rust and gypsy moth (Figure 1 A-C), are native to Europe and therefore their interaction occurs in natural ecosystems, either in summer with the first generation of gypsy moth or in autumn if the gypsy moth produced a second generation. While black poplar and the poplar leaf rust have a close, specialized association, the gypsy moth is a generalist herbivore and might also feed on other hosts. Its broad host range allows the gypsy moth to choose between various food sources, which makes it especially interesting to study its behavior, consequences and underlying mechanisms.

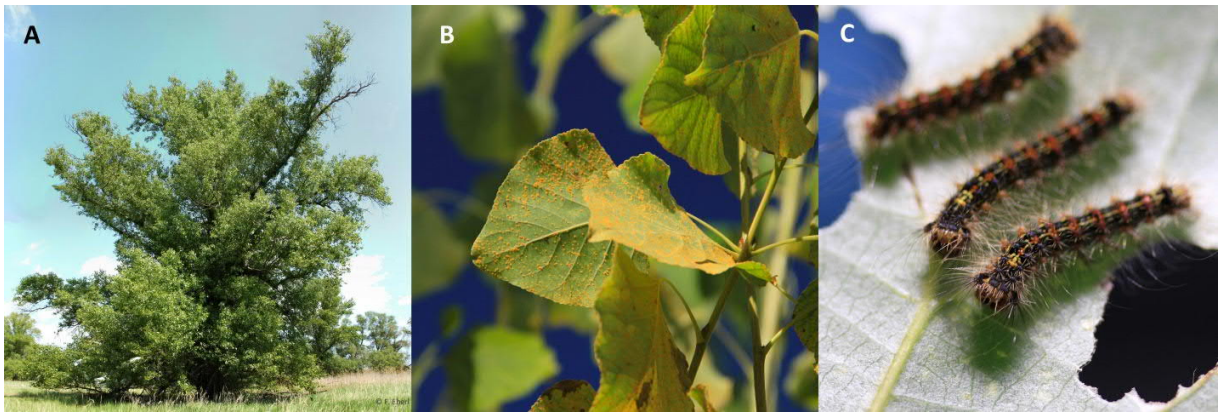

Figure 1. The three study species used in this thesis. A. Black poplar (*Populus nigra*) in its natural habitat, a floodplain forest. B. Poplar leaf rust (*Melampsora larici-populina*) sporulating on the abaxial side of black poplar leaves. C. Gypsy moth (*Lymantria dispar*) larvae feeding on a leaf of black poplar.

1.5. Aim of the thesis

Trees shape forest ecosystems as they harbor and interact with a huge diversity of other organisms. Despite the importance of woody plants in forest ecosystems, studies on the chemically mediated interactions of plants with other organisms have been predominantly conducted on short-lived herbaceous species. Additionally, complex interactions of plants with two or more antagonistic species as they appear in natural systems are still poorly understood, especially in trees.

The aim of this thesis was therefore to investigate the tripartite interactions between a woody plant species (black poplar, *Populus nigra*) and two different antagonists, a fungal pathogen (poplar leaf rust, *Melampsora larici-populina*) and an insect herbivore (gypsy moth, *Lymantria dispar*). The project focused on the molecular and chemical changes in the tree's physiology and metabolism after fungal infection alone as well as during simultaneous fungal and insect attack. The consequences of a host tree infection for herbivores were evaluated and explained by illuminating aspects of direct and indirect effects between plant pathogens and herbivores.

2. OVERVIEW OF MANUSCRIPTS

This thesis is based on the following manuscripts.

2.1. Manuscript I

Friend or foe?

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

Franziska Eberl, Christin Uhe, Sybille B. Unsicker

Published in *Fungal Ecology* (2018), in press. doi: 10.1016/j.funeco.2018.04.003.

Summary

This review summarizes the literature on the interaction of foliar fungi, trees and insects. First, the fungal colonization process and the molecular and physiological responses to it by trees are discussed for both endophytes and pathogens. Next, the effects of fungal colonization on the preference, performance and abundance of herbivorous insects on trees are reviewed, and factors influencing these tripartite interactions are highlighted. Finally, experimental and methodological approaches for future research are suggested.

Author Contributions

Conceptualization of article:	FE (35 %), CU, SBU
Literature research:	FE (40 %), CU, SBU
Manuscript writing:	FE (40 %), CU, SBU
Figure preparation:	FE (100 %)
Table preparation:	FE (70 %), CU

2.2. Manuscript II

Rust infection of black poplar trees reduces photosynthesis but does not affect isoprene biosynthesis or emission

Franziska Eberl, Erica Perreca, Heiko Vogel, Louwrance Wright, Almuth Hammerbacher, Daniel Veit, Jonathan Gershenzon, Sybille B. Unsicker

Published in *Frontiers in Plant Science* (2018), Volume 9: 1733; doi: 10.3389/fpls.2018.01733

Summary

In this study, we investigate the effect of leaf rust infection in black poplar trees on the emission of isoprene, a compound long associated with plant resistance to abiotic stresses. Using LI-COR measurements, rust infection was shown to cause an immediate and drastic decrease in photosynthetic activity and stomatal conductance mediated by phytohormonal regulation. Surprisingly, the biosynthesis and emission of isoprene, which is formed by the photosynthesis-dependent methylerythritol 4-phosphate pathway in the chloroplasts, was not affected by rust infection. However, the mevalonate pathway, an alternative cytosolic pathway of terpenoid biosynthesis, showed increases in biosynthetic gene transcripts and products in rust-infected leaves. A contribution of fungal metabolism to these terpenoid products is possible, but has to be verified by future research.

Author Contributions

Conceived project:	FE (40 %), LW, AH, JG, SBU, EP
Designed experiments:	FE (70 %), EP, DV
Performed experiments:	FE (70 %), EP, LW, DV
Chemical analysis:	FE (50 %), EP
Bioinformatics analysis:	HV
Data analysis:	FE (60 %), EP
Manuscript writing:	FE (80 %), EP, AH, JG, SBU

2.3. Manuscript III

Leaf rust infection reduces herbivore-induced volatile emission in black poplar and attracts a generalist herbivore

Franziska Eberl, Almuth Hammerbacher, Jonathan Gershenzon, Sybille B. Unsicker

Published in *New Phytologist* (2017), Volume 220 (3): 760-772; doi: 10.1111/nph.14565.

Summary

In this study the effect of simultaneous herbivore and pathogen attack on black poplar trees and the interaction between the two antagonists was investigated. Trees were challenged with a biotrophic leaf rust fungus and then a generalist leaf-chewing herbivore in sequence. Rust infection attenuated the biosynthesis and emission of herbivore-induced volatiles, which are an important part of the anti-herbivore defense in trees. This attenuation was mediated by the antagonistic crosstalk between the phytohormones induced by the two attackers. Rust infection alone also increased the emission of volatiles which influenced the behavior of the insect herbivores. Herbivores were attracted to rust-infected trees as well as to rust spores alone, showing a direct interaction between the two antagonists. This study demonstrates the presence of phytohormonal crosstalk in trees, as well as direct and indirect effects of a fungal pathogen on herbivorous insects.

Author Contributions

Conceived project:	FE (50 %), AH, JG, SBU
Designed experiments:	FE (90 %), AH, SBU
Performed experiments:	FE (100 %)
Chemical analysis:	FE (100 %)
Data analysis:	FE (100 %)
Manuscript writing:	FE (80 %) AH, JG, SBU

2.4. Manuscript IV

Co-evolution in a threesome?

The tripartite interaction of fungi, plants and herbivores

Franziska Eberl, Maite Fernandez de Bobadilla, Michael Reichelt, Almuth Hammerbacher, Jonathan Gershenzon, Sybille B. Unsicker

In preparation for *Nature Communications*

Summary

Having observed behavioral changes of herbivores towards poplar tree volatiles after rust infection, we here investigated the consequences of rust infection for the herbivores. Gypsy moth larvae in the early instars preferred to feed on black poplar leaves infected with rust compared to uninfected controls. The caterpillars further displayed selective feeding behavior for fungal tissue. This was true for spores of the rust fungus, as well as for mycelium of mildew, and was observed in gypsy moth and another related species. The sugar alcohol mannitol, which accumulates in infected leaves and fungal spores, was identified as a feeding stimulant for gypsy moth larvae. Further, gypsy moth larvae developed faster when feeding on rust-infected compared to uninfected trees. Elevated amino acid levels and high nitrogen content in the fungal spores appear to be responsible for this fitness advantage. This study proves strong evidence for the direct effects of fungal pathogens on herbivores sharing the same woody host.

Author Contributions

Conceived project:	FE (70 %), AH, JG, SBU
Designed experiments:	FE (80 %), MFdB, SBU, JG
Performed experiments:	FE (90 %), MFdB
Chemical analysis:	FE (90 %), MR
Data analysis:	FE (90 %), MFdB
Manuscript writing:	FE (90 %) AH, JG, SBU

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Friend or foe?

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

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Abstract

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

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

Keywords: Biotrophic pathogen, defense mutualism, folivore, mutualistic fungus, trophic interactions, necrotrophic pathogen, pathosystem, tree defense

Introduction

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

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

indirect effects of tree-inhabiting fungi on herbivorous insects. Finally, we make suggestions for future research directions.

Fungus-tree interactions

Pathogenic fungi

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

Fungal diseases are widespread in plant systems, although a successful infection requires the synergy of a susceptible host, a virulent pathogen and favorable environmental conditions (Ferreira *et al.* 2006). Plants can recognize a pathogen attack either by general pathogen-associated molecular patterns, provoking pattern-triggered immunity (Naidoo *et al.* 2014), or by a specific interaction involving the gene-for-gene theory (Flor 1971, Ferreira *et al.* 2006), also termed as effector-triggered immunity (Naidoo *et al.* 2014). In the latter interaction, the plant receptor (product of *R* (resistance) gene) binds to the product of the *avr* (avirulent) gene from the pathogen. If both the *R* gene of the plant and the corresponding *avr* gene in the pathogen are

present, it will be considered an incompatible interaction: the plant defense mechanisms will be triggered early and no infection will be established (Dangl & Jones 2001, Ferreira *et al.* 2006). If one of the two components is missing, or not functioning, a compatible interaction occurs, thus leading to successful disease development (Ferreira *et al.* 2006). In poplar trees (*Populus sp*.) the nucleotide-binding-site leucine-rich-repeat (NBS-LRR) family, an important class of *R* genes, is larger than in other angiosperm genomes. For example, *Arabidopsis* possesses 178 NBS-LRR genes, whereas *Populus trichocarpa* has 402 NBS-LRR genes (Duplessis *et al.* 2009). Within the *P. trichocarpa* genome, this gene family is one of the largest families, making up about 1% of the total number of genes (Duplessis *et al.* 2009). This suggests that early pathogen recognition may be more important for long-lived, large-sized trees than for smaller annual plants.

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

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

The pathogen will try to evade recognition by the host and continuously counteract the defense mechanisms, making the infection process an ongoing battle between fungus and plant. The production of the sugar alcohol mannitol, an acyclic polyol, for example was reported in biotrophic rust fungi (Voegele *et al.* 2005) as well as the necrotrophic fungus *Alternaria* (Jennings *et al.* 2002). Its antioxidant properties might help the pathogen to quench ROS produced by its host plant. Some pathogens also release enzymes that target the host plant cell wall and hydrolyze the long-chain carbon polymers (Ferreira *et al.* 2006). Biotrophic fungi are even able to shift source-sink relationships within the host, so that infected tissue becomes a sink to which carbon sources are transported (Ferreira *et al.* 2006, Voegele & Mendgen 2011). Necrotrophic fungi can harm the plant in a more aggressive way by releasing phytotoxic compounds (Evidente & Motta 2001). The red-band needle blight-causing fungus *Dothistroma pini* for example produces a red-colored toxin with a similar anthraquinone structure as aflatoxin (Bradshaw 2004). It is activated by light and leads to the production of damaging oxygen radicals. Alkaloids, which are well known from grass diseases involving ergot alkaloids (Miedaner & Geiger 2015),

also play a role in tree-pathogen interactions. The conifer pathogen *Sphaeropsis sapinea* produces sapinopyridione which causes yellowing and dieback of young cypress trees (Evidente *et al.* 2006). Plant defense mechanisms have been intensivley studied, but the activity of pathogen-derived responses should not be underestimated and needs further attention in future studies.

Endophytic fungi

Tree endophytes that mainly consist of Ascomycota and Basidiomycota (Petrini 1986) belong to the non clavicipitaceous group of endophytes (Rodriguez *et al*. 2009) and occur in above-ground plant tissues and in roots, distinguishable from mycorrhiza by lacking external hyphae (Mandyam & Jumpponen 2005, Yuan *et al.* 2007). Like fungal plant pathogens hyphae of germinating endophytic spores can penetrate directly through leaf cuticle, stomata or other leaf openings, but unlike pathogen infection, endophytes undertake a quiescent state after infection (Sieber 2007). Apart from random distribution of fungal spores e.g. *via* wind dispersal, the occurrence and distribution of endophytes is assumed to be affected by the genetic structure of the host plant (Hata & Futai 1996, Collado *et al.* 1999, Ahlholm *et al.* 2002, Helander *et al.* 2006) and/or large- and small-scale climate conditions (Carroll & Carroll 1978, Arnold & Herre 2003, Arnold & Lutzoni 2007). Unterseher *et al.* (2007) showed that the fungal endophyte distribution in trees also varies within the tree crown e.g. depending on whether leaves are sun-exposed or not. Furthermore, this study revealed that the endophyte species *Apiognomonia errabunda* exhibits distinct seasonal patterns in occurrence, with high abundance in young leaves in spring and very low abundance in older leaves in autumn. The authors of this study also suggest a relationship between the distribution of fungal endophytes and the accumulation of antifungal secondary metabolites in the tree throughout the growing season (Unterseher *et al.* 2007). Our knowledge of which specific factors determine the distribution and occurrence of fungal endophytes in treetops is limited, specifically with respect to species-specific requirements. It has been shown

that some fungal endophytes benefit their host by enhancing plant growth (Khan *et al.* 2016). Bullington & Larkin (2015) speculated that early colonization with beneficial fungal endophytes allow better plant growth and thus promoting plant defense. For instance, re-inoculation of *Preussia* sp. in the host tree *Boswellia sacra* leads to enhanced plant growth and accumulation of photosynthetic pigments (Khan *et al.* 2016). In another study, the plant growth promoting siderophore ferrirubin was isolated from *Talaromyces pinophilus,* an endophytic fungus colonizing the strawberry tree (*Arbutus unedo*, Vinale *et al.* 2017). Endophytic yeast fungi isolated from the stems of poplar trees were shown to produce plant hormones which directly increase plant growth (Doty 2011). In general our knowledge of the consequences of endophyte infection for tree performance and tree defense is scarce and limited to only a few studies on single-species interactions. Under natural conditions, however, the foliage of trees is colonized by numerous microbial endophytes at the same time. Investigating whether the consequences of complex multi-species interactions for tree performance and defense can be predicted from simple single-species interactions should be the focus of studies in the future.

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

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

Direct and indirect effects of fungi on tree-insect interactions

The effect of plant-inhabiting fungi on herbivorous insects is either direct, from the fungus to the insect, or indirect *via* fungus-inflicted changes of the host plant. Direct effects can be provoked by

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

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

Tree pathogen effects on herbivorous insects

The effects of tree-pathogenic fungi on insect herbivore performance and behavior reported in the literature are inconsistent. A few studies report positive responses of herbivorous insects to pathogen infection (Johnson *et al.* 2003, Milanović *et al.* 2015, Eberl *et al.* 2017), while others document detrimental effects (Eyles *et al.* 2007, Zargaran *et al.* 2012, Busby *et al.* 2015) or no effect (Kellogg *et al.* 2005). General conclusions on the effect of tree-pathogens on insect

herbivore performance and behavior cannot be drawn. The outcome of such multi-trophic interactions for insects seems to be dependent on many factors, and the interplay of these factors is not yet well understood. First, the identity of the fungus and the insect plays an important role, as seen in birch (Ahlholm *et al.* 2002) and in oak, where the composition of the arthropod community changed in response to mildew infection (Tack *et al.* 2012). Similarly, the species and genotype of the host tree, which both determine the susceptibility to biotic stressors, is an important predictor of the impact on herbivores (Busby *et al.* 2015). Furthermore, environmental conditions strongly influence the effect of tree-pathogen infection on insect herbivores. In Austrian pine (*Pinus nigra*), for example, the fertility level determines whether infection with the necrotrophic fungus *Sphaeropsis sapinea* has a slightly positive (high nitrogen), no (medium nitrogen) or a negative (low nitrogen) effect on the survival of sawfly (*Neodiprion sertifer*) larvae (Eyles *et al.* 2007). Focusing on the fungal infection, there are also variables influencing trophic interactions. In the well-studied willow-rust system the behavior of the willow leaf beetle *Plagiodera versicolora* depends on the spatial distance of the beetle to the pathogen infection site (Simon & Hilker 2005) as well as on the time since the onset of infection (Peacock *et al.* 2003, Simon & Hilker 2005). In *Botrytis*-infected wine, the importance of the disease severity was also shown, by monitoring the choice and oviposition of a lepidopteran moth (Rizvi & Raman 2015). A central problem for deducing generalities from tree-pathogen-insect studies are differences in experiment methodology and the varying level of observation (**Figure 1, Table 1**). Several studies have been conducted under field conditions at the level of tree populations (Bashford 2002, Zargaran *et al.* 2012, Funamoto & Sugiura 2017), whereas other studies focused on detached single leaves from trees grown under laboratory conditions (Simon & Hilker 2003, Rizvi & Raman 2015) or collected from the field (Lappalainen *et al.* 1995, Ahlholm *et al.* 2002, Tack *et al.* 2012, Milanović *et al.* 2015). Some studies also performed experiments with different pathogen and insect species in individual trees (Peacock *et al.* 2003, Kellogg *et al.* 2005, Simon & Hilker 2005, Eyles *et al.* 2007, Busby *et al.* 2015, Eberl *et al.* 2017). On the insect

side, mostly behavioral responses (Mondy *et al.* 1998, Peacock *et al.* 2003, Simon & Hilker 2005, Moskowitz & Haramaty 2012, Busby *et al.* 2015, Rizvi & Raman 2015) and performance (Ahlholm *et al.* 2002, Simon & Hilker 2003, Mondy & Corio-Costet 2004, Kellogg *et al.* 2005, Eyles *et al.* 2007) in response to pathogen infection in trees have been assessed. Other studies have observed the arthropod community structure, i.e. species richness and abundance, as a consequence of pathogen infection in trees (Tack *et al.* 2012, Zargaran *et al.* 2012, Busby *et al.* 2015, Funamoto & Sugiura 2017). Currently, there is still little information on the molecular mechanisms for tree-pathogen-insect interactions, such as phytohormone signaling (Xu *et al.* 2009, Naidoo *et al.* 2013), gene expression patterns (Duplessis *et al.* 2011, Büchel *et al.* 2012), or general phytochemical patterns including primary as well as secondary metabolites. Studies that combine behavioral or performance assays with a molecular approach to explain the ecological patterns seen in these multi-trophic interactions are even rarer. Johnson *et al.* (2003) observed a positive correlation between the abundance of aphids (*Euceraphis betulae*) and the necrotrophic pathogen *Marssonia betulae* occurring in trees of a natural birch population. In addition, choice and performance tests supported the positive interaction between these two antagonists. Finally, the enhanced aphid performance could be explained by elevated levels of free amino acids that were found in infected birch leaves. More recently, Eberl *et al.* (2017) reported reduced emission of plant volatiles, an indirect anti-herbivore defense, in rust-infected and herbivore-attacked poplar trees. The diminished emission could be explained by an antagonistic phytohormonal crosstalk and a downregulation of biosynthetic genes. Furthermore, an olfactometer experiment revealed that gypsy moth (*Lymantria dispar*) caterpillars were more attracted to the odor of their food plant *Populus nigra* when it was infected with the leaf rust fungus *Melampsora larici-populina* than to the odor of non-infected control trees. The caterpillars were also attracted to the smell of rust spores alone, showing even a direct interaction between insects and fungus (Eberl *et al.* 2017). A clear plant-mediated effect was observed in oak (Milanović *et al.* 2015). In this study, the pathogen *Phytophthora plurivora* infected the roots

below ground, whereas the herbivores *Lymantria dispar* fed on aboveground tissue of the same tree. Here, the larvae performed better on leaves of infected trees due to a higher protein content compared to uninfected trees. These examples illustrate that numerous factors, such as species identity temporal and spatial patterns as well as environmental conditions, determine the effect of a plant pathogen on plant-feeding insects. To disentangle the role of individual factors, future studies should start manipulating one factor at a time while keeping the other factors as stable as possible. In this way, we can understand the impact of single factors and have a basis to extend studies manipulating two or more factors, to also reveal interactive effects.

Tree endophyte effects on herbivorous insects

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

Carroll & Carroll (1978) were the first to suggest that endophytic fungi benefit coniferous trees by making the needles less palatable for herbivorous insects. A study with Spanish elm seedlings (*Cordia alliodora*) showed that leaf cutter ants (*Atta colombica*), when given a choice, prefer to cut leaves from trees with low over high endophyte density (Bittleston *et al.* 2011), suggesting that high loads of endophytes in tree leaves generate costs to the ants. *Phialocephala scopiformis* isolated from evergreen White spruce (*Picea glauca*) is probably the best studied

endophytic fungus in the context of tree-insect interactions. This fungal species produces the yellow pigment rugulosin, known to negatively affect the performance of herbivorous insects such as the spruce budworm (*Choristoneura fumiferana*) and the hemlock looper (*Lambdina fiscellaria*) (Miller *et al.* 2002, Miller *et al.* 2008, Sumarah *et al.* 2008, Sumarah & Miller 2009, Sumarah *et al.* 2010). Furthermore, numerous other compounds isolated from endophytes in conifer needles were shown to have toxic effects on the spruce budworm, suggesting that these fungi play a crucial role in tree defense against insect herbivores (reviewed by Sumarah & Miller 2009). More recently, Vinale *et al.* (2017) showed that the survival of the pea aphid *Acyrthosiphon pisum* was negatively affected by the bioactive metabolite 3-O-methylfunicone isolated from *Talaromyces pinophilus*, an endophytic fungus of the Strawberry tree (*Arbutus unedo*). This result suggests that chemicals isolated from tree-inhabiting endophytic fungi may also play a role in protecting crop species against their insect pests in the future.

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

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

Concluding remarks

Leaf-colonizing fungi can produce chemical compounds or modify the host plant metabolism and thus directly and indirectly affect the interaction of plants with insect herbivores. So far, the majority of studies investigating the tripartite interaction between fungi, plants and insects have been performed in short-lived annuals within an agricultural context, and mostly with plant pathogens, but rarely endophytes. The consequences of fungal colonization for the interaction of long-lived woody perennials with insect herbivores are barely known and our mechanistic understanding of the role of pathogenic and endophytic fungi for tree-insect interactions is incomplete. Neither pathogenic fungi nor endophytic fungi in trees have shown consistently negative or positive effects on insect herbivores in the studies reviewed for this manuscript (**Table 1**). Descriptive studies have shown that arboricolous fungi can shape the diversity and composition of insect herbivore species but the underlying molecular mechanisms for these community level effects are barely understood. So far, the taxonomic identification of endophytic fungi in trees has been restricted to easily cultivable species and thus numerous uncultivable species have been overlooked (Arnold 2007, Hyde & Soytong 2008, Unterseher *et al.* 2011). In the future, high-throughput sequencing techniques should be applied to detect less abundant and

uncultivable taxa (Rajala *et al.* 2014). Furthermore, novel in vitro culturing techniques should be developed to reveal fungal metabolite profiles and biosynthetic pathways, also of so far non-cultivable species (e.g. biotrophic pathogens). Non-targeted metabolomics and proteomics of fungi *in vitro* and *in planta* will help to reveal fungus-derived compounds and fungus-inflicted metabolite changes in trees. A detailed knowledge of the biosynthetic pathways and metabolite profiles of single fungal species in trees will then allow us to study the consequences of fungal colonization for insect herbivores at the molecular level. Methodologically this is certainly challenging as it requires sterile trees to be infected with a single fungal species to start off with. Experiments with only one fungal species at a time are automatically restricted to young trees propagated from sterile tissue cultures under laboratory conditions, as trees under natural conditions are usually colonized by microbes. Although single-species interactions with immature trees do not reflect real-world scenarios, we argue that very controlled studies are essential to gain comprehensive mechanistic knowledge of the role of fungi in tree-insect interactions. To validate the results from laboratory studies, performing descriptive and experimental studies under natural field conditions are recommended.

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Figures

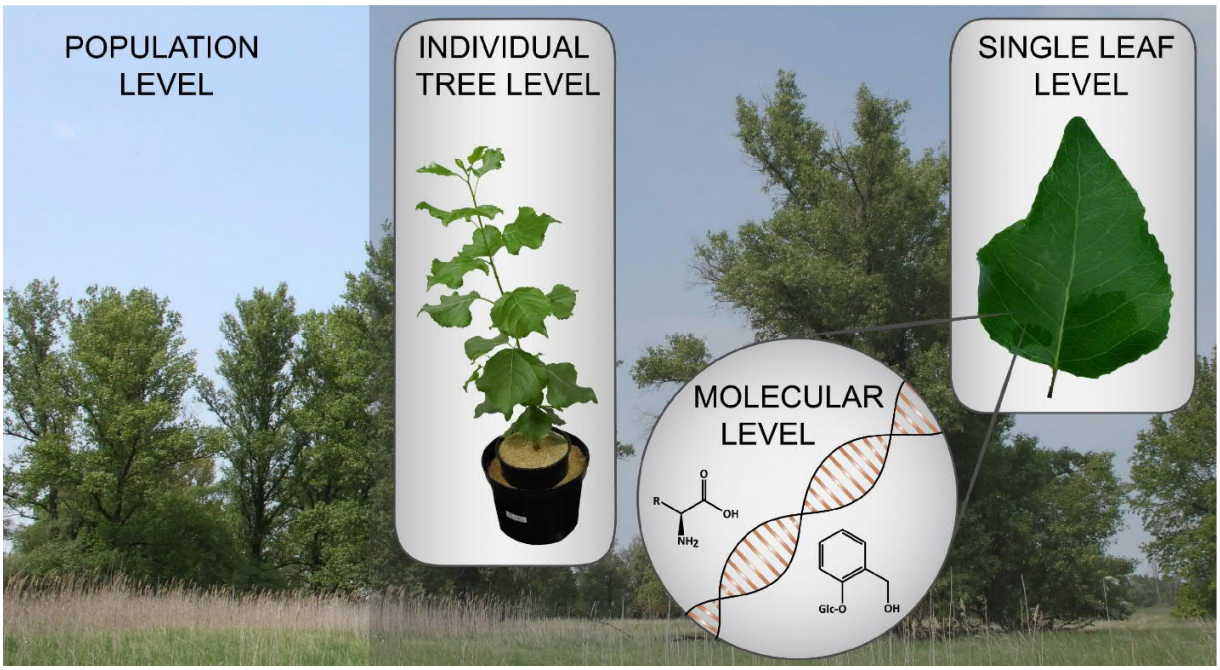

Figure 1: Different levels of observation used in the studies focusing on fungus-tree-insect interactions. Populations are considered in field and common garden surveys, other studies work on the level of individual trees, in the field as well as in laboratory studies. Setups using single leaves – attached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

Tables

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

Fungal life strategy	Tree species	Fungal species	Insect species	Insect feeding guild	Parameter	Effect on insect	Location	Level	Reference
biotroph	*Populus nigra*	*Melampsora larici-populina*	*Lymantria dispar*	chewing	behavior	+	L	tree, molecular	(Eberl *et al.* 2017)
	Salix viminalis	*Melampsora epitea*	*Phratora vulgatissima*	chewing	behavior	-	L	tree	(Peacock *et al.* 2003)
	Salix x cuspidata	*Melampsora allii-fragilis*	*Plagiodera versicolor*	chewing/ skeletonizing	performance	-	L	leaf	(Simon & Hilker 2003)
	Salix x cuspidata	*Melampsora allii-fragilis*	*Plagiodera versicolor*	chewing	behavior	-	L	tree, leaf	(Simon & Hilker 2005)
	Quercus spp.	*Erysiphe alphitoides*	*Cynipidae*	galling	abundance	-	F	population	(Zargaran *et al.* 2012)
	Quercus robur	*Erysiphe alphitoides*	arthropod community	all	abundance	+/- [a]	F	population	(Tack *et al.* 2012)
			Tischeria ekebladella	mining	behavior	+	F	leaf	
			Acronicta psi	chewing	performance	-	F	leaf	
	Betula pubescens	*Melampsoridium betulinum*	*Epirrita autumnata*	chewing	performance	- / 0 [b]	F	population, leaf	(Lappalainen *et al.* 1995)
	Betula pubescens	*Melampsoridium betulinum*	*Epirrita autumnata*	chewing	performance	0	F	leaf	(Ahlholm *et al.* 2002)
			Deporaus betulae	rolling	abundance	0	F	tree	
			Eriophyes rudis	galling	abundance	0	F	tree	
			Arge sp.	chewing	performance	0	F	leaf	

Fungal life strategy	Tree species	Fungal species	Insect species	Insect feeding guild	Parameter	Effect on insect	Location	Level	Reference
			Priophorus pallipes	chewing	performance	0	F	leaf	
			Dineura pullior	skeletonizing	performance	-	F	leaf	
	Cinnamomum yabunikkei	*Melanopsichium onumae*	arthropod community	spore & gall feeding	abundance	+	F	population	(Funamoto & Sugiura 2017)
	Acacia dealbata	*Uromycladium spp.*	arthropod community	all	abundance	+	F	population	(Bashford 2002)
necrotroph	*Populus spp.*	*Drepanopeziza populi*	arthropod community	all	behavior	-	F	tree	(Busby *et al.* 2015)
					abundance	-	F	tree	
	Quercus rubra	*Phytophthora plurivora*	*Lymantria dispar*	chewing	behavior	+	F + L	population, leaf	(Milanović *et al.* 2015)
					performance	+	F + L	leaf, molecular	
	Betula pendula	*Marssonia betulae*	*Euceraphis betulae*	piercing-sucking	abundance	+	F	population	(Johnson *et al.* 2003)
					behavior	+	F	leaf	
					performance	+	F	leaf, molecular	
	Pinus nigra	*Sphaeropsis sapinea*	*Neodiprion sertifer*	chewing	performance	- / 0 [c]	F	tree	(Eyles *et al.* 2007)
endophyte	*Populus tremula*	*Aureobasidium sp.*	*Phratora vitellinae*	chewing	behavior	-	F	tree	(Albrectsen *et al.* 2010)[d]
	Quercus garryana	*Discula quercina*	*Besbicus mirabilis*	galling	performance	-	F	leaf	(Wilson & Carroll 1997)
			Bassettia ligni	galling	performance	0	F	leaf	
	Quercus emoryi	*Asteromella sp.*	*Cameraria sp.*	mining	performance	0	F	leaf	(Faeth & Hammon

Fungal life strategy	Tree species	Fungal species	Insect species	Insect feeding guild	Parameter	Effect on insect	Location	Level	Reference
									1997)
		Plectophomella sp.	*Cameraria sp.*	mining	performance	0 / - [e]	F	leaf	
	Betula pubescens	*Fusicladium sp.*	*Epirrita autumnata*	chewing	performance	- [f]	F	leaf	(Ahlholm *et al.* 2002)
			Deporaus betulae	rolling	abundance	-	F	tree	
			Eriophyes rudis	galling	abundance	0	F	tree	
			Arge sp.	chewing	performance	0	F	leaf	
			Priophorus pallipes	chewing	performance	0	F	leaf	
			Dineura pullior	skeletonizing	performance	0 [f]	F	leaf	
		Melanconium sp.	*Epirrita autumnata*	chewing	performance	0	F	leaf	
			Deporaus betulae	rolling	abundance	0	F	tree	
			Eriophyes rudis	galling	abundance	0	F	tree	
			Arge sp.	chewing	performance	0	F	leaf	
			Priophorus pallipes	chewing	performance	0	F	leaf	
			Dineura pullior	skeletonizing	performance	+	F	leaf	
	Embothrium coccineum	various species	arthropod community	all	behavior	-	F	population, tree	(Gonzalez-Teuber 2016)
	Cordia alliodora	unknown	*Atta colombica*	cutting	behavior	-	L	tree	(Bittleston *et al.* 2011)
	Arbutus unedo	*Talaromyces pinophilus*	*Acyrthosiphon pisum*	piercing-sucking	performance	-	L	molecular [g]	(Vinale *et al.* 2017)
	Picea rubens	unknown	*Choristoneura fumiferana*	chewing	performance	-	L	molecular [h]	(Sumarah *et al.* 2010)
	Picea glauca	*Phialocephala*	*Choristoneura*	chewing	performanc	-	L	leaf,	(Miller *et al.*

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

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

4. MANUSCRIPT II

Rust infection of black poplar trees reduces photosynthesis but does not affect isoprene biosynthesis or emission

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Rust Infection of Black Poplar Trees Reduces Photosynthesis but Does Not Affect Isoprene Biosynthesis or Emission

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Poplar (*Populus* spp.) trees are widely distributed and play an important role in ecological communities and in forestry. Moreover, by releasing high amounts of isoprene, these trees impact global atmospheric chemistry. One of the most devastating diseases for poplar is leaf rust, caused by fungi of the genus *Melampsora*. Despite the wide distribution of these biotrophic pathogens, very little is known about their effects on isoprene biosynthesis and emission. We therefore infected black poplar (*P. nigra*) trees with the rust fungus *M. larici-populina* and monitored isoprene emission and other physiological parameters over the course of infection to determine the underlying mechanisms. We found an immediate and persistent decrease in photosynthesis during infection, presumably caused by decreased stomatal conductance mediated by increased ABA levels. At the same time, isoprene emission remained stable during the time course of infection, consistent with the stability of its biosynthesis. There was no detectable change in the levels of intermediates or gene transcripts of the methylerythritol 4-phosphate (MEP) pathway in infected compared to control leaves. Rust infection thus does not affect isoprene emission, but may still influence the atmosphere *via* decreased fixation of CO_2.

Keywords: biotrophic pathogens, disease, isoprenoids, MEP pathway, non-mevalonate pathway, plant hormones, Salicaceae, stomatal conductance

INTRODUCTION

Poplar (*Populus* spp.) trees are deciduous, woody plants that are widely distributed in the northern hemisphere (Stanton et al., 2010; Isebrands and Richardson, 2014). In their natural habitat, which consists of floodplain forests and riverbanks, they are of significant ecological importance as host plants for an enormous diversity of microbes, insects, and other organisms. Apart from that, poplar trees have gained increased economic attention in recent years as fast growing sources of wood, plywood, paper, and biofuels when grown in short-rotation coppices (Karp and Shield, 2008). The fast growth, which also favors agronomical use, combined with the availability of the sequenced genome (Tuskan et al., 2006) and the possibility of clonal reproduction, makes this tree

an excellent model organism for woody plant research. However, natural populations as well as plantations of poplar regularly suffer from severe infections by biotrophic rust fungi (Polle et al., 2013). *Melampsora* rusts are among the most devastating diseases for poplars worldwide (Pei and Shang, 2005; Wan et al., 2013; Štochlová et al., 2016). An epidemic outbreak of rust disease can cause drastic losses over 50% in biomass production[1] (Gérard et al., 2006), premature defoliation and even mortality in young stands (Aylott et al., 2008; Benetka et al., 2011; Polle et al., 2013). Upon rust infection, poplar trees activate the salicylic acid (SA) defense signaling pathway (Azaiez et al., 2009; Eberl et al., 2018), as in many other plants infected by biotrophic pathogens (Glazebrook, 2005) leading to an enhanced production of phenolic secondary metabolites and the expression of pathogenesis-related proteins (Miranda et al., 2007; Rinaldi et al., 2007; Azaiez et al., 2009; Chen et al., 2014; Ullah et al., 2017). However, studies connecting rust infection with primary physiological processes are rare, even though it can be assumed that the biotrophic lifestyle of the rust fungus will unbalance the host's primary metabolism dramatically (Berger et al., 2007; Voegele and Mendgen, 2011). Rust infection also modifies volatile emissions of poplar, leading to increased release of monoterpenes (C_{10}) and sesquiterpenes (C_{15}) (Eberl et al., 2018). In addition, poplar trees emit large amounts of isoprene (C_5). This small volatile hydrocarbon is emitted by a number of plant species, most of them woody. Poplar trees are amongst the strongest isoprene emitters known (Logan et al., 2000; Laothawornkitkul et al., 2009). In the atmosphere, isoprene is involved in the formation of ozone and hydroxyl radicals, and hence plays an important role in atmospheric chemistry (Sharkey et al., 2008). Even though isoprene is emitted in considerable amounts that exceed the emission of all other biogenic volatiles (Guenther et al., 1995), its biological function is still not well understood. Isoprene is hypothesized to increase the thermotolerance of plants, protect against ozone and oxygen radicals, and act as a "safety valve" for dissipating energy under high light conditions (Loreto and Velikova, 2001; Sharkey et al., 2008; Laothawornkitkul et al., 2009).

The biosynthesis of isoprene occurs in the chloroplasts *via* the methylerythritol 4-phosphate (MEP) pathway (Logan et al., 2000), an alternate route for isoprenoid production to the mevalonate (MVA) pathway, which is located in the cytoplasm. The MEP pathway is present in higher plants, algae and some bacteria, but not in fungi and animals (Hemmerlin et al., 2012). Both pathways produce dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IDP), the universal building blocks for isoprenoid formation (Rodríguez-Concepción and Boronat, 2015). The regulation of both pathways occurs at different levels, ranging from transcriptional control of genes encoding biosynthetic enzymes to post-translational modifications of pathway enzymes (Hemmerlin, 2013). The MEP pathway is tightly connected to photosynthesis, not only spatially by sharing the same compartment, but also metabolically. The MEP pathway consumes metabolic intermediates, energy and reducing equivalents drawn directly from the light and dark reactions; in return it produces the photosynthetic pigments, chlorophylls and carotenoids, and phytohormones (Hemmerlin et al., 2012). One of these hormones is abscisic acid (ABA) which controls stomatal opening and mediates responses to drought stress (Acharya and Assmann, 2009). However, ABA is also known to be involved in defense reactions against biotic stressors, but usually acts as an antagonist to SA-mediated signaling cascades (Ton et al., 2009; Cao et al., 2011).

In the past, most studies on isoprene emission have focused on its biosynthesis, its effects on atmospheric chemistry and its involvement in plant interactions with the abiotic environment. For poplar, 40% of all the articles on isoprene emission since 1990 studied its relation to abiotic environmental factors[2] (January 2018), while only 3% studied its relation to biotic environmental factors (Brilli et al., 2009; Müller et al., 2015; Jiang et al., 2016).

We therefore investigated the effect of the pathogenic rust fungus *Melampsora larici-populina* on isoprene emission from black poplar trees (*Populus nigra*). We monitored temporal changes of photosynthesis and isoprene emission during infection and investigated underlying physiological mechanisms by analyzing aspects of leaf chemistry, physiology, and transcriptional changes.

MATERIALS AND METHODS

Experimental Material

Black poplar (*P. nigra* L.) trees were grown from hardwood cuttings (photosynthesis experiment and isoprene experiment) or softwood cuttings (transcriptome experiment) obtained from different tree genotypes growing in a common garden in Isserstedt, Germany (50°57′28.5″N 11°31′17.4″E). One genotype was used for the transcriptome experiment as well as for the photosynthesis and isoprene measurements, whereas two different genotypes were additionally used for photosynthesis measurements. The cuttings were potted in 2 l- pots, grown in the greenhouse (18/20°C, night/day, relative humidity 60%, natural light with 9–14 h photoperiod, supplement light for 12 h, SON-T Agro; Philips, Andover, MA, United States) and transferred to an environmental chamber [18/20°C, night/day; relative humidity 60%; photoperiod 16 h, MT 400 (Eye, Uxbridge, United Kingdom)] 2 days before the onset of the experiment. All three experiments (photosynthesis experiment, isoprene experiment and transcriptome experiment) were conducted separately at different time points but under the same temperature and humidity conditions. All trees were used ca. 4 months after potting and had reached a height of about 0.5 m. Trees used for photosynthesis and isoprene measurement did not show any noticeable shoot growth at time of experiment.

Uredospores of the biotrophic poplar leaf rust fungus (*M. larici-populina* Kleb.) were obtained from naturally infected black poplar trees growing in the above-mentioned common

[1] https://ohioline.osu.edu/factsheet/plpath-tree-8

[2] www.webofknowledge.com

garden. The identity of the fungus was verified by using specific primers for the internal transcribed spacer region of *M. larici-populina* as described in Eberl et al. (2018). The pathogen was amplified by infecting 1-year-old trees, and after 2–3 weeks uredospores were harvested with a scalpel and a brush. Spores were stored at −20°C until the start of the experiment either dried over silica overnight (photosynthesis experiment and transcriptome experiment) or used within 2 months after harvesting (isoprene experiment). Plants were inoculated with the fungus by spraying a mixture of water and spores (dry: 1 mg ml^{-1} and fresh: 1.5 mg ml^{-1}) on the abaxial side of each leaf (approximately 1 ml per leaf) and covering each tree with a polyethylene terephthalate (PET) bag (Bratschlauch, Toppits, Minden, Germany), which was kept closed for 1 day to ensure sufficient humidity for spore germination. Control groups received the same treatments but were sprayed with water only. First sporangia in the rust-infected trees were visible on the abaxial side of the leaves at 7 dpi (days post-infection) (**Supplementary Figure S1**), which matches the time course of infection in the literature (Hacquard et al., 2011). Under natural conditions individual poplar leaves are usually exposed to many cycles of rust infection. We decided to investigate only a single cycle of infection to better determine which stage of infection influences plant processes.

Photosynthesis Measurements

Photosynthetic parameters were measured on the second mature leaf (counting from the apex) of six trees from both groups ("control" and "rust-infected"; $n = 6$) at six different time points: 1 day before rust infection (−1 dpi), 4 h post-infection (hpi), 1, 7, and 10 dpi. Due to unexpectedly high CO_2-levels in the air supply (**Supplementary Figure S3**) at one measurement time (4 dpi), we excluded this data point from further analysis. All measurements were conducted at the same time of the day (9 am–12 pm) except for 4 hpi (1 pm–4 pm). The leaf was put into a custom-made single leaf chamber [chamber: polyoxymethylene, lid: poly(methyl methacrylate), openings sealed with sponge rubber; for picture see **Supplementary Figure S2**], which was connected to a LI-6400XT Portable Photosynthesis System (LI-COR, Lincoln, NE, United States). For each treatment a separate chamber was used to avoid contamination by fungal spores of the control leaves. The air supply for the LI-6400XT was filtered through active charcoal and humidified to 12%. An LED lamp (850 PAR at leaf position; 5 W, warm white and cool white; Roschwege GmbH, Greifenstein, Germany; for spectrum see **Supplementary Figure S4**) was placed over the leaf chamber as light source. The two different treatments were measured alternatingly to avoid temporal effects for one of the groups. The leaf was allowed to equilibrate to the LED light for 10 min before it was connected to the LI-6400XT, and photosynthetic parameters (photosynthetic rate, stomatal conductance and intercellular CO_2) were measured for 10 min. The photosynthetic rate and stomatal conductance were normalized to the leaf area, which was determined with Photoshop CS5 (Adobe, San Jose, CA, United States) from a picture taken at the end of the experiment with a reference field of known size. As the trees were not actively growing during the experiment, the leaf size was assumed to be the same throughout the whole experiment. After the last measurement, the leaf was flash-frozen in liquid nitrogen for phytohormone and sugar analysis.

Chemical Analyses

Phytohormones and sugars were analyzed from 10 mg freeze-dried, ground leaf material of the photosynthesis experiment ($n = 6$). Phytohormone analysis was carried out on an LC/MS/MS system as previously described (Eberl et al., 2018). Data were processed using ANALYST 1.5.2 (AB Sciex, Framingham, MA, United States) and hormones were quantified relative to the peak area of their corresponding standard (D_4-salicylic acid and D_6-abscisic acid; Santa Cruz Biotechnology, Dallas, TX, United States). For sugar analysis, extracts were diluted 1:10 with water prior to analysis on an Agilent 1200 HPLC system (Agilent, Santa Clara, CA, United States) coupled to an API 3200 tandem mass spectrometer (AB Sciex). The analytes were separated on an hydrophobic interaction liquid chromatography (HILIC)-column (apHera NH_2 Polymer; 15 × 4.6 mm, 5 μm; Supelco, Bellefonte, PA, United States) with a water/acetonitrile gradient (flow, 1.0 ml min^{-1}), for more details see Madsen et al. (2015). The data were processed using ANALYST 1.5.2 (AB Sciex) and the compounds were quantified using an external standard curve. For this, a mixture of glucose, fructose and sucrose (Sigma-Aldrich, St. Louis, MO, United States) was analyzed at six different concentrations ranging from 20 to 1.25 μg ml^{-1}.

Isoprene Measurements

Isoprene emission from rust-infected and control trees was measured from the second mature leaf from the apex in six trees of each treatment ($n = 6$). The same conditions, setup and time points (from 1 to 10 dpi) as for the photosynthesis experiment were used, but the material of the single leaf chamber was changed to aluminum (**Supplementary Figure S2**) to avoid volatile contamination, while the lid was still made from poly(methyl methacrylate). The ambient temperature of 20°C used in our experiments is lower than the temperature applied in comparable studies on isoprene emission (e.g., Brilli et al., 2007) but better simulates the conditions that trees of this poplar species and the pathogen experience in the field. Furthermore, the relative humidity was reduced to 6% in order to avoid excessive transpiration inside the chamber (due to a larger leaf size compared to the photosynthesis experiment). Isoprene emission was analyzed with a proton transfer reaction mass spectrometer (PTR-MS; Ionicon Analytik, Innsbruck, Austria). A detailed description of the PTR-MS can be found in Lindinger and Jordan (1998). Before starting the analysis the PTR-MS was calibrated by using the Gas Calibration Unit, a system generating clean air mixed with precise flows of an isoprene gas standard (Ionicon Analytik). The capillary line of the PTR-MS was connected with the outflow of the leaf chamber. The proton transfer reactions occurred in the reaction chamber (drift tube) between the primary ion H_3O^+ coming from the ion source, and the isoprene in the sampled air. In the drift tube the pressure was in the range of 2.3 mbar

and the E/N ratio (electric field/particle density) was 137 Td (1 Td = 10^{-17} V cm^2). Isoprene was monitored at the mass signal 69 (*m/z*). The raw count-rate signal intensity () of the isoprene was normalized (ncps) to the cps sum of the primary ion and water cluster, and to the drift tube pressure. Each leaf was given a 10 min equilibration period in the leaf chamber under LED light. Then the leaf chamber was connected to the PTR-MS to monitor isoprene emission for 15 min. This time was enough to reach steady-state conditions of isoprene emission. The average of cps during the steady-state period was used to calculate the emission rate, from which the background (empty leaf chamber, **Supplementary Figure S2**) was subtracted. Isoprene emission was normalized to the leaf area, which was determined by a picture taken at the beginning of the experiment. As the trees were not actively growing during the experiment, the leaf size was assumed to be the same throughout the whole experiment. Leaf area was calculated as described in "Photosynthesis measurements." After the last measurement (10 dpi), the second mature leaf was flash-frozen immediately in liquid nitrogen for metabolite analysis.

Analysis of MEP Pathway Metabolites

Leaves sampled from the isoprene experiment (n = 6) were ground in liquid nitrogen and then lyophilized. The MEP pathway metabolites (see **Figure 5** legend) were extracted twice with a 250 µl solution of 50% acetonitrile containing 10 mM ammonium acetate, pH 9.0, using 5 mg dry tissue. After vortexing and micro-centrifugation, 200 µl of the supernatant from both extracts were combined, transferred into a new 1.5 ml tube and dried under a stream of nitrogen gas at 40°C. The residue was dissolved in 100 µl of 10 mM ammonium acetate, pH 9.0, and, after vortexing, 100 µl of chloroform was added. The upper aqueous phase, separated by centrifugation, was transferred into a new tube and diluted with 1 volume of acetonitrile. After centrifugation for 5 min to remove any precipitate, the supernatant was transferred to an HPLC vial. MEP pathway metabolites were analyzed on an Agilent 1260 Infinity HPLC system (Agilent) connected to an API 5000 triple quadrupole mass spectrometer (AB Sciex). A 5 µl portion of the extract was injected and the metabolites were separated on a HILIC XBridge Amide column (150 × 2.1 mm, 3.5 µm; Waters, Milford, MA, United States) with a HILIC guard column containing the same sorbent (3.5 µm, 10 × 2.1 mm) and a SSITM high pressure pre-column filter (Sigma-Aldrich) using two solvents: 20 mM ammonium bicarbonate adjusted to pH 10.5 with ammonium hydroxide (solvent A) and 80% acetonitrile containing 20 mM ammonium bicarbonate, pH 10.5 (solvent B). The solvent gradient profile started with 100% of solvent B which decreased to 60% in the first 15 min, followed by an isocratic gradient with solvent B. Separation was performed at 25°C with a flow rate of 500 µl min^{-1}. The mass spectrometer operated in negative ionization mode with ion spray voltage −4500 eV, turbo gas temperature 700°C and nebulizer gas 70 psi. The MEP pathway metabolites were analyzed using the MRM conditions described by Wright et al. (2014). The metabolite concentrations were calculated by using external standard curves, and were normalized to the [^{13}C]-labeled internal standards of each intermediate (González-Cabanelas et al., 2016) added to the extract after the first extraction step.

Leaf Pigment Analysis

Leaves sampled from the isoprene experiment at 10 dpi were ground in liquid nitrogen and 50 mg of fresh tissue was extracted in light-protected tubes with 1 ml of acetone by shaking for 6 h at 4°C in the dark. After centrifugation for 5 min at 2350 g at 4°C, 800 µl of the extract was transferred into a new light-protected tube and 200 µl of water was added. After spinning the samples for 1 min at 5000 rpm at 4°C, they were transferred to brown glass vials for analysis on an Agilent 1100 Series HPLC with UV/VIS diode array detector. The detector was set at 445 nm for the detection of carotenoids and at 650 nm for the chlorophylls. The pigments were separated on a Supelcosil column LC-18 (7.5 cm × 4.6 mm × 3 µm; Sigma-Aldrich) using an acetone (solvent A)/1 mM $NaHCO_3$ (in water, solvent B) gradient with a flow rate of 1.5 ml min^{-1}. The initial mobile phase consisted of 65/35% (v/v) solvent A/solvent B. Then, solvent A was linearly increased to 90% within 12 min and to 100% over 8 min. 100% solvent A was kept for 2 min and then decreased to 65% again within 3 min. Quantification was done using external standard curves. Authentic standards of the chlorophylls and β-carotene (Santa Cruz Biotechnology) were analyzed in a range from 0.1 to 0.00625 mg ml^{-1}. Lutein, neoxanthin, and violaxanthin were assumed to have the same response factor as β-carotene.

Transcriptome Analysis

To investigate transcriptional changes in black poplar leaves upon rust infection, eight trees grown from green cuttings were selected and half of them were inoculated with *M. larici-populina* uredospores. Leaves from the trees of both treatments ("control" and "rust-infected"; n = 4) were harvested 8 dpi and flash-frozen in liquid nitrogen. RNA was isolated with the InviTrap Spin Plant Mini Kit (Stratec Biomedical AG, Birkenfeld, Germany), including a DNase digestion (DNase kit, Qiagen, Hilden, Germany). RNA concentration and quality was analyzed with a NanoDrop 2000c spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany) and the RNA 6000 Nano Kit on a Bioanalyzer (Agilent). Sequencing was done at the Max Planck-Genome-Centre (Köln, Germany) on a HiSeq 2500 (Illumina, San Diego, CA, United States) with 9 Mio reads 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 v9.1[3]. The same software was used for *de novo* transcriptome assembly, combining two 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 *P. nigra*

[3]http://www.clcbio.com

contained 81,580 contigs (sets of overlapping sequence segments that together represent a continuous region of the original RNA). Minimum contig size was 250 bp with an N50 contig size of 1320 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 v4.1[4]. Based on the BLAST hits, the contigs were designated as being of either plant or fungal (i.e., *M. larici-populina*) origin. To assess transcriptome completeness, we performed a BUSCO[5] (Benchmarking Universal Single-Copy Orthologs) analysis by comparing our assembled (plant-derived only) 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. Our assembled transcriptome was determined to be 87.8% complete and only 3.6% of the BUSCO genes were missing. Digital gene expression analysis was carried out using CLC Genomics Workbench v9.1 to generate BAM (mapping) files, and QSeq Software (DNAStar Inc., Madison, WI, United States) 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) corrected for multiple testing using the Benjamini–Hochberg procedure to check the false discovery rate (FDR). A gene was considered significantly differentially expressed if the FDR-corrected *p*-value was less than 0.05. Fisher's exact test was used as part of BLAST2GO to identify the overrepresentation of GO terms among lists of differentially expressed genes between treatment groups. The GO-enriched bar charts were simplified to display only the most specific GO terms by removing parent terms representing existing child terms using the function "Reduce to most specific terms" in BLAST2GO. A GO term was considered significantly enriched if the *p*-value corrected by FDR control was less than 0.05.

Statistics

All data were tested for statistical assumptions, i.e., normal distribution and homogeneity of variances. Whenever necessary, the data were log-transformed (salicylic acid, DMADP + IDP). For the photosynthetic parameters and the isoprene emission a two-way repeated measures ANOVA was performed using "time" as a within-subject factor and "rust infection" as a between-subject factor. For end-point measurements (phytohormones, sugars, MEP pathway metabolites and carotenoids) an independent student's *t*-test was performed for each compound. Correlations between photosynthetic parameters and phytohormones were tested using bivariate Pearson's *r* correlation. All statistical analyses were performed with SPSS 17.0 (SPSS, Chicago, IL, United States).

[4]http://www.blast2go.de

[5]http://busco.ezlab.org

RESULTS

Photosynthesis Decreases After Rust Infection but Sugar Levels Are Unaffected

In order to investigate the influence of fungal infection on photosynthesis in black poplar, we measured photosynthetic parameters at various times during the development of rust infection in the leaves.

The photosynthetic assimilation rate in uninfected control trees was generally stable at 9–12 μmol CO_2 m^{-2} s^{-1} during the time course of measurements (**Figure 1A**). The photosynthetic rate in rust-infected poplar trees, however, dropped by approximately 50% within the first 4 hpi from 12.1 ± 1.32 to 6.1 ± 0.86 μmol m^{-2} s^{-1}. This reduction persisted throughout the later time points. The factor "rust infection" strongly influenced the photosynthetic rate, as also

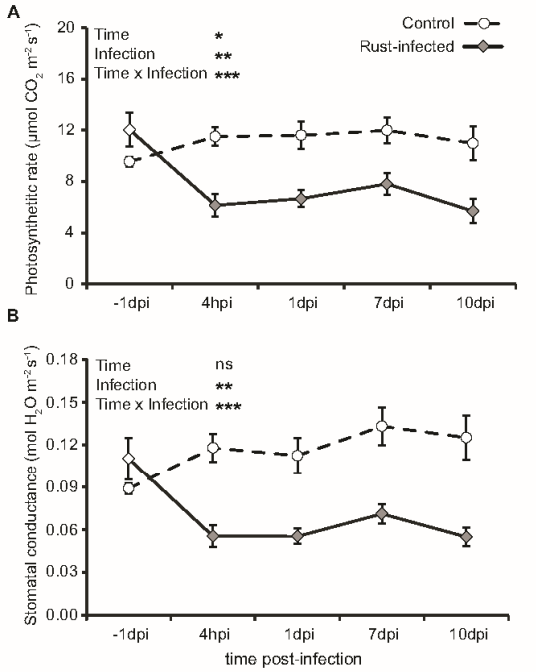

FIGURE 1 | Photosynthetic parameters of rust-infected black poplar trees (filled symbols) and uninfected controls (open symbols) at different time points after infection (dpi = days post-infection, hpi = hours post-infection; −1 dpi = 1 day before infection). Measurements were made on the second mature leaf counting from the apex. Shown are means ± SEM (n = 6) for **(A)** photosynthetic rate (μmol CO_2 m^{-2} s^{-1}) and **(B)** stomatal conductance (mol H_2O m^{-2} s^{-1}). Results from repeated measures ANOVA using the factors "Time" (time points before and post-infection), "Infection" (rust infection), and the interaction "Time × Infection" are given as insets in the graphs (ns = not significant; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

shown statistically [repeated measures ANOVA: $F_{(1, 10)} = 15.524$, $P = 0.003$]. However, there was also a significant interaction of time and rust infection [repeated measures ANOVA: $F_{(4, 40)} = 8.891$, $P < 0.001$]. The patterns of stomatal conductance were similar to those observed for photosynthetic assimilation rate. Control plants without rust infection showed a slight increase in stomatal conductance throughout the experiment from 0.09 ± 0.003 mol m^{-2} s^{-1} at the first measurement to 0.13 ± 0.012 mol m^{-2} s^{-1} during the last three measurements (**Figure 1B**). Rust fungus infection decreased stomatal conductance in poplar trees by more than 50% as early as 4 hpi, when it declined from 0.11 ± 0.015 to 0.06 ± 0.005 mol s^{-1} m^{-2}. As the infection progressed, the stomatal conductance of rust-infected trees remained at around 50% of that measured from uninfected controls. The effect of "rust infection" was statistically highly significant [repeated measures ANOVA: $F_{(1, 10)} = 19.815$, $P = 0.001$], as was the interaction of time and rust infection [repeated measures ANOVA: $F_{(4, 40)} = 10.665$, $P < 0.001$]. However, the intercellular CO_2 levels did not differ between the different time points, nor between the two treatments, i.e., rust-infected and control black poplar leaves (**Supplementary Figure S5**).

After observing the reduction in photosynthetic assimilation rate in rust-infected poplar leaves, we analyzed glucose, fructose and sucrose levels in leaves harvested at 10 dpi. For these sugars, no significant changes in concentration were observed after infection of leaves by the rust fungus (**Table 1**).

Our results show that photosynthetic activity is downregulated in poplar leaves right after the onset of infection with the rust fungus, and remains lower over the course of infection without affecting soluble sugars.

Salicylic Acid (SA) and Abscisic Acid (ABA) Increase in Rust-Infected Leaves

To analyze phytohormones that might be involved in regulating anti-pathogen defense and photosynthesis, leaves were collected at 10 dpi.

Salicylic acid increased fivefold in rust-infected black poplar leaves compared to uninfected controls [331 ± 45 ng g^{-1} DW in controls; 1580 ± 390 ng g^{-1} DW in rust-infected trees, **Figure 2A**], which is highly significant [Student's t-test, $t_{(10)} = -4.687$, $P = 0.001$]. ABA, a phytohormone regulating stomatal opening, among other processes, also increased in rust-infected black poplar leaves, reaching a level twice as high as in the control leaves (36.2 ± 6.10 ng g^{-1} DW in controls; 72.1 ± 11.34 ng g^{-1} DW in rust-infected trees, **Figure 2B**). Additionally, ABA levels in rust-infected leaves correlated negatively with stomatal conductance and photosynthetic rate measured at 10 dpi (**Table 2**). In control leaves, however, no such correlation could be observed.

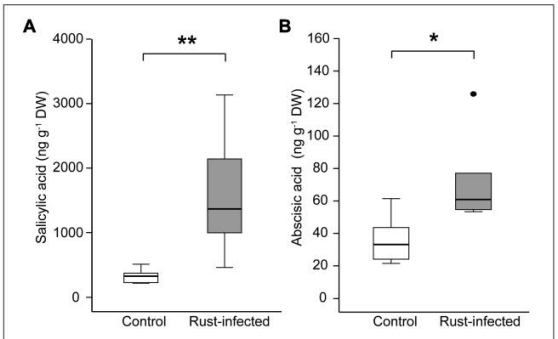

FIGURE 2 | Levels of the phytohormones **(A)** salicylic acid and **(B)** abscisic acid in leaves from rust-infected black poplar trees (filled symbols) and uninfected controls (open symbols) 10 days post-infection. Measurements were made on the second mature leaf counting from the apex. Boxplots show the median with upper and lower quartile ($n = 6$) and dots for outliers; asterisks indicate statistically significant differences between groups (independent Student's t-test, $^*P < 0.05$, $^{**}P < 0.01$).

Taken together, we could show that rust infection increased SA as well as ABA in poplar leaves, whereas the latter shows a negative relation to photosynthetic parameters.

Rust Infection Does Not Affect Isoprene Emission

To evaluate the effect of pathogen infection on isoprene emission, we monitored leaves of infected and uninfected young poplar trees using a PTR-MS at different time points before and during infection with the rust fungus (*M. larici-populina*).

The isoprene emission from uninfected controls showed slight variations throughout the time course of the experiment, ranging from 2.7 to 3.4 nmol m^{-2} s^{-1}. Infection with the rust did not significantly change emission of isoprene compared to control trees or compared to the levels measured before the pathogen inoculation (**Figure 3**). The fluctuations over time were similar to those in uninfected controls, leading to a significant statistical effect of "time" [repeated measures ANOVA: $F_{(4, 40)} = 1.475$, $P = 0.031$]. However, there was no significant effect of "infection" or the interaction "time × infection" on isoprene emission. This shows that rust infection does not affect the emission of isoprene from black poplar trees.

TABLE 1 | Soluble sugar levels in control and rust-infected black poplar trees 10 days post-infection expressed in mg g^{-1} dry weight.

	Control	Rust-infected	*t*	*P*
Glucose	1.31 ± 0.19	1.36 ± 0.17	−0.204	0.843
Fructose	2.02 ± 0.51	2.03 ± 0.36	−0.012	0.991
Sucrose	35.53 ± 3.76	41.44 ± 3.09	−1.213	0.253

Measurements were made on the second mature leaf counting from the apex. Shown is mean ± SEM (n = 6) and t- and P-value of Student's t-test.

TABLE 2 | Correlation between phytohormone levels and photosynthetic parameters of control and rust-infected black poplar trees 10 days post-infection (data from **Figures 1**, **2**).

	Control		Rust-infected	
	Photosynthetic rate	**Stomatal conductance**	**Photosynthetic rate**	**Stomatal conductance**
Salicylic acid	$\rho = -0.384$	$\rho = -0.589$	$\rho = -0.537$	$\rho = -0.661$
Abscisic acid	$\rho = -0.257$	$\rho = -0.186$	**$\rho = -0.987$*****	**$\rho = -0.969$****

*Measurements were made on the second mature leaf counting from the apex. ρ, Pearson correlation coefficient. Significant correlations are highlighted in bold and marked with asterisks (***$P < 0.001$; **$P < 0.01$; $n = 6$).*

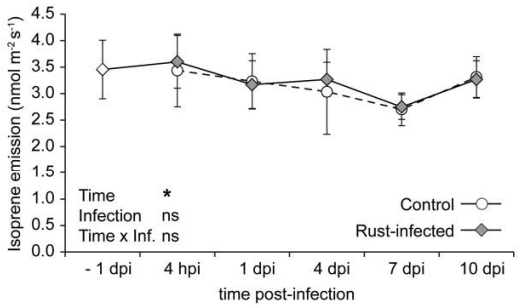

FIGURE 3 | Isoprene emission from leaves of rust-infected black poplar trees (filled symbols) and uninfected controls (open symbols) at different time points after infection (dpi = days post-infection, hpi = hours post-infection; −1 dpi = 1 day before infection). Measurements were made by proton-transfer reaction mass spectrometry (PTR-MS) on the second mature leaf counting from the apex. Shown are means ± SEM ($n = 6$). Results from repeated measures ANOVA using the factors "Time" (time points before and post-infection), "Infection" (rust infection) and the interaction "Time × Infection" are given in the graph (ns = not significant; *$P < 0.05$).

Rust Infection Did Not Influence the Genes, Intermediates or Most Products of the MEP Pathway

We analyzed intermediates of the MEP pathway and levels of the photosynthetic pigments, chlorophylls and carotenoids. Like isoprene, these are also produced from DMADP and IDP originating from the MEP pathway. The analyzed leaves were sampled together with any fungal spores and mycelium present in infected leaves. The MEP pathway intermediates, DXP, MEP, CDP-ME, and MEcDP, were present at similar levels in rust-infected and control leaves (**Figures 4A–D**). However, the amount of DMADP and IDP (DMADP + IDP), the final products of both the MEP and MVA pathways (not separable in our LC-MS analysis), were significantly higher in rust-infected leaves [Student's t-test, $t_{(10)} = -3.503$, $P = 0.006$] (**Figure 4E**). The concentration of DMADP + IDP in rust-infected leaves was more than double that of control leaves (4.1 ± 0.46 nmol g^{-1} FW in controls; 9.3 ± 1.60 nmol g^{-1} FW in rust-infected trees). On the other hand, many of the major isoprenoids that are known to be produced from MEP pathway-derived C_5 units, including the carotenoids, lutein, neoxanthin, violaxanthin, and chlorophylls a and b, did not change after rust infection (**Table 3**). β-Carotene, however, increased by more than 50% in leaves from rust-infected poplars compared to uninfected controls (0.59 ± 0.072 mg g^{-1} FW in controls; 0.93 ± 0.090 mg g^{-1} FW in rust-infected; Student's t-test, $t_{(10)} = -3.100$, $P = 0.011$). The orange-colored uredospores of the rust fungus *M. larici-populina* were also analyzed separately to determine if these contained any carotenoids, and β-carotene, but none of the other poplar carotenoids, was found (**Supplementary Table S1**). The concentration of β-carotene was more than threefold higher in fungal spores than in uninfected poplar leaves.

DMADP and IDP, the C_5 building blocks of all isoprenoids, can be produced by either the plastidic MEP pathway or the cytosolic MVA pathway (Rodríguez-Concepción and Boronat, 2015). To determine whether rust infection could influence either pathway, we carried out transcriptome analysis of uninfected and rust-infected black poplar trees. No differential expression of genes involved in the MEP pathway was observed (**Figure 5**). However, genes encoding biosynthetic enzymes of the MVA pathway increased in expression in rust-infected leaves compared to uninfected controls (**Figure 5**). Two enzymes catalyzing early steps of the MVA pathway, acetoacetyl-coenzyme A thiolase and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase, had especially higher RPKM values (reads per kilobase of transcript per million mapped reads) in leaves infected with the rust fungus. The transcriptome analysis also identified MVA pathway transcripts of fungal origin, likely from *M. larici-populina*. Contigs annotated as fungal HMG-CoA synthase and all downstream MVA pathway enzymes were detected in the transcriptome of rust-infected leaves (**Supplementary Table S2**), but not in control leaves.

Thus rust infection did not affect the levels of intermediates of the MEP pathway. However, the amounts of the isoprenoid building blocks DMADP + IDP increased, as a result of up-regulation of the MVA pathway. In addition, there was an increase in β-carotene, which likely arose from the isoprenoid biosynthesis by the rust fungus itself.

DISCUSSION

Melampsora rusts are among the most devastating pathogens of poplar trees (Pei and Shang, 2005). We investigated the effect of rust infection (*M. larici-populina*) on photosynthesis and the emission of isoprene in black poplar (*P. nigra*).

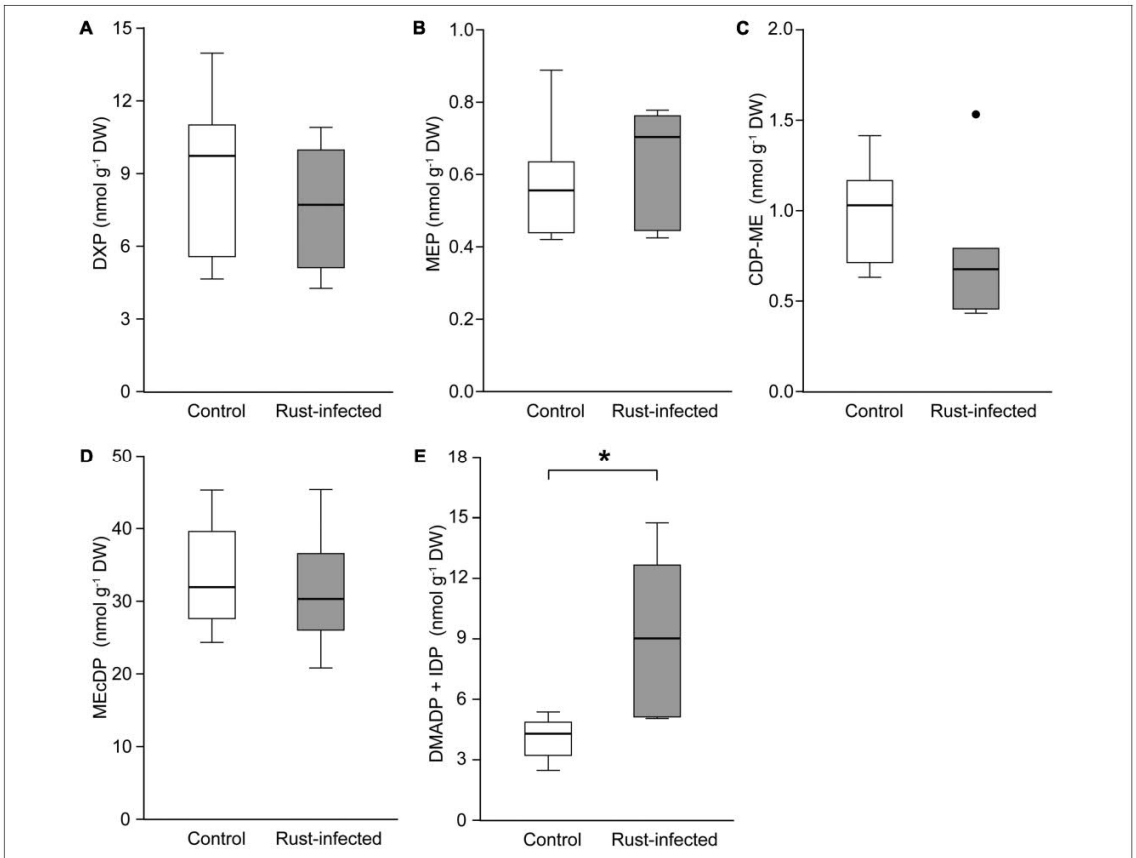

FIGURE 4 | Concentrations of intermediates of the methylerythritol-4-phosphate (MEP) pathway in leaves of rust-infected black poplar trees (filled bars) and uninfected controls (open bars) 10 days post-infection. Boxplots show the median with upper and lower quartile as bars (n = 6) and dots for outliers; asterisks indicate statistically significant differences between groups (independent Student's t-test, $^*P < 0.05$). Intermediates are shown in the order occurring in the pathway: **(A)** 1-deoxy-D-xylulose-5-phosphate (DXP); **(B)** 2*C*-methyl-D-erythritol-4-phosphate (MEP); **(C)** 4-diphosphocytidyl-2*C*-methyl-D-erythritol (CDP-ME); **(D)** 2*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MEcDP); **(E)** dimethylallyl diphosphate and isopentenyl diphosphate (DMADP + IDP, not separable in our analysis).

TABLE 3 | Carotenoid and chlorophyll levels in control and rust-infected black poplar trees 10 days post-infection expressed in mg g^{-1} fresh weight.

	Control	Rust-infected	*t*	*P*
β-Carotene	0.59 ± 0.07	0.93 ± 0.09	−3.100	**0.011**
Lutein	1.43 ± 0.05	1.35 ± 0.06	0.978	0.351
Neoxanthin	0.31 ± 0.01	0.29 ± 0.01	1.572	0.147
Violaxanthin	0.28 ± 0.03	0.32 ± 0.02	−1.204	0.256
Chlorophyll *a*	1.01 ± 0.05	0.92 ± 0.01	1.762	0.133
Chlorophyll *b*	0.68 ± 0.03	0.63 ± 0.01	1.467	0.192

Measurements were made on the second mature leaf counting from the apex. Shown is mean ± SEM (n = 5–6) and t- and P-value of Student's t-test. Bold numbers indicate significant differences.

Our results indicate that black poplar actively downregulates photosynthesis when it is subjected to rust infection. Yet despite the spatial and metabolic connections between photosynthesis and isoprene formation, the emission of isoprene was completely unaffected by the presence of the rust fungus. Consistent with this, fungal infection also did not change the expression of biosynthetic genes or levels of intermediates of the MEP pathway, the route to formation of the C_5 units used in isoprene biosynthesis. However, an increase in the quantity of DMADP and IDP was observed, which may be attributed to the increased

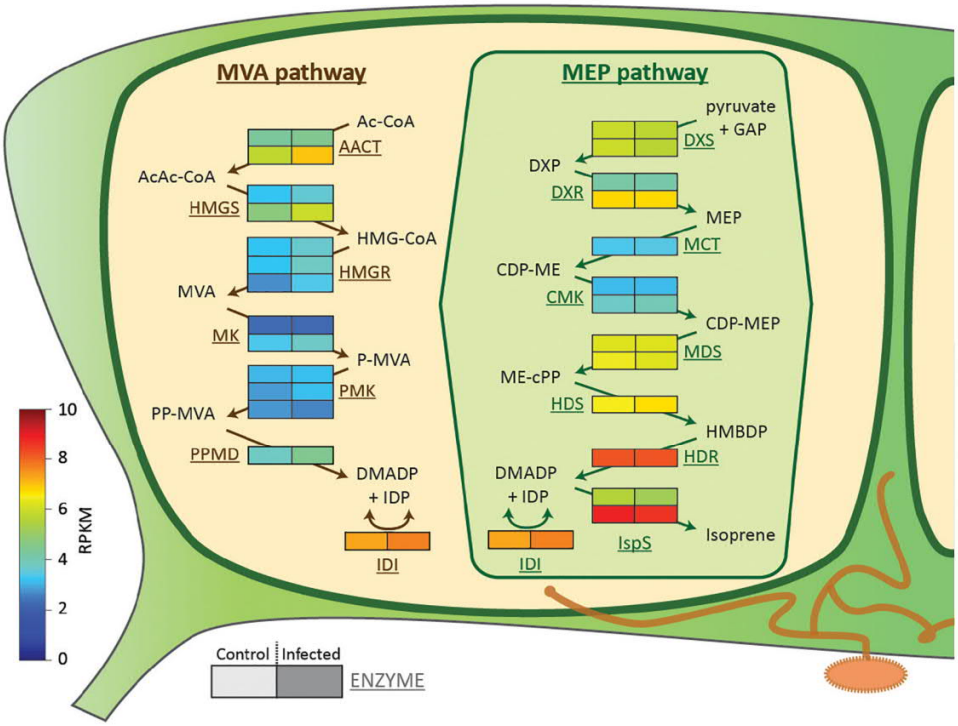

FIGURE 5 | Expression of biosynthetic genes of the two isoprenoid pathways in black poplar leaves from rust-infected trees ("Infected," right box) compared to uninfected controls ("Control," left box). Shown are RPKM (reads per kilobase of transcript per million mapped reads) values of contigs annotated as enzymes of the MVA and MEP pathways (n = 4 biological replicates). Each row with two boxes (Control and Infected) represents one contig. **Metabolites** (in black): acetyl coenzyme A (CoA) (Ac-CoA), acetoacetyl coenzyme A (AcAc-CoA), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), mevalonate (MVA), mevalonate-5-phosphate (P-MVA), mevalonate-5-diphosphate (PP-MVA), dimethylallyl diphosphate (DMADP), isopentenyl diphosphate (IDP), D-glyceraldehyde-3-phosphate (GAP), 1-deoxy-D-xylulose-5-phosphate (DXP), 2*C*-methyl-D-erythritol-4-phosphate (MEP), 4-diphosphocytidyl-2*C*-methyl-D-erythritol (CDP-ME), 4-diphosphocytidyl-2*C*-methyl-D-erythritol-2-phosphate (CDP-MEP), 2*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MEcDP), and 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate (HMBDP). **Enzymes** (in brown/green): AcAc-CoA thiolase (AACT), HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGR), mevalonate kinase (MK), phosphomevalonate kinase (PMK), diphosphomevalonate decarboxylase (PPMD), isopentenyl-diphosphate isomerase (IDI), DXP reductoisomerase (DXR), MEP cytidyltransferase (MCT), CDP-ME kinase (CMK), MEcDP synthase (MDS), HMBDP synthase (HDS), and HMBDP reductase (HDR).

activity of the MVA pathway (the alternative route to producing C_5 isoprenoid units) in poplar or the fungus itself.

Rust Infection Drastically Reduces Black Poplar Photosynthesis

Despite the central importance of photosynthesis in supplying plants with carbon, energy, and reducing equivalents, we observed an immediate and sustained decrease in photosynthetic activity in rust-infected black poplar leaves of nearly 50%, from 4 hpi to 10 dpi (**Figure 1**). Infection of poplar with *Melampsora* leaf rust was earlier reported to be associated with a marked reduction in photosynthesis (Zhang et al., 2010, 2016; McKown et al., 2014; Jiang et al., 2016; Gortari et al., 2018), but the detailed temporal dynamics of this reduction had not been studied. In willow, a similar pattern of decreased net photosynthetic rate was observed after rust infection, but stomatal conductance changed only at late time points (Toome et al., 2010). A negative impact of rust on transcripts of photosynthetically relevant genes in poplar was also reported at 6 dpi (summarized in Major et al., 2010). Interestingly, we did not observe any changes in the intercellular CO_2 concentration (C_i) after rust infection (**Supplementary Figure S5**). A decreased C_i would be expected when the assimilation rate decreases after stomatal closure due to the lowered availability of CO_2. The lack of decrease of C_i in our study might be explained by temporal dynamics, i.e., a fast reduction and subsequent equilibration of C_i before the first measurement. Alternatively, a signal – most likely phytohormonal – might have regulated stomatal conductance and assimilation rate simultaneously, so that reduced uptake and reduced consumption of CO_2 would balance out without any net effect on the C_i. A decline in stomatal conductance

and assimilation rate with stable C_i has been observed before after herbivory (Meza-Canales et al., 2017). Also, the influence of biotic stress-related phytohormones on photosynthesis has been shown in other systems (Popova et al., 1988; Tang et al., 2017). A transcriptional analysis conducted at early time points of infection would help to better understand the physiological mechanisms leading to the rapid decline in photosynthesis.

In addition to the decrease in photosynthesis we observed elevated levels of SA and ABA (**Figure 2**) in poplar leaves upon rust infection. SA is known to play a central role in plant defense against biotrophic organisms such as rusts or mildew, and induces a hypersensitive response, the expression of pathogenesis-related genes and other responses (Derksen et al., 2013). ABA, on the other hand, is primarily known to mediate responses to abiotic stresses such as drought (Tuteja, 2007) and to control stomatal closure (Acharya and Assmann, 2009). Stomata are natural openings through which the rust fungus can enter the intercellular spaces of the leaf within the first 6 h after inoculation (Hacquard et al., 2011). Yet rapid closure of stomata might prevent the pathogen from entering, as was observed in tomato leaves infected by *Pseudomonas syringae* (Melotto et al., 2006). A similar mechanism might occur in poplar leaves and would explain the fast decrease in stomatal conductance and the increased levels of ABA observed in infected tissue. Phytohormone analysis of earlier time points and exogenous application of ABA and SA will help to disentangle the signaling networks between these two hormones and control of photosynthesis. Considering the fast response of photosynthetic parameters to rust infection and the changes in phytohormone content, we infer an active control of stomatal closure by the plant. Previous work suggested that a mechanical disturbance by fungal hyphae could also be involved in reducing stomatal conductance (Jiang et al., 2016), perhaps especially at later time points.

Soluble Carbohydrate Levels Are Maintained in Rust-Infected Leaves

Although rust infection triggered a drastic decrease in the photosynthetic assimilation rate of poplar (**Figure 1**), soluble sugar content did not decrease in rust-infected compared to uninfected control leaves (**Table 1**). Given the rapid production of sucrose and hexose sugars as photosynthetic assimilates, it is surprising that their levels were not affected by the decline in photosynthesis. Soluble sugars can also originate from breakdown of storage carbohydrates or transport from other tissues. Since biotrophic pathogens utilize hexose sugars from their hosts (Voegele and Mendgen, 2011), it is assumed that infected tissues become carbon sinks even when photosynthetically active in order to satisfy the increased demand for carbon (Berger et al., 2007). This would require the mobilization of carbohydrates from other parts of plant. Such mobilization might account for the decline in wood production in poplar observed on rust infection, which causes significant economic losses in infected plantations (Frey et al., 2005; Wan et al., 2013). Another explanation for the maintenance of constant sugar levels in rust-infected leaves with a concurrent decrease in assimilation rate could be the reduced export of sugars. Further work is needed to elucidate the mechanisms responsible for maintaining sugar levels under these conditions.

Isoprene Emission Is Not Affected by Rust Infection

Isoprene is emitted in large amounts by poplar and other tree species, and it is assumed to protect leaves from heat stress or reactive oxygen species (Sharkey et al., 2008). However, the role of isoprene under biotic stress is poorly studied. We observed no influence of rust infection on isoprene emission from black poplar (**Figure 3**). Similar patterns of stable isoprene emission were observed under drought stress conditions (Pegoraro et al., 2004; Brilli et al., 2007), which also trigger ABA-mediated stomatal closure. On the other hand, studies on the effects of insect herbivory or mechanical wounding have reported variable outcomes, showing either an increased (Brilli et al., 2011), stable (Müller et al., 2015), decreased (Brilli et al., 2009; Jardine et al., 2013; Jiang et al., 2018), or time-dependent (Loreto and Sharkey, 1993; Loreto et al., 2006; Portillo-Estrada et al., 2015) isoprene emission patterns after stress application.

In contrast to our results, another study investigating the influence of rust infection on poplar trees observed lower isoprene emissions from infected compared to uninfected trees (Jiang et al., 2016) possibly due to the more severe level of infection. Although in our study rust significantly decreased photosynthetic parameters and altered hormone levels, our infected leaves did not have necrotic lesions (**Supplementary Figure S6**). Such necrotic lesions, however, were present on the leaves used by Jiang et al. (2016), and may have induced the reduction in isoprene emission. Necrosis leads to premature death of cells and hence could reduce the area of living tissue with which the plant synthesizes isoprene. The stability of isoprene emission under various stress conditions suggests that this compound is of vital importance to the physiology of isoprene-emitting plants. This importance might be due to a direct effect of isoprene, for example, by reducing oxidative stress, or an indirect effect by maintaining flux through the MEP pathway (Logan et al., 2000), which provides essential compounds for plant metabolism. After many years of research, the physiological role of isoprene is still to a large extent unknown.

Involvement of Plant and Fungal MVA Pathways in Isoprenoid Production in Infected Leaves

Considering the tight metabolic connections between photosynthesis and the MEP pathway, we expected to observe lower MEP pathway activity after rust infection due to the reduction in photosynthesis. However, the transcription of genes encoding biosynthetic enzymes of the MEP pathway did not change after rust infection (**Figure 5**). In addition, the stable levels of MEP pathway intermediates (**Figures 4A–D**) suggest a constant metabolic flux through the pathway despite

fungal infection. Consistent with this, the levels of the chlorophylls and most carotenoids, the main non-volatile products of the MEP pathway, did not change after rust infection (**Table 3**). Constant levels of the chlorophylls in leaves after rust infection were also recently reported in another poplar species (Gortari et al., 2018). However, the levels of DMADP and IDP (quantified together in our LC-MS analysis) increased after infection (**Figure 4E**). DMADP and IDP can be produced by both the plastidic MEP pathway and the cytosolic MVA pathway (Hemmerlin et al., 2012). Since the cells in the leaf were disrupted for chemical analysis, the DMADP and IDP present in both cellular compartments were analyzed simultaneously. An increased transcript abundance of genes involved in the early steps of the MVA pathway (**Figure 5**) suggests the increased DMADP + IDP levels observed in infected tissue might be derived from this pathway. This explanation is also supported by the recently reported increased emission of sesquiterpenes upon rust infection of poplar (Eberl et al., 2018). Alternatively, the increased DMADP + IDP levels could have resulted from the fungal metabolism in rust-infected tissue. Future research using ^{13}C-labeled CO_2 (Ghirardo et al., 2014) or glucose (Hemmerlin et al., 2012) could elucidate the biosynthetic origin of the increased DMADP + IDP.

In addition to higher DMADP + IDP-levels we also found higher amounts of β-carotene in rust-infected leaves compared to controls (**Table 3**). When rust spores were analyzed separately for carotenoids, high concentrations of β-carotene but no other carotenoids were found (**Supplementary Table S1**). We then analyzed the transcriptome of infected poplar leaves for *Melampsora*-specific genes involved in isoprenoid biosynthesis and found transcripts for genes encoding all steps of the MVA pathway (**Supplementary Table S2**). In contrast to plants where carotenoids are produced *via* the MEP pathway, their biosynthesis in fungi occurs *via* the MVA pathway (Disch and Rohmer, 1998). Taken together, it is likely that *M larici-populina* produces β-carotene in its hyphae and spores. The fungus *M. larici-populina* in the infected tissue is therefore the most probable cause of the increased β-carotene and DMADP + IDP levels. The fungus might use this pigment in spores to attract spore dispersers (Cano et al., 2013) or in hyphae to scavenge oxygen radicals (Davoli and Weber, 2002) that are produced by plants as defenses against infection (Ferreira et al., 2006).

CONCLUSION

Our study provides new insight into the impact of a widely distributed biotrophic pathogen on photosynthesis and isoprene formation in a poplar species. The hormone-mediated closure of stomata upon infection diminishes the photosynthetic activity of infected leaves and hence reduces the ability of the tree to assimilate new carbon. However, infected leaves maintain stable carbohydrate levels and continue to emit isoprene at unchanged rates despite carbon consumption by the pathogen. Most likely, infected leaves import soluble sugars from elsewhere in the tree. In the long term, rust disease may therefore result in reduced biomass production by poplar resulting in significant declines in plantation yield. However, since the level of isoprene emission was not affected, the influence on atmospheric chemistry will likely be minimal.

AUTHOR CONTRIBUTIONS

FE designed the experiments and wrote the article. FE and EP performed the experiments and analyzed the data except for the transcriptome. HV analyzed the transcriptome data. DV designed and constructed the experimental equipment. LW, AH, SU, and JG conceived the project and complemented writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01733/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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5. MANUSCRIPT III

Leaf rust infection reduces herbivore-induced volatile emission in black poplar and attracts a generalist herbivore

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Research

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Leaf rust infection reduces herbivore-induced volatile emission in black poplar and attracts a generalist herbivore

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Summary

• Plants release complex volatile blends after separate attack by herbivores and pathogens, which play many roles in interactions with other organisms. Large perennials are often attacked by multiple enemies, but the effect of combined attacks on volatile emission is rarely studied, particularly in trees.

• We infested *Populus nigra* trees with a pathogen, the rust fungus *Melampsora larici-populina*, and *Lymantria dispar* caterpillars alone and in combination. We investigated poplar volatile emission and its regulation, as well as the behavior of the caterpillars towards volatiles from rust-infected and uninfected trees.

• Both the rust fungus and the caterpillars alone induced volatile emission from poplar trees. However, the herbivore-induced volatile emission was significantly reduced when trees were under combined attack by the herbivore and the fungus. Herbivory induced terpene synthase transcripts as well as jasmonate concentrations, but these increases were suppressed when the tree was additionally infected with rust. Caterpillars preferred volatiles from rust-infected over uninfected trees.

• Our results suggest a defense hormone crosstalk upon combined herbivore pathogen attack in poplar trees which results in lowered emission of herbivore-induced volatiles. This influences the preference of herbivores, and might have other far-reaching consequences for the insect and pathogen communities in natural poplar forests.

Introduction

One way plants communicate with other species is by releasing volatile organic compounds (VOCs). These substances can prime other plants for a herbivore attack (Karban *et al.*, 2000) or recruit natural enemies of attacking herbivores, which predate on or parasitize the attackers (Arimura *et al.*, 2005; Mumm & Dicke, 2010; McCormick *et al.*, 2014b). On the other hand, VOCs also act directly by deterring herbivores (Unsicker *et al.*, 2009), inhibiting pathogen development (Mendgen *et al.*, 2006) or serving as airborne guides for herbivores to find their preferred host plants (McCormick *et al.*, 2016).

Most knowledge on how plant VOCs respond to the biotic environment was obtained from studies with a single species of attacker. Yet under natural conditions, simultaneous occurrence of different attackers, pathogens as well as herbivores, is much more common. Only a few studies have investigated the effect of combined attacks on plant volatiles so far (Ponzio *et al.*, 2013). Rostás *et al.* (2006), for example, showed that maize seedlings infected with a necrotrophic pathogen emitted fewer VOCs than uninfected seedlings after herbivory. However, pathogen infection enhanced volatile emission (Cardoza *et al.*, 2002) or had no effects (Ponzio *et al.*, 2014) in other systems. Nearly all of the studies on simultaneous attacks have been conducted on herbaceous plants. In comparison with herbaceous plant species, woody plants are usually larger with longer life spans and so can suffer simultaneous attack by herbivores and pathogens at many points throughout their lives. The formation of wood and new leaves each year requires huge energy resources that have to be taken into account when considering growth–defense tradeoffs, more so than for annual plants that just produce one generation of leaves. Thus, the fitness of trees depends not only on seed production but also on annual growth (Holopainen, 2011), which requires a more sophisticated fine-tuning of growth and defense. But how woody plants allocate resources to defense when attacked by multiple enemies has not been investigated. Because of their greater size, woody plants also play an important role in structuring terrestrial ecosystems as they harbor a huge diversity of arthropod and microbial species. Nonetheless, woody plants are rarely used in scientific studies on complex biotic interactions.

Different plant attackers may elicit different defense mechanisms by triggering specific signaling pathways involving defense hormones. Typically necrotrophic pathogens and chewing insects induce the jasmonic acid (JA) pathway, whereas biotrophic pathogens and piercing-sucking insects induce the salicylic acid (SA) pathway (Pieterse & Dicke, 2007). When JA- and

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SA-inducing attackers infest a plant simultaneously or subsequently, both hormone pathways are initially triggered and then interact with each other. The outcome of such signaling crosstalk is usually antagonism, although there are examples of synergistic or independent interactions (Derksen *et al.*, 2013). In particular, JA signaling is repressed by simultaneous or prior activation of SA signaling (Caarls *et al.*, 2015).

Jasmonic acid signaling often stimulates an induction of VOCs following herbivore damage (Rodriguez-Saona *et al.*, 2001; McCormick *et al.*, 2014b). For example, poplar trees release a large blend of herbivore-induced volatiles that includes terpenes, green leaf volatiles, aromatics (dominated by benzenoids), and nitrogenous compounds (mostly aldoximes and nitriles) (McCormick *et al.*, 2014a). For terpenes, the major components of the poplar volatile blend (Irmisch *et al.*, 2014), a principal factor regulating emission is the transcriptional control of terpene synthases (Arimura *et al.*, 2005; Irmisch *et al.*, 2014). These enzymes catalyze the biosynthesis of terpenes via the conversion of widespread prenyl diphosphate substrates to olefins and alcohols (Degenhardt *et al.*, 2009). While the ability of herbivory to stimulate terpene biosynthesis by inducing terpene synthase transcription is well known, there is no information about how this regulation is influenced by a second attacker. Moreover, as defense hormone signaling has been nearly exclusively studied in herbaceous plants (Caarls *et al.*, 2015), the regulation of defense mechanisms in woody plants is poorly understood (Eyles *et al.*, 2010).

The aim of our study was therefore to investigate the volatile emission of poplar and its regulation when trees are attacked by multiple enemies. For our experiments, we used black poplar (*Populus nigra*), an endemic species in European riparian ecosystems. Its ecological role as host for many microbial and insect species and the close relation to the genome-sequenced *P. trichocarpa* (Tuskan *et al.*, 2006) make it an excellent organism to study complex biotic interactions. The main pathogens restricting poplar growth in natural systems as well as in plantations all over the world are rust fungi. In particular, *Melampsora larici-populina* has enormous ecological and economic importance as a result of its wide distribution and significance in biomass reduction (Benetka *et al.*, 2011; Wan *et al.*, 2013). Another naturally occurring antagonist of poplar trees is the gypsy moth, *Lymantria dispar*. Endemic to Europe, this generalist feeding species is well known as an invader in North America, where caterpillars defoliate large areas of broadleaf forests (Wilson, 2016). In our study, poplar trees were challenged with rust infection, gypsy moth herbivory or a combination of both attackers under controlled laboratory conditions. We collected volatiles from the trees and used chemical and molecular analyses to reveal the underlying regulatory mechanisms. We also investigated how rust infection affected gypsy moth caterpillar host selection. Previous feeding experience and herbivore damage have been shown to affect the host choice of gypsy moth caterpillars (McCormick *et al.*, 2016), but the influence of a phytopathogenic fungus has not been studied yet.

Our results show that both rust and caterpillars induce volatile emission in black poplar trees as separate attackers. However, the combination of both attackers did not result in an additive effect, but in a suppression of herbivore-induced volatile emission by the rust. We found that this suppression is transcriptionally regulated, and we show evidence for an antagonistic crosstalk between defense hormones. Furthermore, we found that gypsy moth caterpillars preferred the odor of rust-infected trees to that of uninfected trees.

Materials and Methods

Plants

Nine genotypes of black poplar (*Populus nigra* L.) were cultivated as stem cuttings of trees grown in a common garden near Jena, Germany. These genetically distinct trees were derived from a natural black poplar population in northeastern Germany (Küstrin-Kietz, 52°34′1″N, 14°38′3″E). Stem cuttings potted in 1 l (olfactometer experiment) or 2 l pots (volatile experiment) filled with a 1 : 1 mixture of sand and soil (Klasmann potting substrate; Klasman-Deilmann, Geeste, Germany) were grown in a glasshouse (18 : 20°C, night : day, humidity 60%, natural light with 11–14 h photoperiod, supplement light for 12 h; lamps, 400 W (SON-T Agro; Philips, Andover, MA, USA)). Two days before the onset of the experiment the plants were transferred to a climate chamber (18 : 20°C, night : day; humidity 60%; photoperiod 16 h). Owing to an aphid infestation, trees for the volatile experiment were treated once 1 wk before the experiment with 2% Neudosan Blattlausfrei (potash soap formulation; Neudorff GmbH KG, Emmerthal, Germany), and were washed with water subsequently. The trees used in the olfactometer experiment were not infested and were therefore not treated.

Insects

Gypsy moth (*Lymantria dispar* L.) caterpillars were hatched from eggs obtained from Hanna Nadel (US Department of Agriculture, Buzzards Bay, MA, USA) and reared on artificial diet (MP Biomedicals LLC, Illkirch, France) in a climate chamber (14 : 10 h, light : dark; 20°C; 60% humidity) until the start of the experiment.

Pathogen

Uredospores of the biotrophic poplar leaf rust fungus (*Melampsora larici-populina* Kleb.) were obtained from naturally infected black poplar trees growing near Jena, Germany. Amplification was done by infecting (see the Plant treatments and leaf sampling subsection) 1-yr-old trees and harvesting uredospores at 2–3 wk post-infection with a scalpel and brush. Spores were dried over silica under vacuum overnight and stored at −20°C until the start of the experiment. The identity of the fungus was verified by using specific primers for the internal transcribed spacer (ITS) region of *M. larici-populina* (Supporting Information Table S1) as described later in the Determination and quantification of fungal genomic DNA subsection.

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Plant treatments and leaf sampling

Five-month-old black poplar trees of nine different genotypes were split into four treatment groups, with all genotypes evenly distributed among the groups. Each genotype represented one biological replicate. Trees of the rust and rust + herbivory treatments were inoculated with the rust fungus *M. larici-populina*. The inoculum consisted of a spore–water mixture (1 mg spores ml^{-1}) and was sprayed on the abaxial side of each leaf (1 ml per leaf). Polyethylene terephthalate (PET) bags (Bratschlauch, Toppits, Minden, Germany) were wrapped around the trees and kept closed for 2 d. Trees of the control and herbivory treatments were treated in the same way but sprayed with water only. In the herbivory and rust + herbivory treatments eight fourth-instar *L. dispar* caterpillars were placed in a PET bag (30 × 60 cm) on the middle section of the trees (six to eight leaves basipetal from the third fully expanded most apical leaf) 12 d postinfection (dpi). The trees in the control and rust treatments also received PET bags but no caterpillars. The remaining parts of the plants were also enclosed in PET bags to avoid volatile-mediated signaling among the plants. Throughout the whole experiment, all bags were flushed with dry air via Teflon tubes to reduce transpiration.

Forty hours after the start of the herbivory treatment, the volatiles of the treated middle section leaves, with caterpillars still present, were collected and the leaves were harvested. All leaves were photographed to determine leaf area loss by caterpillar feeding and fungal disease severity (Table S2), weighed and flash-frozen in liquid nitrogen.

Volatile collection and analysis

Volatiles were collected in a push–pull system for 4 h. Active charcoal-filtered air was pushed into the system at a flow rate of 1.0 l min^{-1}, and pumped out over PoroPak volatile traps (Analytical Research Systems, Inc., Gainesville, FL, USA) at a flow rate of 0.6 l min^{-1}. The volatile traps were eluted twice with 100 µl dichlormethane, containing nonyl acetate (Sigma Aldrich; 10 ng μl^{-1}) as internal standard (IS).

Volatiles were qualitatively and quantitatively analyzed with an Agilent (Santa Clara, CA, USA) 6890 series gas chromatograph (injection, 1 µl splitless; flow, 2 ml min^{-1} constant; temperature, 45 to 180°C at 6°C min^{-1} and then to 300°C at 100°C min^{-1}) coupled to either a flame ionization detector (FID; operated at 300°C) or an Agilent 5973 series mass spectrometer (MS; transfer line temperature, 270°C; quadrupole temperature, 150°C; source temperature, 230°C; electron energy, 70 eV; 4.49 scans s^{-1}; 33–350 amu), respectively. The volatile blend was separated on a DB-5MS column (30 m × 0.25 mm × 0.25 µm; Agilent) with H_2 (FID) or He (MS) as carrier gas. Peak integration was done with Agilent ChemStation software. In order to identify the compounds, their mass spectra were matched with reference spectra from databases (Wiley 275, NIST 98, Adams 2205), or compared with those of authentic standards. The amount of each compound was determined from GC-FID data based on the peak area in relation to the IS peak area. The relative response factor was computed with authentic standards or estimated with the effective carbon number concept, and normalized to FW and duration of collection (Danner *et al.*, 2011).

Collection and analysis of fungal volatiles

Spores from *M. larici-populina* were carefully collected from infected trees at 21 dpi. The spores were incubated overnight with preconditioned polydimethylsiloxane (PDMS) tubes (Kallenbach *et al.*, 2014) in 1.5 ml brown glass vials (VWR International, Radnor, PA, USA). The PDMS tubes were analyzed by GC-MS coupled to a thermodesorption unit (Shimadzu, Duisburg, Germany). Desorption was achieved by He flow (60 ml min^{-1}) at 200°C for 8 min in a glass tube (Supelco; Sigma Aldrich) and the analytes were trapped on a Tenax (Buchem BV, Apeldoorn, the Netherlands) adsorbent trap at −20°C. By heating the trap to 230°C within 10 s, the analytes were injected onto the column. The column and settings for GC-MS were identical to those described in the Volatile collection and analysis subsection. The compounds were identified by comparing their mass spectra with those of authentic standards or with reference spectra from databases (Wiley275, NIST98) using GCMS Solution v.4.20 (Shimadzu).

Volatiles released from spores of three different trees were qualitatively similar but quantitatively different.

Determination and quantification of fungal genomic DNA

Genomic DNA was isolated from freeze-dried leaf material using the Invisorb Spin Plant Mini Kit (Stratec Biomedical AG, Birkenfeld, Germany) according to the manufacturer's manual. The DNA was quantified with a NanoDrop2000c Spectrophotometer (Peqlab Biotechnology GmbH, Erlangen, Germany) and diluted to 100 ng μl^{-1}.

Poplar *ACTIN2*-specific primers (Ramírez-Carvajal *et al.*, 2008) were used for normalization and primers specific to *M. larici-populina*'s ITS region (Table S1) were used to quantify fungal DNA. The reaction mixture contained Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent), DNA (1 µl) and forward and reverse primer (10 µmol each). The quantitative PCR was performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad) with the following parameters: 95°C (3 min), 40 cycles of 95°C (30 s) + 60°C (30 s), melt curve from 53 to 95°C. Data were analyzed using Bio-Rad CFX Manager 3.1 ($\Delta\Delta c_q$). A nontemplate control was included in each run and primer efficiencies were tested. A gel electrophoresis was performed to verify the amplicon length.

Determination of transcript abundance

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). cDNA was prepared from the RNA

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using SuperScript II reverse transcriptase and oligo-dT primers (Invitrogen) and diluted 1 : 5 with water. The cDNA was used in a quantitative real-time PCR for assessing the transcript abundance using the same conditions, calculations and controls as for fungal genomic DNA. *ACTIN2* was used for normalization of the transcript abundance of the genes of interest.

Defense hormone analysis

Lyophilized ground leaf material (10 mg) was extracted with 1 ml methanol containing the following internal standards: D_4-salicylic acid (40 ng ml^{-1}; Santa Cruz Biotechnology, Dallas, TX, USA), D_6-jasmonic acid (JA) (40 ng ml^{-1}; HPC Standards GmbH, Cunnersdorf, Germany), ^{13}C-jasmonoyl-isoleucine (8 ng ml^{-1}; synthesis described in Kramell *et al.* (1988), using ^{13}C-isoleucine; Sigma Aldrich). The extracts were analyzed on an Agilent 1260 Infinity high-performance liquid chromatography system (Agilent) coupled to an API 5000 tandem mass spectrometer (AB Sciex, Framingham, MA, USA). The analytes were separated on a C18-column (XDB-C18, 50 × 4.6 mm × 1.8 µm; Agilent) using a formic acid (0.05% in water)/acetonitrile gradient (flow, 1.1 ml min^{-1}) and detected via multiple reactions monitoring (MRM) in negative ionization mode (ion spray at −4500 eV and 700°C); more information can be found in Vadassery *et al.* (2012). Data were processed using Analyst 1.5.2 (Applied Biosystems, Foster City, CA, USA) and hormones were quantified relative to the peak area of their corresponding standard. Catabolites of JA and jasmonoyl-isoleucine (JA-Ile) were quantified relative to D_6-JA or ^{13}C-JA-Ile, respectively.

Olfactometer experiments

The behavior of caterpillars towards volatile blends and individual compounds was assessed in a four-arm arena (see Fig. S1 for detailed description). In each arena, one early-instar *L. dispar* caterpillar (starved overnight) was placed in the center and its movement was recorded for 8 min. Recording and video analysis were done with EthoVision XT software (Noldus Information Technology, Wageningen, the Netherlands). When a caterpillar did not move for at least 4 min, the trial was excluded from data analysis, resulting in unequal replicate numbers. Each caterpillar was used once and represented one biological replicate.

To test the caterpillar response to the headspace from rust-infected and uninfected *P. nigra* trees, 12 leaves of the middle section of one uninfected control tree and one rust-infected tree (14 dpi) were enclosed in PET bags. Charcoal filtered air (1.5 l min^{-1}) was pumped into each bag and the air flowing out (1.0 l min^{-1}) was directed into the arms of the arena.

Based on a Random Forest analysis of volatile emission (see the Statistical analysis subsection later for more details) and commercial availability, individual compounds were chosen for further olfactometer experiments.

Individual compounds were applied via dispensers containing 200 µl solution in hexane (for details, see Fig. S1). The concentrations of the compounds were chosen to mimic the natural emission rate of young rust-infested black poplar trees of 1 m height (Table S3). For solvent control, 200 µl pure hexane was used. To test the caterpillar behavior towards the volatile blend released from rust fungus spores, 25 mg of fresh *M. larici populina* spores were carefully collected with a brush and then placed into a dispenser. Here, pure air was used as control in the arena.

Statistical analysis

All statistical analyses, except the Random Forest analysis, were performed with Spss 17.0 (SPSS, Chicago, IL, USA). Whenever necessary, data were transformed to meet statistical assumptions such as normality and homogeneity of variances for parametric testing (two-way ANOVA, paired Student's *t*-test). When these assumptions were not fulfilled, nonparametric testing was performed: Kruskal–Wallis tests were applied for volatile emissions and transcript abundance, Mann–Whitney *U*-tests for volatile emission, and Wilcoxon signed-rank tests for the olfactometer results (rust spores, 1-octen-3-ol, benzaldehyde, (*E*)-β-caryophyllene).

To identify volatiles distinguishing the blend of rust-infected vs uninfected black poplar trees, 'Random Forest', a machine learning algorithm (Breiman, 2001), was applied using the software MetaboAnalyst 3.0 (Xia *et al.*, 2015). With this multivariate statistical tool, $n = 5000$ bootstrap samples were drawn with eight (control vs rust) or nine (herbivory vs rust + herbivory) individual compounds chosen randomly at each node. The importance of each volatile for the distinction of the blends is expressed as the mean decrease in accuracy (MDA) and the chance of the compound being improperly classified is expressed as the out-of-bag (OOB) error rate. With the 'Random Forest' algorithm, large datasets with more variables (volatile compounds) than sample size and with autocorrelation between the variables (as is the case for plant volatiles produced by common biosynthetic pathways) can be analyzed (Ranganathan & Borges, 2010).

Results

Herbivory stimulates volatile emission more than fungal infection does, but fungal infection suppresses herbivore-induced emission

To study the effect of multiple attackers on the volatile emission of a tree species, we collected volatiles from black poplar (*P. nigra*) trees after single and combined attack by a pathogenic rust fungus and an herbivorous caterpillar.

Black poplar trees with rust (*M. larici-populina*) infection alone showed an increase (*c.* fourfold) in the rate of emission of total volatiles compared with uninfected controls (Table S4; Kruskal–Wallis, $\chi^2 = 24.096$, $P < 0.001$), which was mostly a result of four- and sixfold higher mono- and sesquiterpene emissions, respectively (Fig. 1a,b; Kruskal–Wallis, monoterpenes, $\chi^2 = 22.711$, $P < 0.001$; sesquiterpenes, $\chi^2 = 17.675$, $P < 0.001$). The emission of aromatic compounds also slightly increased (Fig. 1c). Additionally we identified two C_8-compounds, 1-octen-3-ol and 3-octanone, which were exclusively released from rust-infected trees (Fig. 1f). When analyzing the volatile emission of *M. larici-*

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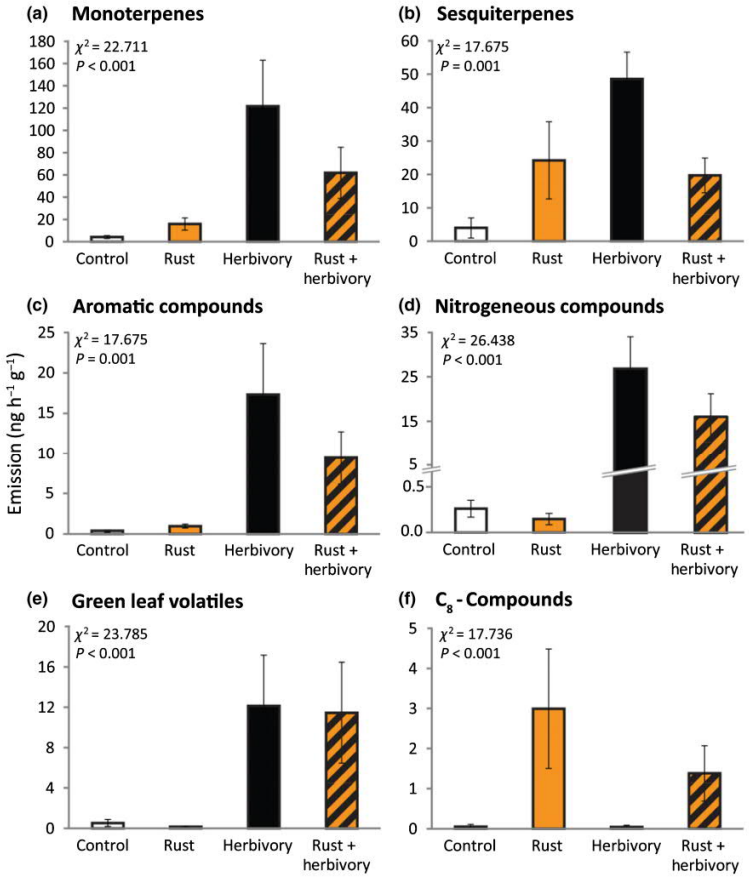

Fig. 1 Influence of rust infection (rust), gypsy moth caterpillar herbivory (herbivory) or both treatments combined (rust + herbivory) on rate of volatile emission from *Populus nigra* trees compared with uninfected, undamaged controls. *Lymantria dispar* larvae were allowed to feed for 2 d directly before and during the 4 h volatile collection. Trees were infected with rust fungus (*Melampsora larici-populina*) 12 d before caterpillar feeding. (a–f) Volatiles were sorted by compound classes (a complete list of each class is given in Supporting Information Table S4). Mean ± 1 SE (n = 8–9). Results of Kruskal–Wallis tests are shown at the top left.

populina spores separately, these and other C_8-compounds were found to be emitted by the rust fungus itself (Fig. 2). The emission of nitrogenous compounds and green leaf volatiles did not change after rust infection or showed a tendency to decrease (Fig. 1d,e).

The influence of herbivory by gypsy moth (*L. dispar*) caterpillars on black poplar volatile emission was much stronger than that of rust infection with an increase of over 30-fold compared with undamaged controls. The release of major volatile classes such as mono-, homo- and sesquiterpenes and aromatic compounds (Figs 1a–e, 3f) increased between 12-fold (sesquiterpenes) and 250-fold ((*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-DMNT, a homoterpene; Kruskal–Wallis, $\chi^2 = 29.005$, $P < 0.001$). In contrast to rust infection, caterpillar feeding also induced the emission of nitrogenous compounds and green leaf volatiles (Kruskal–Wallis, nitrogenous compounds, $\chi^2 = 26.438$, $P < 0.001$; green leaf volatiles, $\chi^2 = 23.785$, $P < 0.001$). However, caterpillar feeding did not affect the emission of C_8-compounds as there was no quantitative difference in the emission of these compounds from rust-infected trees with or without herbivory (Mann–Whitney *U*-test, $P = 0.606$).

When rust infection and herbivory occurred at the same time, rust infection suppressed caterpillar-induced volatile emission from black poplar trees. Trees attacked by both the pathogen and the herbivore emitted two- to three-fold less total volatiles than trees with caterpillar damage only, even though the herbivore damage was the same (Table S2). Among compound classes, sesquiterpenes, in particular, declined (Fig. 1b; Table S5), but monoterpenes, aromatics, and nitrogenous compounds also showed a lower emission (Fig. 1a,c,d). Green leaf volatile emission of herbivore-damaged trees was not affected by rust infection (Fig. 1e).

Overall, the treatment combining leaf rust fungus and gypsy moth caterpillars did not lead to an additive effect on the volatile emission. Rather, rust infection attenuated the caterpillar-induced volatile emission, especially of terpenoids.

Fungal suppression of herbivore-induced terpenoid emission is regulated at the transcript level

As terpenoids made up the biggest portion of the black poplar volatile blend and displayed pronounced changes under separate

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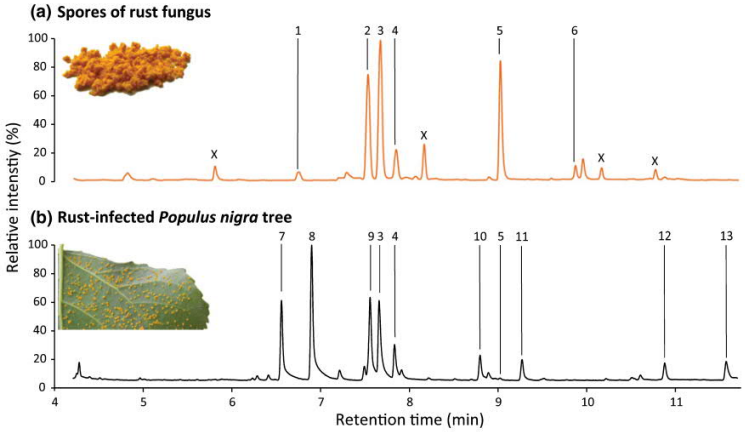

Fig. 2 Comparison of the headspace of isolated spores of the rust fungus (*Melampsora larici-populina*) (a) with a rust-infected *Populus nigra* tree (b). Volatile compounds were collected on polydimethylsiloxane tubes (a) or PoroPak adsorbent (b) and analyzed with GC-MS. 1, 5-Methyl-3-heptanone; 2, 1,5-octandien-3-ol; 3, 1-octen-3-ol*; 4, 3-octanone*; 5, benzyl alcohol*; 6, 2-octen-1-ol; 7, α-pinene*; 8, camphene*; 9, β-pinene*; 10, limonene*; 11, (*E*)-β-ocimene*; 12, (*E*)-4,8-dimethyl-1,3,7-nonatriene*; 13, camphor*; X, contamination. Identity of compounds marked with '*' was confirmed by comparison of mass spectra and retention times with those of authentic standards in this or earlier work (Clavijo McCormick *et al.*, 2014a); identity of other compounds was determined by comparison of their mass spectra with databases.

and combined attack, the transcript abundance of a number of terpene synthase genes was analyzed to learn more about how terpene emission is regulated. The terpene synthases selected for this study were described in the literature previously (Table S1) and are involved in the biosynthesis of all three classes of terpenoids found in poplar volatiles: monoterpenes (PtTPS3 (linalool synthase) and PnTPS1 (camphene synthase, a multiproduct enzyme)); homoterpenes (PnTPS2 ((*E*)-nerolidol synthase) produces the precursor of DMNT); and sesquiterpenes (PtTPS1 (germacrene D synthase, a multiproduct enzyme), PtTPS2 ((*E, E*)-α-farnesene synthase) and PtTPS9 ((*E*)-β-caryophyllene synthase, a multiproduct enzyme)).

During rust infection these transcript abundances were either similar (*PtTPS3*, *PtTPS9*) or lower (*PnTPS2*, *PtTPS1*) than those of uninfected controls (Fig. 3a–d), in contrast to the higher emission of the metabolites produced by the encoded enzymes (Fig. 3e–h). The emission of linalool, (*E*)-DMNT as well as the sesquiterpenes produced by PtTPS1 and PtTPS9 increased after rust infection (Kruskal–Wallis, linalool, $\chi^2 = 16.236$, $P = 0.001$; (*E*)-DMNT, $\chi^2 = 29.005$, $P < 0.001$; PtTPS1 products, $\chi^2 = 17.972$, $P < 0.001$; PtTPS9 products, $\chi^2 = 12.178$, $P = 0.007$). Caterpillar feeding, on the other hand, strongly induced both the transcript abundance of the terpene synthases and the emission of the corresponding metabolites (Fig. 3). The transcript abundances increased strongly (Fig. 3a–d) – 15-fold for *PtTPS9*, 20-fold for *PtTPS3*, 150-fold for *PnTPS2*,and 200-fold for *PtTPS1* (Kruskal–Wallis, *PtTPS9*, $\chi^2 = 11.911$, $P = 0.008$; *PtTPS3*, $\chi^2 = 18.019$, $P < 0.001$; *PnTPS2*, $\chi^2 = 29.724$, $P < 0.001$; *PtTPS1*, $\chi^2 = 29.100$, $P < 0.001$) – as did the emission of the corresponding products (Fig. 3e–h).

When rust fungus infection and caterpillar feeding were combined, the result was lower transcript abundance for most terpene synthases compared with caterpillar feeding alone (Fig. 3a–d). *PtTPS3*, *PnTPS2*, and *PtTPS1* had lower transcript abundance with added rust infection, which is reflected in the emission of their products linalool, (*E*)-DMNT, and the products of PtTPS1, respectively (Fig. 3e–h). Exceptions to this correlation between transcript abundance and emission of volatile products were camphene synthase (PnTPS1) and (*E,E*)-α-farnesene synthase (PtTPS2) (Fig. S2a–d): *PnTPS1* showed lower terpene synthase transcript but similar product emission levels in rust and caterpillar treatment vs caterpillar treatment alone. *PtTPS2* was unchanged in both transcript and product emission for this same comparison.

In order to determine if there was a general increase or decrease in the metabolic activity of black poplar leaves in the different treatment groups, we measured the concentration of soluble sugars and amino acids. Rust fungus infection had no effect on sugar and amino acid concentrations in black poplar leaves (Table S6).

Taken together, these results show that the presence of rust fungus on caterpillar-infested trees generally reduced terpene emission by reducing the transcriptional induction of terpene synthases caused by caterpillar feeding.

Salicylic acid increases while jasmonates decrease in black poplar trees after combined pathogen and caterpillar attack

To evaluate upstream factors regulating terpene synthase transcription in black poplar leaves, defense hormones and genes involved in hormone biosynthesis and signaling were studied. SA significantly increased in response to rust infection, but was not affected by caterpillar feeding (Fig. 4a; statistical results in Table 1). On the other hand, jasmonates were not affected by rust infection, but increased significantly upon caterpillar

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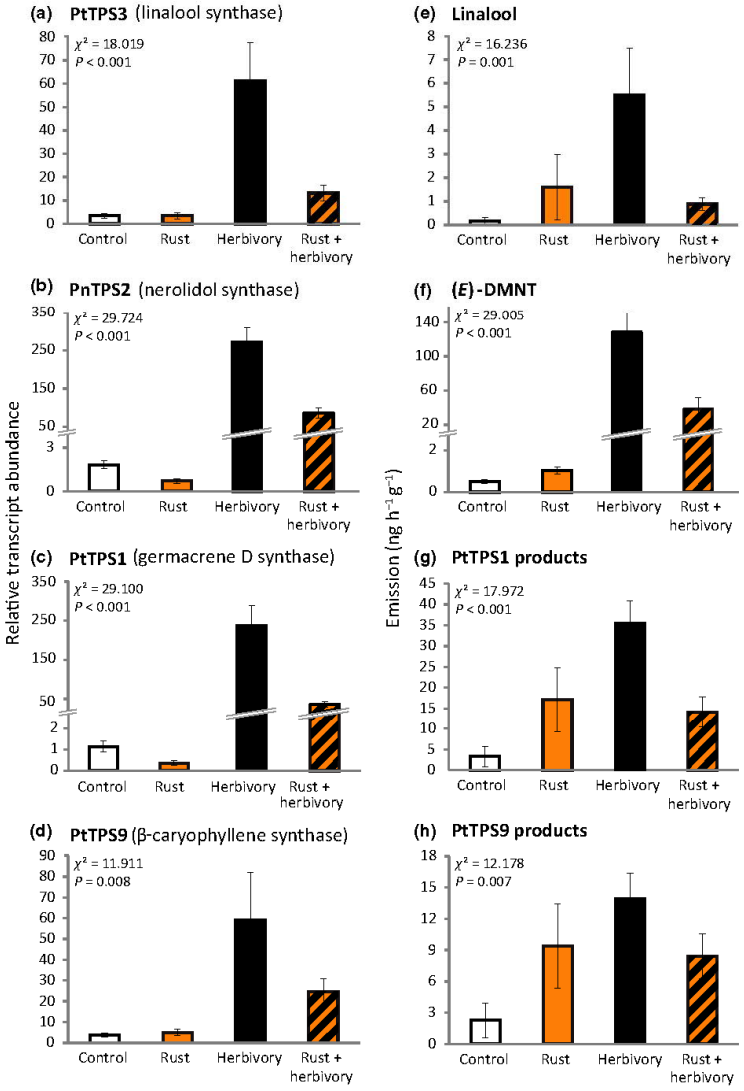

Fig. 3 Transcript abundance of terpene synthase genes and emission of the products of their encoded enzymes from *Populus nigra* trees treated with rust fungus (rust), gypsy moth caterpillars (herbivory), both attackers (rust + herbivory) or untreated (control). *Lymantria dispar* larvae were allowed to feed for 2 d directly before and during the 4 h volatile collection. Trees were infected with rust fungus (*Melampsora larici-populina*) 12 d before insect feeding. (a–d) Transcript abundance of terpene synthase genes previously characterized from *P. nigra* (*PnTPS*) or *Populus trichocarpa* (*PtTPS*) determined by quantitative real-time PCR. Results were normalized to *Actin* and expressed relative to a calibrator sample. (e–h) Emissions of products of the corresponding enzymes were identified and quantified by GC-MS and GC-flame ionization detection, respectively. The product of PnTPS2, (*E*)-nerolidol, is not emitted but is the precursor of (*E*)-4,8-dimethyl-1,3,7-nonatriene ((*E*)-DMNT) (f). PtTPS1 (c) and PtTPS9 (d) are multiproduct enzymes and the sums of their products are presented (f, h). Mean ± 1 SE ($n = 8–9$). Results of Kruskal–Wallis tests are shown at top left.

herbivory (Fig. 4b; statistical results in Table 1). In the combined treatment of rust infection and herbivory, this increase was much lower (Fig. 4b). As jasmonic acid and its derivatives (JA, (+)- and (−)-JA-Ile, hydroxy-JA, hydroxy-JA-Ile, carboxy-JA-Ile) showed the same patterns for all treatments, these compounds were summed and termed 'jasmonates' throughout the manuscript. The significant interaction of rust and herbivory on jasmonate concentrations was also verified by two-way ANOVA (Table 1).

Marker genes for the SA signaling pathway (Derksen *et al.*, 2013; Jiang *et al.*, 2014), *pathogenesis-related gene 1* (*PR-1*) and two WRKY genes (*WRKY 70*, *WRKY 89*) were significantly up-regulated by rust infection (Fig. 4b; Table 1). However, the transcript abundance of *nonexpressor of pathogenesis-related proteins 1* (*NPR1*), which is typically also responsive to SA, did not change after rust infection, despite the increase in SA concentrations. Herbivory alone led to a slight decrease in *NPR1* transcript abundance (Fig. 4c). Allene oxide synthase (AOS) is an enzyme involved in JA biosynthesis, but is also responsive to this hormone via a positive feedback loop (Wasternack & Hause, 2013). *AOS* transcript abundance strongly increased after caterpillar feeding (two-way ANOVA factor 'herbivory', $P < 0.001$). The combined attack of rust and caterpillars resulted in lower *AOS* transcript abundance than did caterpillar feeding alone (Fig. 4d). Taking all treatments together, the transcript

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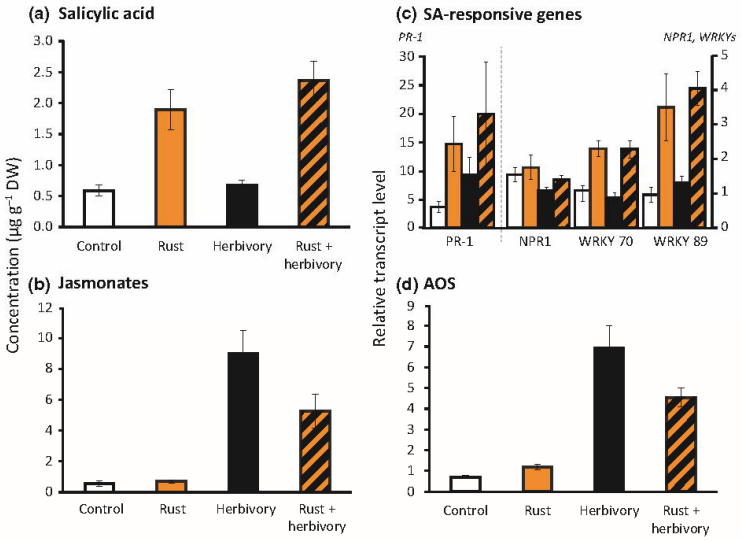

Fig. 4 Concentrations of defense hormones and hormone-related gene transcripts in *Populus nigra* trees that were treated with rust fungus (rust), gypsy moth caterpillars (herbivory), both attackers (rust + herbivory) or untreated (control). *Lymantria dispar* larvae were allowed to feed for 2 d directly before sampling. Trees were infected with rust fungus (*Melampsora larici-populina*) 12 d before insect feeding. (a, b) Defense hormone concentrations in leaves analyzed by LC-MS. (c, d) Transcript abundance of salicylic acid (SA)-responsive genes (*PR-1*, *pathogenesis-related gene 1*; *NPR1*, *nonexpressor of PR proteins 1*) (c) and jasmonate-responsive genes (*AOS*, *allene oxide synthase*) (d). Legend in (c) is the same as that in panels (a, b, d). Results were normalized to *Actin* and expressed relative to a calibrator sample. Mean ± 1 SE (n = 8–9). For results of statistical analyses see Table 1. Jasmonates = sum of jasmonic acid (JA), (+)- and (−)-jasmonoyl isoleucine (JA-Ile), hydroxy-JA, hydroxy-JA-Ile, carboxy-JA-Ile. [Correction added after online publication 18 April 2017: the units on the *y*-axis of (a, b) have been corrected.]

Table 1 Statistical results of a two-way ANOVA or Kruskal–Wallis test (*WRKY 89*) for the concentrations of defense hormones (salicylic acid, jasmonates) and hormone-related gene transcripts (*PR-1*, *NPR1*, *WRKYs*, *AOS*) in *Populus nigra* leaves to estimate the effect of rust infection by *Melampsora larici-populina* ('rust'), caterpillar herbivory by *Lymantria dispar* ('herbivory') and the interaction of both ('rust × herbivory')

	Factor	df_1	df_2	*F*-value	*P*-value[1]
Salicylic acid	Rust	1	31	52.670	**< 0.001**
	Herbivory	1	31	1.784	0.191
	Rust × herbivory	1	31	0.210	0.650
Jasmonates	Rust	1	31	0.001	0.431
	Herbivory	1	31	276.149	**< 0.001**
	Rust × herbivory	1	31	6.656	**0.015**
PR-1	Rust	1	30	6.488	**0.016**
	Herbivory	1	30	1.183	0.285
	Rust × herbivory	1	30	0.483	0.492
NPR1	Rust	1	30	1.680	0.682
	Herbivory	1	30	4.303	**0.047**
	Rust × herbivory	1	30	0.260	0.614
WRKY 70	Rust	1	30	41.525	**< 0.001**
	Herbivory	1	30	0.163	0.689
	Rust × herbivory	1	30	0.106	0.747
WRKY 89	–	3	–	22.242[2]	**< 0.001**
AOS	Rust	1	30	0.311	0.581
	Herbivory	1	30	217.777	**< 0.001**
	Rust × herbivory	1	30	11.767	**0.002**

PR-1, pathogenesis-related gene 1; NPR1, nonexpressor of PR proteins 1; AOS, allene oxide synthase.
[1]Bold numbers indicate statistically significant results.
[2]χ^2-value of Kruskal–Wallis analysis.

abundance of *AOS* was positively correlated with the concentration of jasmonates (Spearman's rank correlation, $\rho = 0.875$, $P < 0.001$).

To verify the role of the SA pathway in the repression of herbivore-induced volatile emission, we treated black poplar trees with methyl salicylate (MeSA) (Park *et al.*, 2007). Trees that were MeSA-treated emitted a lower concentration of volatiles after herbivory than did control trees (Fig. S3). This effect was strongest on sesquiterpene emissions (Kruskal-Wallis, $\chi^2 = 16.662$, $P = 0.001$), similar to what was observed for pathogen infection.

Gypsy moth caterpillars are attracted to volatiles from rust spores

In order to assess the effect of rust-mediated changes in black poplar volatile emission on a herbivore, behavioral assays were performed on gypsy moth caterpillars in an olfactometer. Early-instar caterpillars that were allowed to choose between an uninfected and a rust-infected tree significantly preferred the volatile blend of the rust-infected tree (Fig. 5; paired *t*-test, $P = 0.035$). We conducted a RANDOM FOREST analysis to determine the compounds that most discriminated between the volatile blends of rust-infected and uninfected black poplar trees (Table 2). 3-Octanone, (*E*)-DMNT, (*E,E*)-α-farnesene, benzaldehyde, (*Z*)-ocimene, 1-octen-3-ol, benzyl alcohol, camphene, α-humulene, and (*E*)-β-caryophyllene all increased after rust infection

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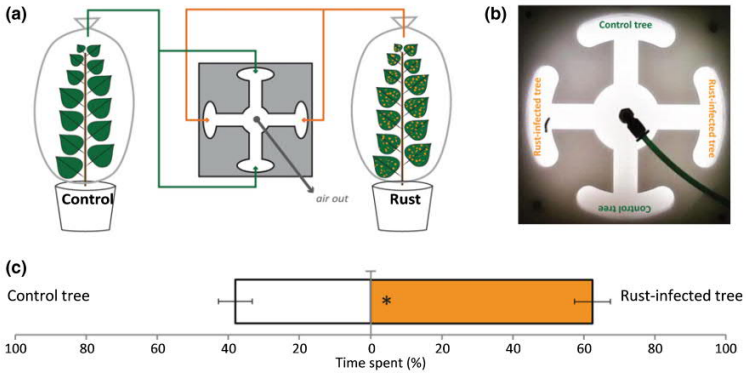

Fig. 5 Preference of *Lymantria dispar* caterpillars towards the headspace of *Populus nigra* trees with or without rust (*Melampsora larici-populina*) infection. (a) Experimental setup for directing the headspace air from the trees into the olfactometer. Headspace air from the trees is pumped out, split and directed to the arms of the olfactometer. In the center of the arena, the air is pumped out to create an air flow. More details are given in the Materials and Methods section under 'Olfactometer experiments'. (b) Cross-section of the four-arm olfactometer from the top, indicating the presence of air from each the treatment. (c) Time spent by the caterpillars in each treatment area. Durations in treatment arms with the same treatment were summed and the time of both treatments was taken as 100%. Mean ± 1 SE ($n = 29$). Paired *t*-test, significant difference is marked with an asterisk (*, $P < 0.05$).

Table 2 Importance ranking of compounds that are most discriminative for the volatile blends of rust-infected and uninfected *Populus nigra* trees, as determined by Random Forest analysis

Rank	Compound	MDA
1	3-Octanone	0.032
2	(*E*)-DMNT	0.022
3	(*E*,*E*)-α-Farnesene	0.013
4	Benzaldehyde	0.009
5	(*Z*)-Ocimene	0.007
6	1-Octen-3-ol	0.007
7	Benzyl alcohol	0.007
8	Camphene	0.006
9	α-Humulene	0.006
10	(*E*)-β-Caryophyllene	0.006

MDA, mean decrease in accuracy.
Out-of-bag (OOB) error = 0.235; classification error: 'control' = 0.111, 'rust-infected' = 0.375.

(Fig. S4). Six of these discriminating volatiles were then tested as single compounds dissolved in hexane in olfactometer assays to establish whether any of them was responsible for the preference of the caterpillars (Fig. 6). Benzyl alcohol, which is emitted by both black poplar leaves and *M. larici-populina* spores (Figs 2, S4), was preferred by the caterpillars to the solvent control (paired *t*-test, $P = 0.032$), but was not as attractive as the complete blend of isolated spores compared with pure air (Wilcoxon signed-rank test, $P = 0.010$). However, none of the other compounds was attractive. The fungus-derived C_8-compounds, 1-octen-3-ol and 3-octanone, and two of the plant-derived compounds, (*E*)-β-caryophyllene and (*E*)-DMNT, were not significantly preferred or avoided by gypsy moth caterpillars. Moreover, benzaldehyde was a repellent, as caterpillars spent significantly more time in the olfactometer areas with the solvent control than in the areas with benzaldehyde (Wilcoxon signed-rank test, $P = 0.031$).

Discussion

Herbivore-induced volatiles may be involved in both direct and indirect plant defense against insect herbivores, and are also used by herbivores for host plant selection (Unsicker *et al.*, 2009; Dicke & Baldwin, 2010). The emission of volatiles has been frequently studied in response to attacks by single enemies, mostly by herbivores, but how it is influenced by multiple enemy attacks is rarely studied (Ponzio *et al.*, 2013). Furthermore, volatile emission and its regulation are far less well understood in woody plants compared with herbaceous species (Eyles *et al.*, 2010). Here we investigated the effect of separate and combined enemy attacks on the volatile emission of black poplar (*P. nigra*) trees, the underlying mechanisms of volatile biosynthesis and insect herbivore behavior. We found that: (a) single attack of either a pathogen or a herbivore triggered the release of plant volatiles, with herbivory inducing greater emission; (b) combined attack of a pathogen and an herbivore resulted in repression of herbivore-induced emission; (c) combined attack caused defense hormone crosstalk; and (d) generalist caterpillars preferred volatiles from pathogen-infected poplar trees.

Induction of poplar volatile emission after attack by rust fungus or gypsy moth caterpillars is regulated in different ways

The infection of black poplar with the biotrophic leaf rust *M. larici-populina* increased the emission of terpenes, aromatics and C_8-compounds (Fig. 1). These C_8-compounds were found to be emitted by the fungus itself (Fig. 2), consistent with previous studies on other fungal species (Combet *et al.*, 2006). The emission of terpenes from plants after pathogen attack has been reported in several other systems, for example, rust-infected willow (Toome *et al.*, 2010) and mildew-infected squash (Tabata *et al.*, 2011). The inhibiting effect of terpenes on pathogens, such

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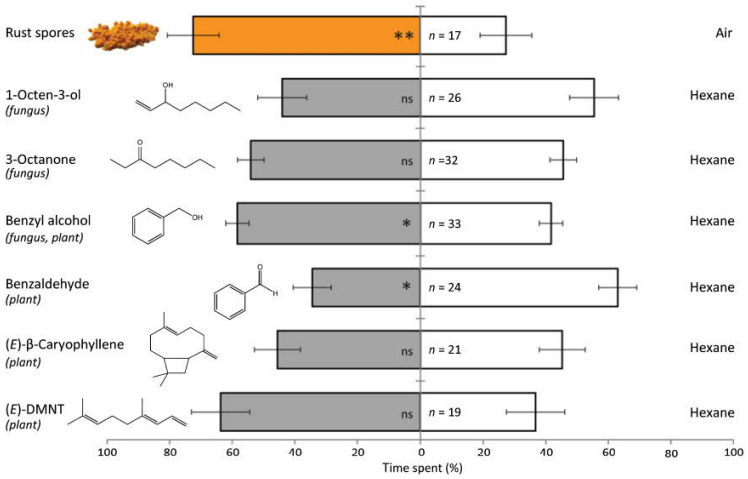

Fig. 6 Behavior of *Lymantria dispar* larvae towards isolated spores of the rust fungus *Melampsora larici-populina* and towards individual volatile compounds emitted by black poplar foliage infected by the rust. The natural origin of each compound is indicated in brackets (for fungal volatiles see Fig. 2). Early-instar caterpillars were observed in a four-arm olfactometer and the time spent in each arm was determined by video analysis. Durations in treatment arms with the same treatment were summed and the time of both treatments was taken as 100%. Compounds were dissolved in hexane and tested against a hexane control. Mean ± 1 SE (*n* indicated in bars). Paired *t*-test or Wilcoxon signed-rank test; significant differences are marked with asterisks (*, $P < 0.05$; **, $P < 0.01$; ns, not significant). (*E*)-DMNT, (*E*)-4,8-dimethyl-1,3,7-nonatriene.

as those causing rice bacterial blight (Lee *et al.*, 2015), Faba-bean rust (Mendgen *et al.*, 2006) and white mold (Cardoza *et al.*, 2002), suggests that terpenes might serve as anti-pathogen defenses. Whether this is also the case in the poplar–rust interaction should be addressed in future work focusing on antifungal defense.

The mechanisms regulating terpene emission in rust-infected trees remain unclear. The transcript patterns of poplar terpene synthases measured do not explain the increased emission in rust-infected trees (Fig. 3). Instead, regulation of upstream enzymes in the methylerythritol phosphate or mevalonate pathways (Rodriguez-Concepcion, 2006) at the transcriptional or post-transcriptional levels (Leivar *et al.*, 2011; Doblas *et al.*, 2013) could explain the increase of poplar volatiles. Alteration in metabolic flux caused by pathogen infection (Toome *et al.*, 2010) or even the fungal mevalonate pathway (Schmidt-Dannert, 2014) might also be involved.

Separate attack by gypsy moth (*L. dispar*) caterpillars also increased the emission of black poplar volatiles, predominantly terpenes, but to a much greater extent than rust infection (Fig. 1). Herbivore-induced volatiles in this species are known to have roles in direct (McCormick *et al.*, 2016) and indirect (McCormick *et al.*, 2014b) defenses.

The regulation of herbivore-induced terpene emission appears to occur at the transcriptional level as terpene synthase transcripts increased dramatically after herbivore damage, closely correlated with the increase in emission (Fig. 3). The control of terpene emission by changes in transcript abundance of terpene synthases was shown previously for *P. trichocarpa* (Danner *et al.*, 2011; Irmisch *et al.*, 2014) and in many other species. Volatile terpenes do not accumulate to any significant extent in leaves after herbivore damage, but are directly released after their biosynthesis (Köllner *et al.*, 2004).

Rust infection decreases herbivore-induced volatile emission

While separate attacks by rust fungus or caterpillars both increased the emission of volatiles from black poplar trees, the combined attack of these two antagonists did not result in an additive effect. Instead, the rust infection attenuated the greater herbivore-induced emission (Fig. 1). Such combined attacks were previously studied only in herbaceous plants. This work showed a range of effects: pathogen infection had negative (Cardoza & Tumlinson, 2006; Rostás *et al.*, 2006; Desurmont *et al.*, 2016), positive (Cardoza & Tumlinson, 2006) or no influence (Ponzio *et al.*, 2014, 2016) on herbivore-induced volatile emission. This underlines the specificity of combined attack scenarios. In the scenario investigated here, one possible explanation for the lower herbivore-induced emission under combined attack may be a tradeoff between anti-pathogen and anti-herbivore defense with lower allocation to anti-herbivore defenses as a result of allocation to anti-pathogen defense. From a regulatory perspective, the decline of volatile emission in pathogen- and herbivore-attacked trees compared with trees attacked only by the herbivore might be ascribed to changes in general metabolic rate. However, sugar and amino acid content did not significantly change in response to rust infection (Table S6). Instead the decrease in volatile emission is better explained by the decrease in transcript abundance of terpene synthases in rust-infected and caterpillar-damaged trees compared with those in trees attacked by the caterpillar alone (Fig. 3). This suggests that emission after combined attack is

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transcriptionally regulated, as is the case after herbivory alone (Danner *et al.*, 2011; Irmisch *et al.*, 2014).

Pathogen attack results in a repression of herbivore-induced jasmonate signaling

Biotrophic pathogens such as rust fungi and chewing insects such as caterpillars induce different defense signaling pathways, involving SA and jasmonate, respectively (Pieterse & Dicke, 2007). To get an insight into possible changes in signaling upon separate vs combined attack, we measured defense hormone concentrations and transcript abundance of genes involved in hormone signaling. Rust infection alone or in combination with caterpillar feeding both increased SA concentrations as well as downstream marker genes (Fig. 4), clearly showing activated SA signaling. However, although caterpillar feeding alone increased the concentrations of JAs, this increase was attenuated when trees were additionally infected with rust, suggesting hormone crosstalk between SA and JAs. An artificial treatment of black poplar trees with methyl salicylate resulted in the same terpene emission patterns as pathogen infection (Fig. S3), underlining a regulatory role of the SA pathway after rust infection and combined rust and gypsy moth attack in black poplar. In recent years, several studies investigated the role of defense hormones in combined attacks, mostly in *Arabidopsis* or tomato, and found examples for synergistic, antagonistic, and independent interactions (Derksen *et al.*, 2013; Caarls *et al.*, 2015). In our system, JA repression by rust can be explained by a lowered rate of biosynthesis, as indicated by a decrease in the transcript abundance of *AOS* (Fig. 4). On the other hand, a decrease in JA signaling downstream of JA biosynthesis could also result in lowered *AOS* transcript abundance as a result of the positive feedback loop in JA signaling (Wasternack & Hause, 2013). In contrast to *AOS*, the transcript abundance of the SA pathway regulator *NPR1* did not change for rust-infected trees with or without herbivory (Fig. 4). Previous studies on herbaceous plants revealed NPR1 to be a key player in the repression of JA signaling by SA (Spoel *et al.*, 2003; Beckers & Spoel, 2006). By contrast, Xue *et al.* (2013) claim that NPR1 is not involved in SA signaling in poplar. Indications for NPR1-independent hormone signaling were also found in *Arabidopsis* (Blanco *et al.*, 2005). *NPR1* induction in our system might be missing as a result of the timing of sampling. We harvested samples at 14 dpi, whereas a study by Rinaldi *et al.* (2007) found a peak in *NPR1* transcription at 1 dpi. Although more research on the signaling mechanism is needed, our study supports the idea of an antagonistic interaction between SA and JA signaling in woody plant species confronted by different modes of attack.

A generalist herbivore is attracted to pathogen-infected trees

Besides their function in plant defense, volatiles can also serve as cues for herbivores to find their host plants. Here we showed that caterpillars of the gypsy moth, a generalist feeder, preferred volatiles from rust-infected vs uninfected black poplar trees (Fig. 5), thus illustrating the biological relevance of pathogen-induced volatile changes. Among individual compounds, caterpillars were slightly attracted to the aromatic volatile benzyl alcohol (Fig. 6). However, this preference was much smaller than the preference for complete blends, such as the headspace of plants or fungal spores (Figs 5, 6). This indicates that a blend of different compounds might be more important than a single compound for the attraction of gypsy moth caterpillars (McCormick *et al.*, 2016). The importance of the caterpillars' choice in this insect species is underlined by the fact that the female moths cannot fly (Wilson, 2016). Hence, the caterpillars may play a bigger role in determining their feeding substrate than do other herbivore larvae. Whether gypsy moth caterpillars gain any advantage from choosing to feed on fungus-infected plants is unclear. As black poplar volatiles have been shown to attract a wasp parasitizing gypsy moth caterpillars (McCormick *et al.*, 2014b), the decline of herbivore-induced volatile emission caused by the rust fungus may reduce enemy pressure on the caterpillars. However, other studies showed that parasitoids possibly prefer hosts reared on pathogen-infected plants (Cardoza *et al.*, 2003) or do not distinguish between pathogen-infected and uninfected plants (Ponzio *et al.*, 2014, 2016). Another advantage to gypsy moth caterpillars in preferring rust-infected leaves might be the chemical constituents of this food source. Increased concentrations of nutrients such as protein, sterols and vitamins in fungal-infected tissue (Hatcher, 1995), as well as lower concentrations of defense compounds, could have selected for the volatile-guided preferences of this insect.

Conclusion

Our results suggest that the combined effects of pathogen and herbivore attack on woody plants might have far-reaching consequences at the community and ecosystem levels. The attenuation of black poplar volatile release by fungal infection could decrease defense against herbivores, reduce attraction of natural enemies, alter herbivore host selection and even modify intra- and interspecific tree communication. Further work is required on mature trees growing under natural conditions as well as on the response of parasitoids to trees under separate and combined attack.

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Author contributions

S.B.U., F.E., and A.H. planned and designed the research; F.E. performed the research and analyzed the data; and F.E., S.B.U., and J.G. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Setup of the olfactometer for behavioral assays with caterpillars.

Fig. S2 Transcript abundance of terpene synthases and the emission of their products.

Fig. S3 Effect of methyl salicylate application on the volatile emission from *Populus nigra*.

Fig. S4 Emission of single compounds used in the olfactometer assay from rust-infected and uninfected trees.

Table S1 Primer sequences used in this study

Table S2 Herbivory damage and pathogen quantification

Table S3 Chemicals and concentrations used in the olfactometer assay

Table S4 Volatile compounds from *Populus nigra* and their classification

Table S5 Importance ranking of herbivore-induced volatiles from trees with and without rust infection

Table S6 Primary metabolites in *Populus nigra* leaves

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6. MANUSCRIPT IV

Co-evolution in a threesome?

The tripartite interaction of fungi, plants and herbivores

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Title:

Co-evolution in a threesome? The tripartite interaction of fungi, plants and herbivores

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6. Manuscript IV

Abstract

When insect herbivores feed on their host plant under natural conditions, they automatically also consume plant-inhabiting microbes. Despite their omnipresence in plants, microbes are rarely considered in studies on insect herbivore behavior and performance. Therefore, we investigated the direct and indirect effects of the pathogen on generalist insect herbivores in black poplar. We analyzed the chemistry of tree and fungal tissues as well as the behavior and performance of the insects. In choice assays the herbivores preferred infected over uninfected plant material and selectively fed on fungal tissue. Moreover, insects reared on infected foliage developed faster than those on uninfected controls, presumably by enhanced nitrogen and amino acid uptake. In case our finding on the selective feeding of generalist herbivores on a plant pathogen is a widespread phenomenon, the definition of a plant feeding insect (herbivore) needs to be reconsidered.

Keywords

biotrophic pathogen, coevolution, generalist, gypsy moth, herbivore, mycophagy, Salicaceae, rust fungus

Introduction

Insects are the most diverse class of organisms with an estimated number of 5.5 million species on Earth (Stork 2018). A huge proportion of insects is associated with plants, often throughout all life stages, from larvae feeding on plant tissues to adults pollinating flowers (Krenn 2010). The co-evolution of herbivorous insects and their host plants has been intensively studied in the past decades (Ehrlich & Raven 1964, Futuyma & Agrawal 2009). However, plants are not isolated organisms; they are closely associated with microbes, which can inhabit literally every organ of a plant (Baldrian 2017). Those plant-associated microbes might develop quiescently without effect, cause diseases or be beneficial for their hosts. Mycorrhiza and plant growth promoting rhizobacteria, for example, were reported to support plants in defending against herbivores (Kaling *et al.* 2018; Van Wees *et al.* 2008), some are even used commercially in plant protection (Ramamoorthy *et al.* 2001). However, it was recently reported that microbes can also benefit the insects instead of their host plants. *Acinetobacter sp.*, associated to aspen foliage can detoxify anti-herbivore defense compounds, thereby supporting folivorous insects (Mason *et al.* 2014). In their natural environment, plants are colonized by numerous microbial species and thus microbes or microbial metabolites are frequently ingested by herbivorous insects (Eberl *et al.* 2018). Therefore, it is even more remarkable that microbes are neglected in the vast majority of previous studies on plant-insect interactions. Here, we therefore want to broaden the view on plants into ecological units that consist of the plant matrix and microbial cells. This aspect will consequently affect the definition of plant-feeding insects as herbivores. To what extend can herbivores be considered as facultative or obligate fungivores? Do they ingest fungal tissue accidentally or intentionally to increase their performance? Apart from direct effects by ingestion of microbial cells or metabolites, herbivores can be affected indirectly through microbe-inflicted changes in the plant chemistry or physiology. Microbes can induce, among other changes, degradation of phenolic compounds (Zhao *et al.* 2018), alterations in source-sink relationships (Berger *et al.* 2007) or activation of defense hormone signaling (Glazebrook 2005). However, it is often difficult to disentangle direct and indirect interactions between plant-inhabiting microbes and plant-feeding insects as both plant and microbial tissue are ingested simultaneously.

Woody plant species can harbor an enormous diversity of simultaneously occurring insect and microbial species due to their large dimensions, long life span and chemical diversity (Lämke & Unsicker 2018). We therefore chose to work with the deciduous tree species black poplar

(*Populus nigra*), a tree-feeding insect, gypsy moth (*Lymantria dispar*) and the pathogenic rust fungus *Melampsora larici-populina*, to answer the question whether an insect herbivore can benefit from mycophagy. Gypsy moth larvae feed on a broad range of host plants which belong to more than 40 plant families (Robinson *et al.* 2010). This insect is native to Europe, but was accidently introduced into Massachusetts in the 19th century, from where it has been spreading throughout northern America (Hoover 2000). By defoliating millions of hectares of broadleaf forests, this insect became a serious pest with economic and ecological impact in the United States (global invasive species database; Shields *et al.* 2003). Among the favored host trees of the gypsy moth caterpillars are members of the Salicaceae family, such as willows and poplars. The black poplar (*Populus nigra*) is endemic to Europe and therefore represents a natural host for the gypsy moth in its original habitat. Other relatives of the genus *Populus* are distributed all over the northern hemisphere (Isebrands & Richardson 2014; Stanton *et al*, 2010) in natural floodplain forests as well as in plantations where they are grown for economic use (Karp & Shield 2008). Black poplar is colonized by many different endophytes and pathogens (Busby *et al.* 2016; Mason *et al*. 2014), but one of the most devastating microbes is the group of rust fungi (Pei & Shang 2005). Infection with these biotrophic fungi causes a drastic decline in biomass production, premature defoliation and, in young trees, even mortality (Aylott *et al.* 2008; Benetka *et al.* 2011; Polle *et al.* 2013). The most widespread rust fungus on black poplar in Europe is *Melampsora larici-populina*. In repetitive vegetative cycles on poplar leaves it produces of millions of uredospores (Hacquard *et al.* 2011) which then reinfect leaves of the same or a neighbouring tree. As a consequence, *M. larici-populina* displays a longlasting and widespread prevalence on poplar leaves, opening up many temporal and spatial opportunities for the interaction with poplar-feeding herbivores. In a previous study we could demonstrate direct and indirect effects based on attraction of gypsy moth caterpillar to rust spore volatiles and antagonistic defense hormone crosstalk in the host tree *Populus nigra*, respectively (Eberl *et al*. 2017). To reveal ecological consequences of these results we now investigated the preference and performance of gypsy moth larvae on rust-infected and uninfected black poplar leaves. Additionally, we analyzed leaf and fungal tissue to identify chemical cues that explain mechanisms behind these ecological parameters. Our results prove facultative fungivory in generalist herbivores, which results in an accelerated larval development of the insect.

Methods

Plants

Black poplar (*Populus nigra*) trees were grown from stem cuttings obtained from different genotypes growing in a common garden in Isserstedt, Germany (50°57'28.5"N 11°31'17.4"E). For the preference assay a mixture of three different genotypes was used, whereas single clones were used for the time course-preference, chemical analysis, mannitol preference and performance, respectively. Stem cuttings were potted in single 2 l- pots in a 1:5 mixture of sand: soil (Klasmann potting substrate) and grown in the greenhouse (18°C/ 20°C night/ day, humidity 60%, watered and fertilized regularly). Experiments involving fungal infection were conducted in a climate chamber (18°C/ 20°C night/day, humidity 60%, 16 h light (MT 400, Eye, Uxbridge, UK), watered once per day in single trays), where the plants remained for an acclimation phase (2 d) and the time of the experiment.

Fungal infections

Uredospores of the biotrophic poplar leaf rust fungus (*Melampsora larici-populina* Kleb.) were obtained from naturally infected black poplar trees growing in the above mentioned common garden. The identity of the fungus was verified by using specific primers for the internal transcribed spacer region of *M. larici-populina* as described in (Eberl *et al.* 2017). The pathogen was amplified by infecting ½ -year-old trees and 2 – 3 weeks post-infection uredospores were harvested with a scalpel and a brush. Spores were stored at -20°C until the start of the experiment, either dry (drying over silica overnight; preference assay) or fresh (preference over time, chemical analysis, performance). Trees were inoculated with the fungus by spraying a mixture of water and spores (dry: 1 mg ml^{-1}, fresh: 1.5 mg ml^{-1}) on the abaxial side of each leaf (approximately 1 ml per leaf) and covering each tree with a polyethylene terephthalate (PET) bag (Bratschlauch, Toppits, Minden, Germany), which was kept close for one day to ensure sufficient humidity for spore germination. Control groups received the same treatments but with water only. Mildew (*Erysiphales spp.*) infection occured naturally in the greenhouse, so the time since infection cannot be determined for this pathogen.

Insects

Gypsy moth (*Lymantria dispar* L.) caterpillars were hatched from eggs obtained from Hanna Nadel (US Department of Agriculture, Buzzards Bay, MA, USA) and reared on artificial diet (MP Biomedicals LLC, Illkirch, France) in a climate chamber (14 : 10 h, light : dark; 20°C; 60% humidity) until the start of the experiment.

A female rusty tussock moth (*Orgyia antiqua*, L.) was captured in a natural habitat in Jena, Germany, and the offspring of this wild female was used for the preference assay.

Preference assays

Preference of gypsy moth caterpillars was tested in a custom-made arena consisting of a petridish (diameter 9 cm) with eight pins imposed in a circle on the bottom. The bottom was covered with a wet filter paper to prevent desiccation. Leaf discs (diameter 16 mm) were cut with a cork borer from leaves of different treatment groups (control *vs*. rust-infected or mannitol *vs*. no mannitol) and discs from different groups were arranged alternatingly on the pins. A single gypsy moth larvae was placed into the middle of each arena and the petridish was sealed with cotton tape. After 2 d the remaining discs were photographed and the leaf area loss was determined by Photoshop CS5 (Adobe, San Jose, CA, USA).

When different genotypes of trees were used within the treatments, the same genotype was offered in one petridish. For all preference assays the middle aged leaf pool (3^{rd} to 10^{th} leaf from apex) from each tree was used to cut leaf discs.

For the mannitol choice assay leaves were coated with a thin layer (2.5 ml per 100 cm^2 leaf area) of 1.5 % plant agar (Duchefa Biochemie, Haarlem, The Netherlands) containing 0.2 mg ml^{-1} D-mannitol (Roth, Karlsruhe, Germany), which corresponds to 5 µg cm^{-2}.

Test for time-dependent preference

Young black poplar trees were infected with rust (as described in “Fungal infections”) or treated with water only (control group), and leaves were harvested at different time points after infection: 1, 4, 7 and 10 days post-infection. Leaf discs from control and rust-infected leaves were cut and offered in a petridish-arena to 2^{nd} instar larvae of *L. dispar*. The assay and data analysis was performed as described in “Preference assays”.

Chemical analysis

Leaves from rust-infected (10 dpi) and control trees were harvested into liquid nitrogen 6 d after the start of the performance experiment. At this time point larvae were in the second and third instar and had fed on the sampled leaves for 3 d. Uredospores were collected from undamaged, artificially infected trees. Both leaf tissue and spores were lyophilized. The leaf material was ground to fine powder and 10 mg leaf powder was extracted once in 2 ml-tubes with 1 ml methanol containing 0.8 mg ml^{-1} phenyl-β-glucoside (Sigma-Aldrich, St. Louis, MO, USA) as internal standard by shaking twice in a paint shaker for 30 s. Spores were extracted with 0.5 ml of the same solvent in aluminum tubes with steal beads by shaking three times for 5 min to break the spore wall.

Phenolic compounds (salicinoids and flavonoids) were analyzed in a 1:2 diluted (with water) extract by HPLC-UV on a reversed phase column (Nucleodur Sphinx, RP 5µm, Machery-Nagel, Düren, Germany) as described in (Boeckler *et al.* 2013). The phenolic compounds were quantified in relation to the peak area of the internal standard by using the following response factors: 0.449 (salicin), 0.870 (salicortin), 0.647 (homaloside D), 0.624 (salicortin-6-benzoate; Lackner *et al.* in prep.), 0.259 (catechin), 0.178 (proanthocyanidin B 1), 1.871 (rutin).

Amino acids were quantified by LC-MS/MS on a C18-column (XDB-C18, 50 x 4.6 mm x 1.8 µm; Agilent, Santa Clara, CA, USA) after diluting the extract 1:10 with water containing 10 µg ml^{-1} of a mix of $^{15}N/^{13}C$ labeled amino acids (Isotec, Miamisburg, OH, USA). Details on the chromatography and mass spectrometry can be found in Crocoll *et al.* 2016. The amino acids were quantified relative to the peak area of their corresponding labeled amino acids, except for tryptophan (using phenylalanine and applying a response factor of 0.42) and asparagine (using aspartate and a response factor of 1.0).

Soluble sugars and mannitol were analyzed from the 1:10 diluted extract by LC-MS/MS on a hydrophobic interaction liquid chromatography (HILIC)-column (apHera-NH_2 Polymer; 15 × 4.6 mm, 5 µm; Supelco, Bellefonte, PA, USA) as described in Madsen *et al.* 2015. Mannitol was analyzed by multiple reaction monitoring of the following ions: m/z 180.9 → 89.0 (CE -22 V; DP -35 V) All sugars and mannitol were quantified using an external standard curve with authentic standards of glucose, fructose, sucrose, stachyose (all from Sigma-Aldrich), Raffinose (Fluka, Seelze, Germany) and mannitol (Roth).

Nitrogen content was analyzed from 8 – 15 mg dried leaf tissue and spores, with a varioEL elemental analyzer (Elementar Analysensysteme, Langenselbold, Germany).

Performance assay

To monitor the performance of gypsy moth, larvae were transferred to rust-infected or uninfected black poplar trees 2 d after hatching and fed on poplar leaves until pupation. Each larva was kept in a transparent box (12.5 x 12.5 x 5 cm, KlarPac, Hofheim, Germany; perforated on top and bottom for air circulation) installed around a single leaf (detailed information in Fig. S1). Every third day the larvae were weighed and the food trees were exchanged. The trees were inoculated with rust fungus (or water for controls) 7 d before being offered to the larvae. The pupae were weighed and kept on room temperature until emergence of the adults which were sexed and weighed as well.

Statistics

All data were tested for statistical assumptions, i.e. normal distribution and homogeneity of variances. Whenever necessary, data were log-transformed (chemical analysis: flavonoids, mannitol). Preference assays were tested for significance with a paired *t*-test or, if statistical assumptions could not be met, with a Wilcoxon signed-rank test. The performance assay with a time course was evaluated by repeated measures ANOVA. Chemical analysis data were tested with ANOVA and Tukey's post-hoc test, except for amino acids and flavonoids that were evaluated by Games-Howell post-hoc test due to non-homogenous variances. Differences in essential amino acids were evaluated by ANOVA, or Kruskal-Wallis test in case of unfulfilled statistical requirements. All statistical analyses were performed with SPSS 17.0 (SPSS, Chicago, IL, USA).

Results

Young caterpillars prefer pathogen-infected leaves

It has been shown previously that gypsy moth larvae are attracted to rust-infected black poplar trees by olfactory cues (Eberl et al. 2017), but the feeding preference of these generalist herbivores has not been studied so far. We therefore conducted *in vitro* preference assays with leaf discs from rust-infected and uninfected black poplar leaves.

In the two-choice assay with control and rust-infected leaves (Fig. 1 C), second instar caterpillars of gypsy moth (*Lymantria dispar*) consumed almost exclusively the leaf discs from rust-infected trees (Wilcoxon signed-rank test, $P < 0.001$; Fig. 1 A). To test the generality of this observation, we also tested the preference of a closely related species, the rusty tussock moth (*Orgyia antiqua*). Also larvae of this species consumed about twice as much leaf area of the rust-infected than of the healthy leaf discs (Fig. 1 B; Wilcoxon signed-rank test, $P = 0.019$). During the experiments, we observed an interesting feeding behavior for both of the species: the caterpillars selectively fed on sporangia of the rust fungus rather than ingesting them accidently (Fig. S2; Video S1). This behavior was then studied in more detail by observing early instar gypsy moth larvae over a time period of 72 h. Except for one individual, all caterpillars first consumed almost the entire number of sporangia present (95 ± 2 %) before they started feeding on leaf material (Fig. 2).

In order to study the specificity of the insect preference for pathogen-infected leaves, we also conducted a preference assay with mildew-infected black poplar trees. Here, gypsy moth larvae also clearly preferred infected leaves, consuming ca. three times as much leaf area from mildew-infected than from healthy leaves (Fig. 3 A; Wilcoxon signed-rank test, $P = 0.001$). Interestingly, the larvae also first abraded the fungal mycelium growing superficially on mildew-infected leaves (Fig. 3 B) before they consumed the leaf matrix.

When testing the feeding preference of differently aged caterpillars, we found that the gypsy moth larvae lose their preference for rust-infected leaves at later instars (Table S1). While first and second instar preferred feeding on rust-infected leaf discs over uninfected discs (1^{st} instar: Wilcoxon signed-rank test, $P = 0.056$; 2^{nd} instar: paired *t*-test, $P < 0.001$), no trend was visible for third instar larvae (Wilcoxon signed-rank test, $P = 0.657$). The feeding experience of young caterpillars, however, did not influence their preference, as both diet-reared and poplar-reared gypsy moth larvae consumed significantly more rust-infected than uninfected leaf area (diet-reared: paired *t*-test, $P < 0.001$; poplar-reared: Wilcoxon signed-rank test, $P = 0.005$) in a choice assay (Table S1).

Apart from the ontogenetic development of the insect, we also assessed changes of preference in response to the time course of fungal infection. We tested four different time points after inoculation and could see a clear shift in the caterpillar's preference (Fig. 4). At the earliest time point, one day post-infection (dpi), the larvae preferred control leaves over rust-infected leaves (paired *t*-test, $P = 0.009$). Three days later, at 4 dpi, the preference switched towards rust-infected

leaf discs which accounted for 64% of the leaf area fed (paired *t*-test, $P < 0.001$). The preference did not change until the end of the experiment, but became stronger at 10 dpi with 78% of the consumed leaf area being rust-infected (Wilcoxon signed-rank test, $P < 0.001$).

In summary, we found that generalist caterpillars prefer pathogen-infected over uninfected black poplar leaves. Further, the preference of gypsy moth for rust-infected leaves depends on the age of the larva as well as on the progress of fungal infection in the leaf. Young caterpillars even show selective feeding behavior for fungal tissue on infected leaves.

Fungal spores contain high amounts of a sugar alcohol

In order to identify the trait responsible for the caterpillar's feeding preference, rust-infected and uninfected black poplar leaves, as well as fungal spores were chemically characterized. We analyzed phenolic defense compounds and nutritionally relevant compound classes.

Levels of salicinoids, that are characteristic defense compounds in the Salicaceae plant family (Boeckler *et al.* 2011), were not significantly different between uninfected and rust-infected leaves, but were rarely present in the spores (Fig. 5 A; ANOVA: $F = 101.7$, $P < 0.001$). Flavonoids, another group of phenolic compounds, were increased in the leaves after rust infection, but were not detected in the fungal spores (Fig. 5 B; ANOVA: $F = 1538.2$, $P < 0.001$). Free amino acids and nitrogen content were not significantly different between control and rust-infected leaves, but had considerably higher levels in the fungal spores compared to plant tissues (Fig. 5 C, D; ANOVA, amino acids: $F = 29.8$, $P = 0.001$; nitrogen: $F = 33.8$, $P < 0.001$). Soluble sugar levels in black poplar leaves, which were mainly composed of sucrose, did not change significantly upon rust infection (Fig. 5 E). In fungal spores, soluble sugars were present, but in very little amounts compared to the leaves (ca. 2% of concentration in leaves), leading to a significant difference between the different tissues (ANOVA: $F = 164.4$, $P < 0.001$). We also analyzed mannitol, a sugar alcohol known to be produced by some plant pathogens (Voegele *et al.* 2005; Jennings *et al.* 1998; Joosten *et al.* 1990), and observed striking differences between plant and fungal tissue (ANOVA: $F = 212.5$, $P < 0.001$). Mannitol was elevated in black poplar leaves upon infection and accumulated in fungal spores by ca. 20 to 400 times compared to rust-infected and control leaves, respectively (Fig. 5 F). Mannitol was therefore the only compound that had higher levels in both rust-infected leaves and fungal spores compared to control leaves.

To test whether mannitol stimulates feeding in gypsy moth larvae, we coated black poplar leaves with a thin layer of agar containing 0.2 mg ml^{-1} mannitol or not. This corresponds to an increase of 0.75 mg g^{-1} DW, similar to the increase observed in rust-infected leaves (difference to control: 1.04 mg g^{-1} DW). When young caterpillars were offered leaf discs with and without supplemented mannitol in a choice assay, they consumed double as much of the mannitol-supplemented discs (12 ± 1.1 %) as of those without mannitol (6 ± 1.1 %), which resulted in a statistically significant difference (Fig. 6 A; paired *t*-test: $P = 0.001$). We also analyzed mannitol levels in leaf discs after the mildew-preference assay (Fig. 3) and found a strong positive correlation between the leaf area consumed by gypsy moth larvae and the concentration of mannitol in mildew-infected leaf discs but not in control leaf discs (Fig. 6 B; Spearman correlation, mildew-infected: $\rho = 0.575$, $P = 0.002$; control: $\rho = 0.242$, P = ns).

Conclusively, we identified the sugar alcohol mannitol as active feeding stimulant for gypsy moth caterpillars. The levels of this compound were dramatically higher in fungal spores compared to leaf tissue, and were increased in the poplar leaves upon rust infection.

Gypsy moth larvae develop faster on rust-infected leaves

After observing the clear feeding preference of gypsy moth larvae for rust-infected poplar leaves, the fitness consequence of this choice was investigated. Gypsy moth larvae were reared on control or rust-infected black poplar trees from the first instar to their pupation and were weighed in regular intervals.

Overall, gypsy moth caterpillars that were reared on rust-infected trees gained significantly more weight over time than those reared on uninfected controls (Fig. 7 A; two-way ANOVA: factor 'Rust', $P < 0.001$). Larvae on rust-infected trees had double the weight compared to control caterpillars already 3 d after onset of the experiment (control: 6.3 ± 0.31 mg; rust-infected: 13.0 ± 0.77 mg). In this stage (first and early second instar) the larvae were exclusively feeding on the sporangia of the rust fungus (pers. observation). But also at the last time point of monitoring, which was the day of the first pupation (21 d), larvae on rust-infected trees had twice as much as weight as their conspecifics reared on control trees (control: 399 ± 42.5 mg; rust-infected: 787 ± 65.6 mg). However, pupal and adult weight did not differ between the two groups of gypsy moths (Fig. 7 C, D). The increase in larval weight can yet be explained by an accelerated development of

the larvae, as caterpillars on rust-infected trees pupated 3 (♂) to 4 (♀) days earlier than those in the control group (Fig. 7 B), which refers to ca. 10% of the larval development time.

To test whether the high levels of mannitol in the infected tissue might be responsible for the performance differences, we reared young gypsy moth larvae *in vitro* on leaves with or without supplementation of mannitol. There was no difference in larval weight between the caterpillars feeding on mannitol-supplemented or control leaves (Table S2).

We also had analyzed free amino acids in more detail, which were found earlier, just like total nitrogen, to be more concentrated in the fungal spores (Fig. 5 C). We analyzed the composition of amino acids in rust-infected and uninfected leaves, in fungal spores and in the bodies of gypsy moth caterpillars. The amino acid composition in rust-infected leaves and spores resembled more that of the caterpillars rather than uninfected leaves did (Fig. S3). This is especially pronounced by a shift from the negatively charged Glu, which is very dominant in control leaves (49%), to the polar Gln after rust infection (Gln: in controls 7 %, in infected 33%). Caterpillar bodies contained 34% Gln and only 16% Glu in the free amino acids. Focusing on essential amino acids we found significant differences among the different tissues, i.e. rust-infected leaves, control leaves and fungal spores. Here, fungal spores had the highest abundance of essential amino acids (Table 1). Specifically, Leu, Met, Thr, Arg and His were significantly different among tissues. For these amino acids, the lowest levels were found in control leaves and the highest levels in fungal spores (Table 1).

We showed that gypsy moth has a faster larval development when feeding on rust-infected compared to control trees. This difference is not caused by increased mannitol levels in rust-infected leaves, but high nitrogen content, a modified composition of free amino acids and elevated levels of essential amino acids in rust-infected leaves and fungal tissue are likely to be involved in the improved larval performance.

Discussion

At least half of the currently described insect species feed on plants (Schoonhoven *et al.* 2005). Plant tissues, however, are commonly colonized by microbes, such as leaf-inhabiting fungi which are hence ingested by herbivores. Here, we observed selective feeding behavior of the generalist gypsy moth (*Lymantria dispar*) caterpillars on sporangia of the rust fungus *Melampsora larici-populina* and a preference for rust-infected black poplar (*Populus nigra*) leaves over uninfected

controls. The choice of the caterpillars also resulted in a faster larval development, caused by pathogen-mediated changes in the leaf matrix or the fungal material itself. We therefore ask how strictly herbivorous insects can be classified as solely plant-feeding and what role plant-associated microbes play in plant-insect interactions.

Generalist herbivores feed on fungal tissue

Young larvae of the gypsy moth clearly preferred feeding on rust-infected over uninfected black poplar leaves (Fig. 1 A) and also fed selectively on fungal sporangia present on the surface of infected leaves (Fig. 2; Fig. S2 A). The rusty tussock moth (*Orgyia antiqua*), a close relative of *L. dispar* in the family Erebidae, likewise preferred rust-infected leaves and sporangia (Fig. 1 B; Fig. S2 B). Fungivory in Lepidoptera is rarely studied and seems to be limited to a few families, such as Tineidae which have the biggest proportion of fungus-associated species (Rawlings 1984). In the Erebidae and Noctuidae only a few observations on fungus-feeding individuals have been reported (Moskowitz & Haramaty 2012; Yoshimatsu & Nakata 2006), but systemic investigations of this phenomenon are lacking. The fungus-feeding behavior we observed in two species within the Erebidae, however, might indicate that facultative fungivory is more common than previously assumed. Certainly, more observational and behavioral studies in a larger number of species are needed to confirm this hypothesis. However, the actual extent of facultative fungivory among herbivorous inects will be difficult to estimate, since the separation of fungal and plant material under natural conditions is complicated.

The preference of gypsy moth caterpillars for fungal tissue was not fungus species-specific as caterpillars also preferred black poplar leaves infected with powdery mildew, another biotrophic pathogen over uninfected leaves (Fig. 3 A). In this experiment the caterpillars also and selectively fed on the fungal mycelium growing superficially on the leaves (Fig. 3 B). A preference for pathogen-infected plant tissue has so far been reported only for a few arthropod species that are distantly related, such as other Lepidopteran species (Mondy *et al.* 1998; Rizvi *et al.* 2015), aphids (Johnson *et al.* 2003) or mites (Reding *et al*. 2001).

The infection with a pathogenic fungus can provide benefits to the feeding herbivore by repressing anti-herbivore defenses. *Brassica rapa* plants damaged by caterpillars and additionally infected with powdery mildew, for example, had reduced emission of herbivore-induced volatile emissions (HIPVs) and were less attractive to parasitic wasps than only caterpillar-damaged

plants (Desurmont *et al*. 2016). Similarly, infection of herbivore-damaged black poplar trees with rust was also shown to diminish the emission of HIPVs (Eberl *et al*. 2017). Accordingly, the preference for pathogen-infected trees could be an adaptive behavior by the insects to avoid natural enemies. As the effects on indirect defense were based on antagonistic phytohormonal crosstalk between jasmonic acid and salicylic acid (Eberl *et al*. 2017), the life strategy of the pathogen (biotrophic *vs.* necrotrophic) and feeding guild of the herbivore (chewing *vs.* piercing-sucking) probably is important for the result of the interaction (Glazebrook 2005; Walling 2000).

We could also show that the preference of the gypsy moth larvae depends on the progress of fungal infection in the black poplar leaves, changing from control-preference in the earliest time point to strong rust-preference at later time points (Fig.2). The increasing preference at the later stages of infection might be due to a more dense colonization by fungal hyphae, and fungal-specific compounds accordingly, as well as the emergence of sporangia at 7 dpi that were especially preferred by young larvae. The preference shift from control to rust-infected leaves might also be based on temporal changes of the phytohormones jasmonic acid and salicylic acid during rust infection (Ullah *et al.* 2018), as they induce or suppress anti-herbivore defenses, respectively.

Besides the time course of fungal infection, also the ontogeny of the insect is important for its feeding preference. Gypsy moth caterpillars of the second instar strongly preferred rust-infected leaves, regardless of their previous feeding experience (Table S1). The preference of first instar larvae was marginally non-significant ($P = 0.056$), which is probably due to lower sample size compared to second instar larvae. Individuals of the first two instars also selectively fed on fungal material, the rust fungus sporangia and the mildew mycelium, rather than on leaf material (Fig. 2; Fig. 3 B; Fig. S2 A; Video S1). Third instar larvae, however, neither showed a trend of feeding preference towards infected leaves, nor did they display selective feeding on fungal tissue. Gypsy moth caterpillars in early larval stages are most mobile, they can travel long distances by ballooning from one tree to another or within the canopy by making use of silk threads (Leonard 1971). Moreover, early instars usually discriminate stronger between different food sources than late instars do (Browne 1995), most probably because young caterpillars are more vulnerable to parasitization, virus infections, starvation, weather effects and phytotoxins (Zalucki *et al.* 2002; Elkinton & Liebhold 1990).

Mannitol triggers caterpillar feeding preference

Earlier work on gypsy moth has demonstrated attraction of caterpillars towards rust-infected black poplar trees by means of olfaction (Eberl *et al.* 2017). We now also demonstrated a feeding preference towards infected leaves and spores (Fig. 1 A; Fig. 2). In order to identify the responsible chemical cues triggering this preference, the different tissues were analyzed. The chemical characterization of black poplar leaves with and without rust infection and of the rust spores (Fig. 5) revealed mannitol as the most probable explanation for the gypsy moth's feeding preference. This sugar alcohol was elevated in rust-infected leaves compared to controls and accumulated to even higher levels in fungal spores (Fig. 5 F) that were especially preferred by gypsy moth caterpillars (Fig. 2; Fig. S2 A; Video S1). Artificially added mannitol to black poplar leaves also elicited selective behavior in young gypsy moth caterpillars (Fig. 6 A), proving the phagostimulatory effect of mannitol. Further, the mannitol content in mildew-infected leaves strongly correlated with the feeding damage by gypsy moth larvae (Fig. 6 B) and the temporal patterns of preference during rust infection (Fig. 4) might also be caused by an accumulation of mannitol.

The response of insects to mannitol is rarely studied. In contrast to the preference to other carbohydrates such as sucrose, which is widely spread among insects (Schoonhooven & van Loon 2002; Bernays *et al.* 2003; Schiff *et al.* 1989;), mannitol-preference seems to be a very species-specific trait rather than a widespread, general behavior. According to the few studies available on mannitol, the effect of this sugar alcohol ranges from being deterrent (Akeson *et al.* 1970), neutral (Schiff *et al.* 1989) to phagostimulatory (Takada *et al.* 2017). This suggests that the preference for mannitol might have established in some herbivores as adaptive behavior finding favorable food sources that meet their specific requirements, rather than being a general cue for nutrients. In case of gypsy moth, mannitol might be the indication for a fungal infection in its host plant, providing preferable conditions for larval development.

However, the feeding preference could also be explained by multiple variables, one of them being the presence of mannitol. Another possible chemical trait is the negligible amount of salicinoids, which are ca. 100 times less concentrated in the spores than in poplar leaves (Fig. 5 A). These specialized phenolic compounds were suggested to act as feeding deterrents against gypsy moth larvae (Boeckler *et al.* 2014).

Taken together, the chemical analysis of plant and fungal tissue combined with a behavioral assay has identified mannitol as a fungus-specific feeding stimulant for gypsy moth caterpillars. This sugar alcohol explains the insect's preference for fungal spores and for rust-infected over uninfected leaves, but might be synergistic with other chemical traits that support preference.

Pathogen infection of host tree favors caterpillar development

In a no-choice performance experiment we investigated the consequence of the feeding preference for caterpillar fitness. Gypsy moth caterpillars reared on rust-infected black poplar trees gained twice as much weight as individuals in the control treatment (Fig. 7). The difference in weight gain among the groups appeared already in the first and second instars (i.e. 3 – 6 d) and continued along the development. During these early time points larvae on rust-infected leaves mostly fed on the sporangia of the rust fungus rather than on leaf material. It is therefore likely, that the fungal diet provided a better nutrition to the early instar caterpillars than black poplar leaves did. This led to a faster development in the first and second instar of rust-reared caterpillars while the control group required more time to complete these instars and had a lower weight at each time point of measurement. That hypothesis is supported by the data on development time from hatching to pupation. Here, larvae feeding on rust-infected leaves required three (male) to four (female) days less than their conspecifics on uninfected leaves (Fig. 7 B). Shortening the larval development time is an important fitness factor, as it reduces the exposure time to natural enemies and unfavorable environmental conditions, and might be advantageous in feeding competition as well. An accelerated development was also observed in another Lepidopteran species (*Ostrinia nubilis*) feeding on pathogen-infected maize plants (Carruthers *et al.* 1986). But, in general, the effects of plant-pathogens on insect fitness are very diverse (reviewed in Eberl *et al.* 2018; Shikano *et al.* 2017; Hatcher 1995).

We also tried to identify possible factors for the improved larval development in gypsy moths by analyzing chemical parameters of the fungal spores, the preferred food of early instar larvae. Mannitol, which highly accumulated in the fungal spores, did not have any influence on the larval performance (Table S2) and sugar levels were very low in the spores (Fig. 5 E). Phenolic compounds, specifically salicinoids and flavonoids, were nearly absent or not even detected in the spores, respectively (Fig. 5 A, B), which might reduce the detoxification costs of early instar larvae that feed on spores instead of leaves. Salicinoids are chemical defense compounds specific

to members of the Salicaceae family and have been shown to cause detrimental effects on gypsy moth caterpillars in the last instars (Boeckler *et al*. 2016). As the content of these defense compounds in rust-infected leaves is not significantly different from that in control leaves (Fig. 5 A), they might negatively affect older larvae that consume leaf material. We therefore assume nitrogen-containing compounds mainly being responsible for the performance advantage of gypsy moth feeding on rust-infected poplars. The importance of nitrogen for insects has been validated and reviewed thoroughly (Scriber & Slansky 1981; Mattson 1980; Lindroth *et al.* 1997). Especially plant tissue represents a suboptimal nitrogen source due to its comparably now nitrogen content (Martin & Kukor 1984) and the poor availability after digestion due to the huge amount of structural carbon-polymers and an unequal distribution within the plant (White 1993). In rust spores collected from black poplar leaves, the total nitrogen was ca. 50% higher than in plant tissue (Fig. 5 D) and the levels of free amino acids were even more elevated in spores (Fig. 5 C). Similar patterns were also observed for plants infected with other rust species, such as various *Puccinia* species (Ramsell & Paul 1990; Reddy & Rao 1976) and *Uromyces viciae-fabae* (Al-Naemi & Hatcher 2013). In the latter study, increased nitrogen content in the rust-infected host plants were also made responsible for improved fitness of aphids feeding on these plants (Al-Naemi & Hatcher 2013). In our study, fungal spores reached a nitrogen content of 3 %, which corresponds to the nitrogen intake target of gypsy moth (Stockhoff *et al*. 1993). Moreover, half of the ten essential amino acids were found to be significantly higher in fungal spores and infected leaf tissue than in control leaves (Table 1). Among them are histidine, methionine and arginine, which have been described as low in foliar protein and therefore limiting for herbivore nutrition (Barbehenn *et al.* 2012). Further, we found that the composition in infected black poplar leaves was more similar to the composition of caterpillar bodies rather than control leaves (Fig. S3). Consequently, less metabolic cost for biochemical conversions from food source to body homeostasis has to be invested by the insects when feeding on rust-infected leaves compared to control leaves.

Apart from nitrogen and essential amino acids many other factors of fungivory might be beneficial for insect performance. The absence of certain metabolites in fungal material, such as condensed tannins or lignin (Martin 1979; Hatcher 1995), could facilitate in- and digestion for insects. Especially young caterpillars, still having small and weak mandibles, benefit from tissue with little toughness. Further, the presence of minerals, vitamins, especially high levels of B-

vitamins, polyunsaturated fatty acids and a sterol composition different from plants (Martin 1979) could as well contribute to increased fitness of fungus-feeding insects. Some of these micro- or macronutrients in fungal tissue could even be essential for insect development and might be usually provided in nature by plant-inhabiting fungi such as symbionts, endophytes or pathogens. To test this speculation sterile plants are needed to investigate the fitness of insects in the entire absence of plant-associated microbes.

Conclusions

In our study we revealed a positive impact of a fungal phytopathogen on a tree-feeding lepidopteran insect. Fungal infection positively influenced the behavior as well as the performance of the caterpillars, as displayed in selective feeding and accelerated development. Anecdotal evidence for facultative fungivory of herbivores has existed (Moskowitz & Haramaty 2012; Yoshimatsu & Nakata 2006) but was not investigated systematically in the past. We chemically characterized plant and fungal tissue and found evidence for adapted preference towards a fungal-specific compound and favorable nutritional quality of fungal sporangia. In the future, the concept of coevolution between insects and plants should no longer ignore the microbial "hidden players". Including plant-inhabiting fungi and bacteria will be necessary to complete the picture of ecological interactions and their evolution.

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Author contributions

FE, AH, JG and SU conceived the project; FE, SU and AH designed the experiments; FE conducted most experiments and analyzed the data; MF carried out and analyzed the preference assays over time course; MR optimized the method for mannitol analysis; FE wrote the manuscript; MF, AH, JG and SU reviewed and edited the manuscript.

Competing interests

None declared.

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Figures

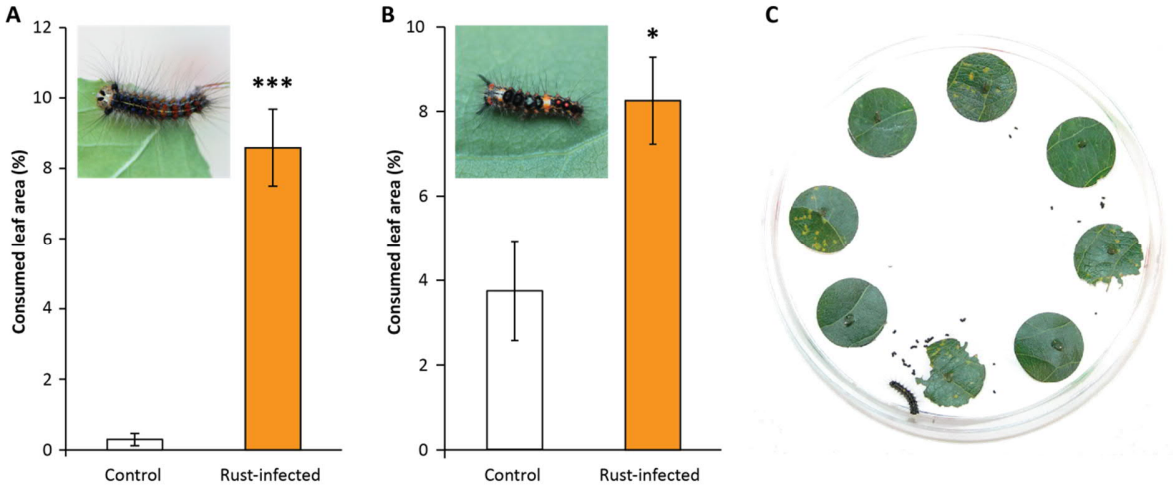

Figure 1. Feeding preference of 2nd instar larvae towards rust-infected leaves. Larvae of gypsy moth (*L. dispar*, A) and rusty tussock moth (*O. antiqua*, B) were allowed to feed on leaf discs of rust-infected (11-12 dpi, filled bars) and uninfected (empty bars) black poplar leaves in a choice assay (C) for 2 d. Preference was evaluated as % consumed area of total leaf area (A). Mean ± SEM (n = 20), Wilcoxon signed-rank test, * $P < 0.05$; *** $P < 0.001$.

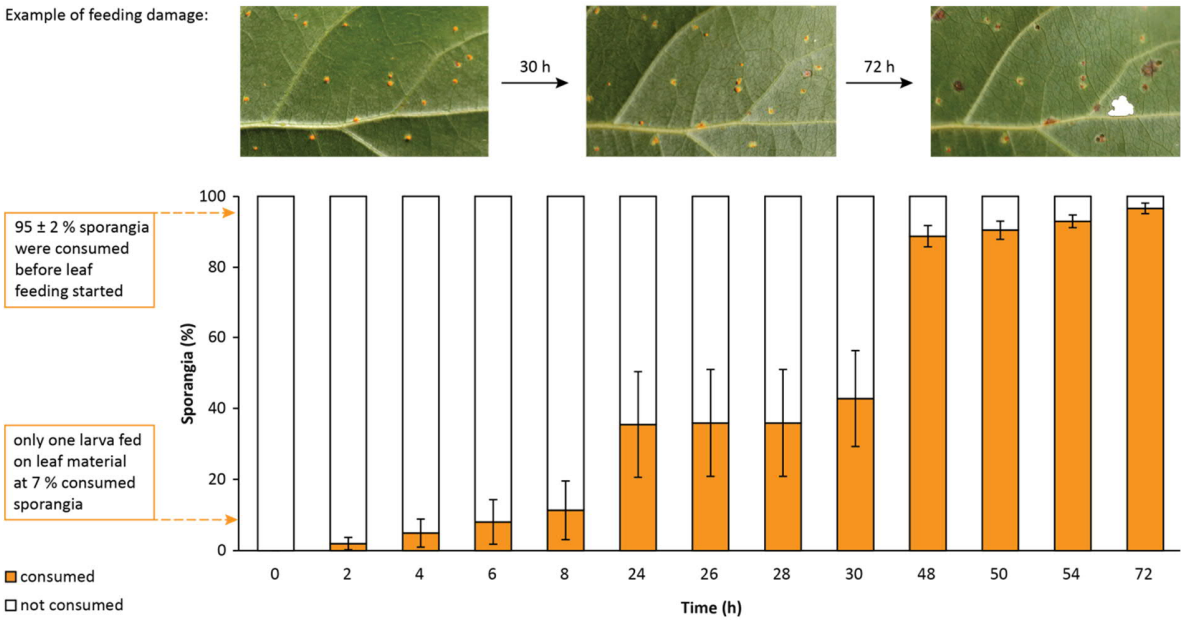

Figure 2. Selective feeding behavior of young gypsy moth larvae on rust fungus sporangia. First instar larvae (change to 2nd instar during experiment) were observed for 72 h on rust-infected black poplar leaves, each larva received one leaf in a separate petridish. Sporangia of the fungus were counted on each leaf before feeding and at the given time points (big graph: orange, consumed sporangia; empty, sporangia still present). First occasion of leaf feeding was documented (left insets), mean ± SEM (n = 7, upper inset) was calculated excluding the outlier (lower inset). An example of the feeding damage on sporangia (30 h) and first occasion of leaf feeding after sporangia were consumed (72 h) is given as pictures (top row).

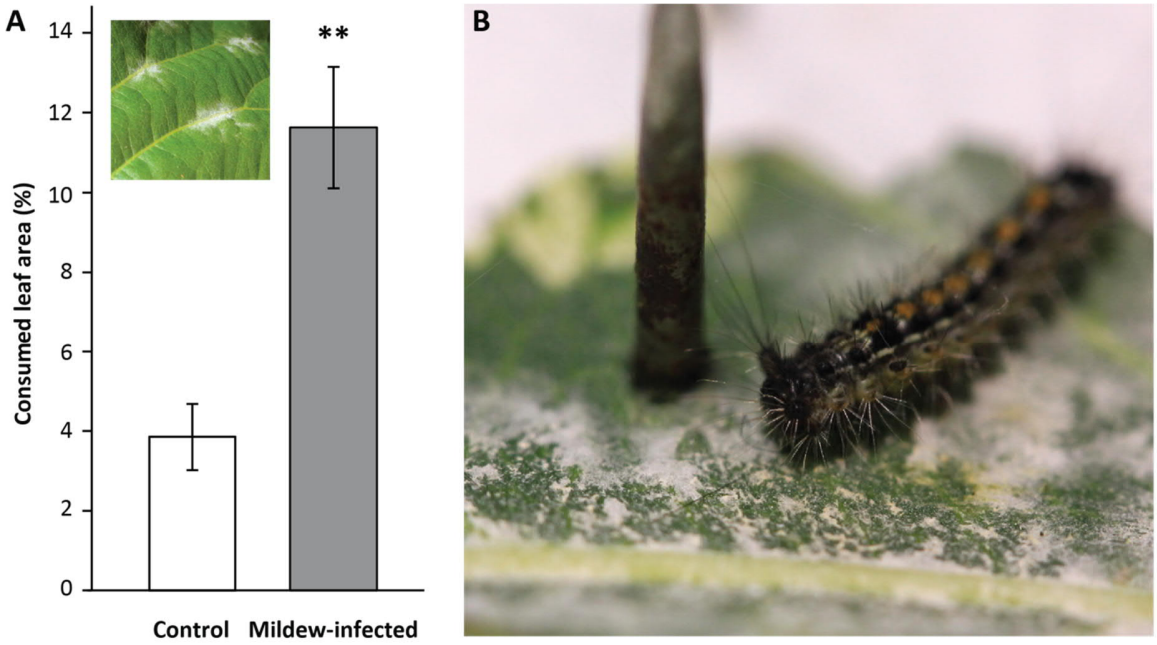

Figure 3. Preference of gypsy moth larvae (2nd instar) towards mildew-infected leaves. Caterpillars were allowed to feed on leaf discs from uninfected (empty bar) and mildew-infected (filled bar) black poplar trees for 2 d (A). Caterpillars preferred to abrade the fungal mycelium first before feeding on the leaf matrix (B). Preference was evaluated as % consumed area of total leaf area. Mean ± SEM (n = 26), paired t-test, ** $P < 0.01$.

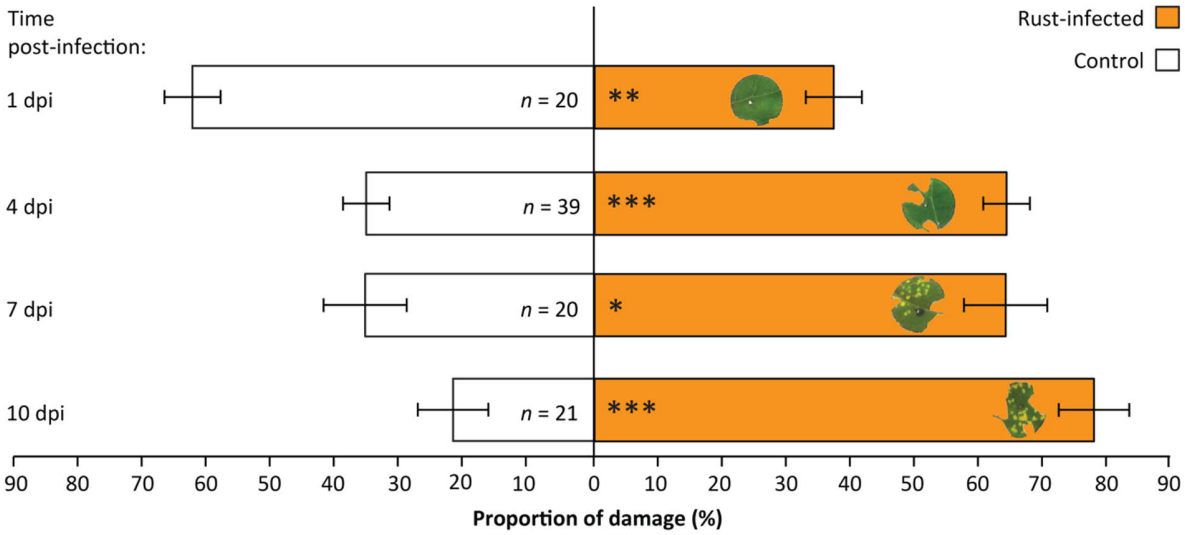

Figure 4. Feeding preference of gypsy moth larvae at different time points of rust infection. Preference assays as described in Fig. 1 were performed at four different days post-infection (dpi) between leaf discs from rust-infected (filled bars) and uninfected (empty bars) black poplar trees. Preference is shown as % damage of total damage. Mean ± SEM (*n* is given in the bars), paired t-test (1 dpi, 4 dpi, 7 dpi) or Wilcoxon signed-rank test (10 dpi),* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

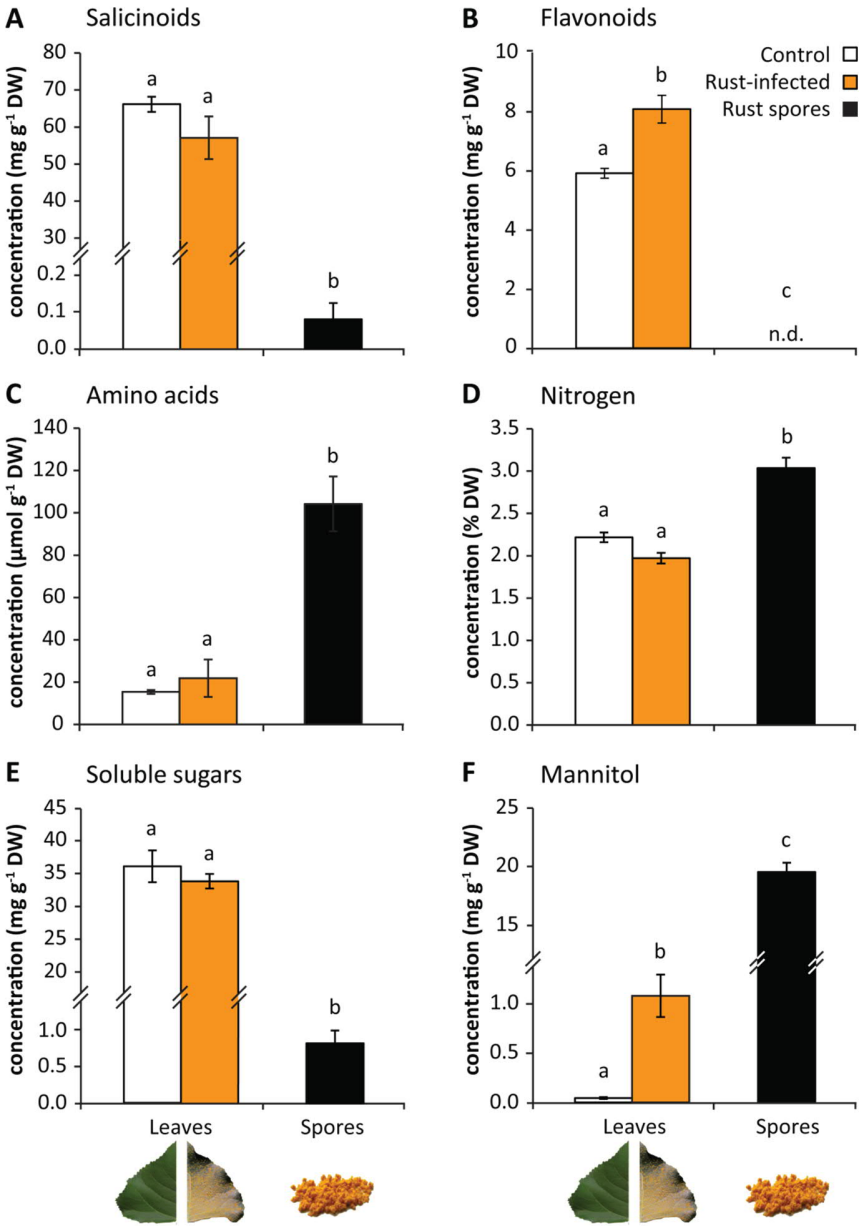

Figure 5. Chemical analysis of plant leaves and fungal spores. Uninfected (empty bars) and rust-infected (orange bars) black poplar leaves as well as separated uredospores of the poplar leaf rust fungus (black bars) were chemically characterized. Presented are salicinoids (A; sum of salicin, salicortin, homaloside D, salicortin-6-benzoate), flavonoids (B; sum of catechin, proanthocyanidin B1, rutin), nitrogen content (C), amino acids (D; sum of free amino acids), soluble sugars (E; sum of glucose, fructose, sucrose, tri- and tetrasaccharides) and the sugar alcohol mannitol (F). Mean ± SEM (n = 3); ANOVA with post-hoc test, different letters indicate significant differences among groups; n.d. = not detected.

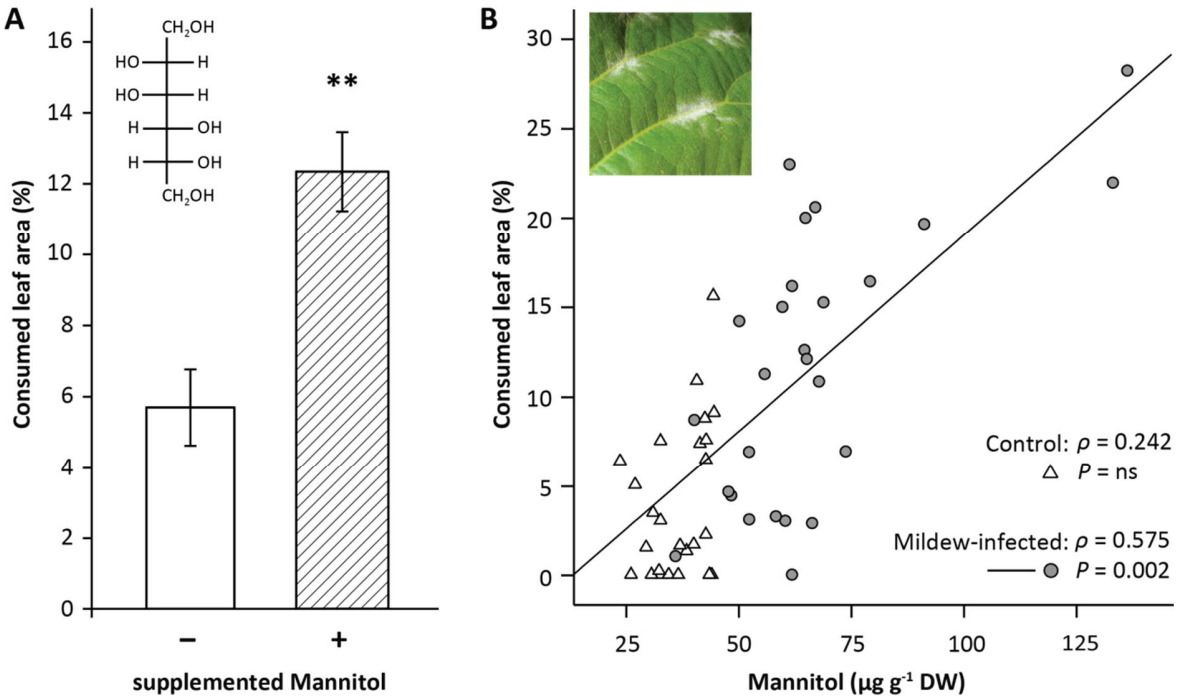

Figure 6. Preference of gypsy moth larvae (2nd instar) towards mannitol. Caterpillars were allowed to choose between leaf discs coated with plant agar with (patterned bar; +) or without (empty bar; –) supplemented mannitol (0.2 mg ml^{-1}; A). Preference was evaluated as % consumed area of total leaf area. Mean ± SEM (n = 20), Wilcoxon signed-rank test, ** $P < 0.01$. Feeding damage in the mildew-preference assay (see Figure 2) correlated with mannitol content in mildew-infected but not in control leaves (B). Spearman's rank correlation (n = 26 for each group).

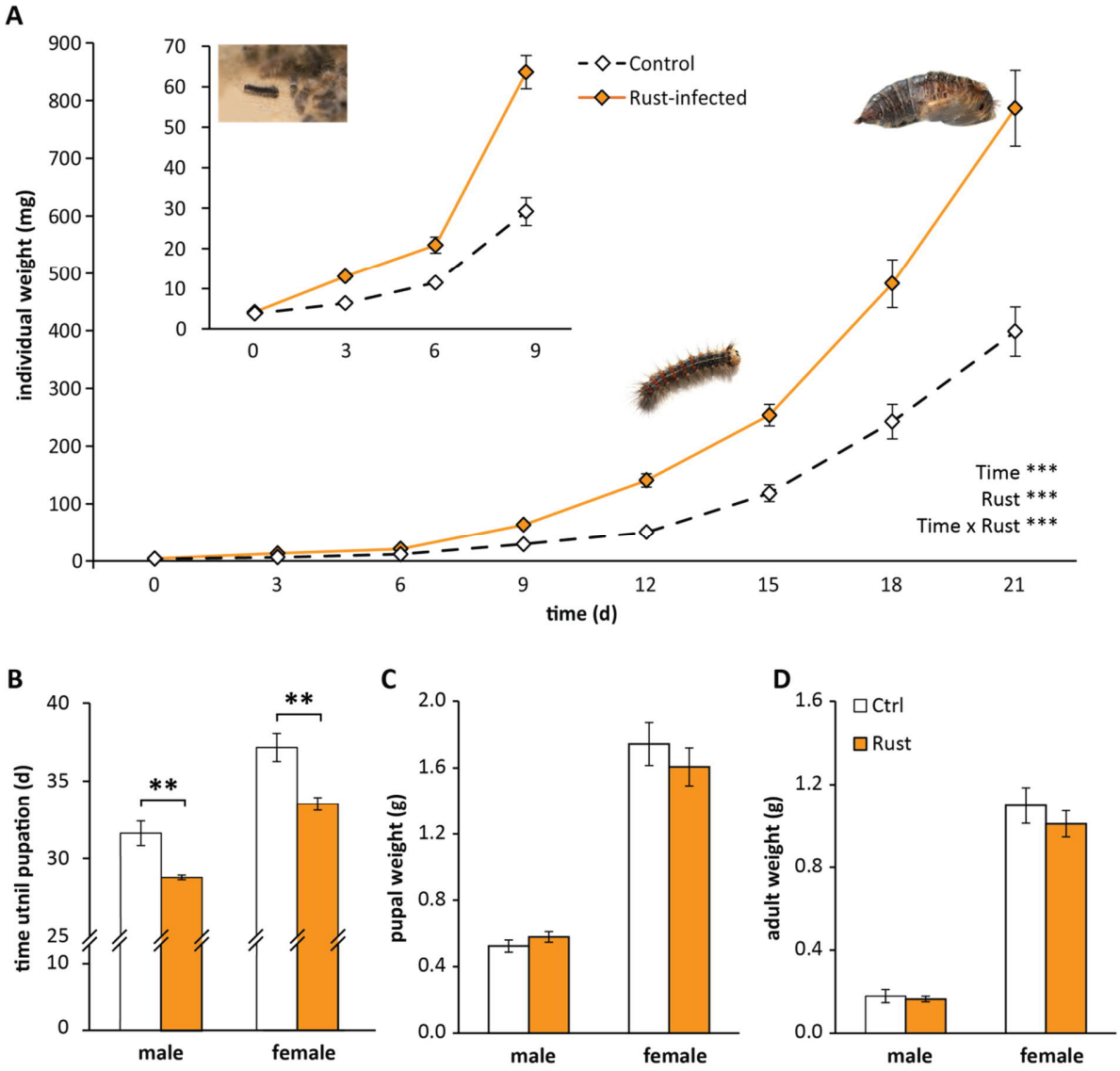

Figure 7. Performance of gypsy moth larvae. Caterpillars were feeding on rust-infected black poplar trees (7 – 10 dpi, leaves were already carrying fungal spores; filled symbols, solid line) or uninfected controls (empty symbols, dashed line) and were weighed every 3 d (A). Data are shown from the onset of the experiment (0 d, i.e. 2 d after hatching) until the beginning of the first pupation (21 d). The inset zooms in for early time points (0 – 9 d), results from repeated measures ANOVA are given for the factors time, infection and their interaction (n = 19 – 20; *** P > 0.001). Larval developmental time (B), pupal (C) and adult weight (D) are shown for male and female gypsy moths after rearing on control and rust-infected trees. Mean ± SEM (n = 8 (control) and 9 (rust-infected) for male; n = 11 for female), Mann-Whitney U-test (time) or Student's t-test (weights); significant differences are marked with asterisks (** P < 0.01).

Tables

Table 1. Levels of essential amino acids (ess. AA) in uninfected controls (Control) and rust-infected black poplar leaves (Infected) and in separated uredospores of the rust fungus (Spores), in µmol g^{-1} DW. Mean ± SEM (n = 3), significance values (*P*) of ANOVA or Kruskal-Wallis test (Phe, Arg, His).

	Val	**Leu**	**Ile**	**Met**	**Phe**	**Trp**	**Thr**	**Arg**	**His**	**Lys**	**ess. AA**
Control	0.21 ± 0.03	0.11 ± 0.02	0.34 ± 0.09	0.013 ± 0.002	0.16 ± 0.01	0.028 ± 0.004	0.34 ± 0.02	0.025 ± 0.003	0.29 ± 0.03	0.010 ± 0.001	1.53 ± 0.19
Infected	0.26 ± 0.02	0.19 ± 0.03	0.24 ± 0.01	0.027 ± 0.008	0.20 ± 0.03	0.086 ± 0.020	0.46 ± 0.06	0.063 ± 0.010	0.40 ± 0.06	0.011 ± 0.002	1.94 ± 0.19
Spores	0.65 ± 0.21	0.55 ± 0.16	0.49 ± 0.14	0.047 ± 0.003	0.21 ± 0.09	0.055 ± 0.011	1.61 ± 0.23	0.226 ± 0.037	1.32 ± 0.23	0.011 ± 0.002	5.17 ± 1.07
P	0.085	**0.035**	0.249	**0.010**	0.837	0.060	**0.001**	**0.027**	**0.039**	0.910	**0.013**

Supplemental information:

Figure S1. Setup of the performance assay for gypsy moth larvae. Trees were put into a stand (A) on which nine single leaf boxes (B) were mounted. Each box was perforated on top and bottom to enable air exchange and prevent condensation of water. One larva per box was allowed to feed for three days until being transferred onto a new tree. All larvae were feeding on leaves (control or rust-infected) until pupation.

Figure S2. Selective feeding on fungal sporangia by 1st instar larvae. Caterpillars of gypsy moth (L. dispar, A) and the related species rusty tussock moth (O. antiqua, B) selectively ingested sporangia of the rust fungus growing on the abaxial side of black poplar leaves.

Figure S3. Composition of free amino acids in poplar leaves, fungal spores and caterpillar bodies. Leaves of black poplar were either rust-infected (10 dpi) or uninfected, for details see *Methods: Chemical analysis*. Uredospores of *Melampsora larici-populus* were separated from leaf material prior analysis. Gypsy moth caterpillar bodies (4^{th} instar) were analyzed after the larvae had fed for 13 d on black poplar leaves. Shown are results for caterpillars that fed on uninfected leaves, which did not differ from those that fed on rust-infected leaves. Total amount of amino acids per dry weight is given below the charts as mean ± SEM (n = 3 for leaves, spores; n = 10 for insects). Amino acids are sorted by type of side chain: aliphatic (purple), aromatic (orange), polar (green), positively charged (red), negatively charged (blue).

Table S1. Performance of gypsy moth larvae under addition of mannitol. Gypsy moth caterpillars were feeding on black poplar (one genotype) leaves coated with plant agar containing mannitol (0.2 mg ml^{-1}; + Mannitol) or not (- Mannitol) as described for mannitol preference assays (*Method: Preference assays*) and were weighted at the beginning of the experiment (d = 1, late 1^{st} /early 2^{nd} instar) and five different time points until reaching the 4^{th} instar (d = 13). Shown is mean ± SEM (n = 20). Repeated measures ANOVA; time: $\boldsymbol{P < 0.001}$; treatment: P = ns, treatment x time: P = ns.

Table S2. Preference of gypsy moth caterpillars in different instars and previous diets. The preference assay with leaf discs of rust-infected *vs.* uninfected black poplar trees, as described in *Methods: Preference assays*, was repeated with 1st, 2nd and 3rd instar larvae. If not stated differently caterpillars were reared on diet. One group of 2nd instar larvae was reared on black poplar leaves prior the preference assay (2nd instar/ poplar). Preference was evaluated as % consumed area of total leaf area (n = 14 for 1st and 3rd instar; n = 20 for 2nd instar, n = 25 for 2nd instar/ poplar), paired t-test (2nd instar) or Wilcoxon signed-rank test (1st instar, 3rd instar, 2nd instar/ poplar).

Video S1. Feeding behavior of young gypsy moth larvae. 1st instar caterpillars selectively feed on sporangia of the poplar leaf rust fungus on black poplar leaves.

7. DISCUSSION

Forest ecosystems are the site of many complex interactions between different organisms, in which trees play a central role due to their large size and long life span. Trees are colonized by fungi and bacteria, infected by viruses and nematodes, and are also the habitat of numerous insects, mites, birds and mammals. Consequently, a tree is at once a host for many different species that might influence each other directly, or indirectly through the changes they elicit in their common host. The host tree on the other hand influences all of its associated species and may do so in a unique way given the collective impact of many different kinds of colonists. The outcomes of multiple interactions on the organisms involved are poorly studied, and it is questionable whether they can be deduced from adding up the effects of the component two-way interactions. As many distinct factors influence multiple interactions (**manuscript I**), their properties and outcomes are hard to predict.

7.1. Rust infection changes physiology and metabolism in black poplar

The colonization of plant tissue by a pathogenic fungus triggers an assortment of changes in the host plant. Many of these changes are defense-related starting from local signaling events, biosynthesis of anti-microbial toxins and the formation of pathogenesis-related (PR)-proteins, leading to cell-wall reinforcement, cell death (hypersensitive response) and systemic signaling, which induces metabolic changes in uninfected parts. In the woody plant-pathosystem of poplar trees (*Populus spp.*) and biotrophic rust fungi (*Melampsora spp.*) many phenomena that were described in herbaceous plants could be demonstrated as well. Rust-infected poplar trees showed increased expression of genes involved in cell signaling (Azaiez *et al*. 2009) and an accumulation of phytohormones, especially of salicylic acid (SA) (Ullah *et al*. 2018; Pfabel *et al*. 2012). The phytohormone accumulation was also validated in the manuscripts of this thesis, showing a 4- to 5-fold increase in levels of SA (**manuscript III, II**, respectively) and a 2-fold increase for abscisic acid (ABA; **manuscript II**). Both phytohormones have recently been shown to enhance resistance against rust disease in black poplar when applied externally (Ullah *et al.* 2018). Furthermore, in **manuscript III**, we showed enhanced expression of SA-responsive genes upon rust infection, specifically the transcription factors WRKY 70 and WRKY 89, as well as one of the PR genes, PR-1. The importance of several WRKY genes in defense signaling in poplar has

been shown before, as they function downstream of SA by activating the transcription of PR genes (Jiang *et al*. 2014; Jiang *et al*. 2017). The expression of PR genes upon rust infection was also demonstrated by many studies (Ullah *et al*. 2018; Chen *et al*. 2014; Azaiez *et al*. 2009; Miranda *et al*. 2007; Rinaldi *et al*. 2007), consistent with the results shown in **manuscript III**. The functions of PR proteins can be diverse, ranging from enzymatic activities (chitinases, glucanase), enzyme inhibition (proteinase inhibitors) to putative membrane-acting mechanisms (defensins, thaumatin-like proteins), but for many PR proteins the mode of action is still unknown (Duplessis *et al*. 2009; Ferreira *et al*. 2007).

Other changes in poplar upon rust infection include alterations in the metabolic profile as a result of gene regulation of biosynthetic pathways (Azaiez *et al*. 2009; Miranda *et al*. 2007). The most dominant changes are increases in the phenolic compounds, such as condensed tannins (Pfabel *et al*. 2012), lignins (Azaiez *et al*. 2009), proanthocyanidins and monomeric flavan-3-ols (**manuscript IV**; Ullah *et al*. 2017; Miranda *et al*. 2007) that show antifungal activity (Ullah *et al*. 2017). However, in this thesis I also showed that another important group of poplar secondary metabolites, volatile organic compounds, was also influenced by rust infection. The volatile blend emitted from rust-infected poplar trees differed substantially from that of uninfected controls, mainly due to increases in C_8 compounds (from fungi), aromatic compounds and terpenoids, especially sesquiterpenes (**manuscript III**). However, in contrast to experiments with only herbivory, the enhanced emission of the terpenoids was not reflected by enhanced gene expression of poplar terpene synthases after rust infection (**manuscript III**). Hence, the biochemical origin of the emitted terpenoids is unclear.

In **manuscript II** we investigated the biosynthetic pathways of C_5 isoprenoid units (dimethylallyl diphosphate, isopentenyl diphosphate), the precursors of terpenoids. The methylerythritol 4-phosphate (MEP) pathway is localized in chloroplasts and produces precursors for isoprene (C_5) and monoterpenes (C_{10}) among other terpenoids, whereas the mevalonate (MVA) pathway is confined to the cytosol and provides precursors for, among others, sesquiterpenes (Hemmerlin *et al*. 2012). The gene expression and metabolic intermediates of the MEP pathway in black poplar did not show any changes after rust infection, whereas genes involved in the early steps of the MVA pathway were expressed at higher levels after infection (**manuscript II**). The emission of the main volatile product of the MEP pathway, isoprene, was not altered, but the C_5 isoprenoid precursors produced by both pathways were significantly higher in infected poplar leaves, most

likely caused by an increased metabolic flux through the MVA pathway. Additionally, the fungus also contributed to the pool of C_5 isoprenoid precursors, as transcripts of terpene biosynthetic genes could be identified in the transcriptome of the rust (**manuscript II**). Apart from the possibility of an active MVA pathway producing C_5 precurors in the rust fungus, the recent identification of fungal terpene synthases in *Melampsora larici-populina* (Wei *et al.*, in prep.) also supports the hypothesis that the increased emission of terpenoids observed in rust-infected poplars (**manuscript III**) derives from the fungus itself rather than from changes in poplar metabolism.

These findings open questions about the ecological relevance of volatile terpenoid production in rust. Many terpenoids have been shown to possess antimicrobial activity (José Alves *et al*. 2013; Hammer *et al*. 2003; Kang *et al*. 1992) and so the emission of terpenoids might have a protective function against fungal hyperparasites that occur on rust fungi (Liesebach & Zaspel 2004; Nasini *et al.* 2004; Sharma & Heather 1978). In another rust fungus, *Uromyces fabae*, which grows on broad bean, the perception of three volatiles promoted the development of the fungus (Mendgen *et al.* 2006). It is possible that a similar mechanism exists in the poplar rust fungus *Melampsora larici-populina* to promote colonization of the host tree. Volatiles can also contribute to long-distance effects by attracting flying insects for dispersal (Sharifi *et al.* 2017). Even though spores of the rust fungus are easily distributed by wind and rain, the attraction of insect vectors could extend its distribution (Friedli & Bacher 2001; Nagarajan & Singh 1990). Other rust-emitted volatiles might fulfill this function as well, for example the C_8 compounds 1-octen-3-ol and 3-octanone that were shown to attract mycophagous ladybird beetles (Tabata *et al.* 2011). Since color can function as visual attractant for insects, too (Prokopy *et al.* 1983), the accumulation of β-carotene in the spores (**manuscript II**), giving them their characteristic orange color, might have been selected for vector attraction as well.

When considering pathogen-mediated changes in plants, less attention is given to changes in the primary metabolism and physiology of the host plants (Berger *et al.* 2007), even though these might impact plant fitness at least as much as the metabolic costs of defense responses. A common pattern found in the literature is the reduction in photosynthetic rate and stomatal conductance in rust-infected poplar leaves (Gortari *et al*. 2018; Jiang *et al*. 2016; Zhang *et al.* 2016; Zhang *et al*. 2010), which we also found in our experiments (**manuscript II**). These observations were supported by transcriptional analyses of rust-infected poplar leaves (Major *et al*. 2010). However, all of the studies conducted so far used late time points after emergence of

the spores (Zhang *et al.* 2016; Zhang *et al*. 2010) or an unknown period of infection in naturally infected trees (Gortari *et al*. 2018; Jiang *et al*. 2016). The novelty of **manuscript II** is therefore the time-resolution of photosynthetic changes after initial infection. Interestingly, the drastic decrease in the photosynthetic parameters appeared already at the first measurement (4 hour post-infection, growth of germ tube) and persisted until the end of the experiment (10 days post-infection, growth of sporangia and possible re-infections). This implies that the mechanism of photosynthetic reduction is based on an active plant-internal signaling process, rather than being just a consequence of mechanical damage of the epidermal cells (Jiang *et al.* 2016) or loss of living tissue by necrosis. Increased levels of ABA, which is known to control stomatal closure (Acharya & Assmann 2009), as reported in **manuscript II** and another study (Ullah *et al.* 2018) indicate the involvement of phytohormones in the regulation of photosynthesis.

As a consequence of reduced photosynthetic activity and sugar uptake by the pathogen, a reduction of leaf carbohydrates seems likely. However, in black poplar leaves infected with rust we could not detect any changes in the concentrations of soluble sugars (**manuscript II**; **manuscript III supplementary data**). During microbial infection, constant sugar levels might be maintained by a reduced export of photosynthetic assimilates or the inversion of source-sink relationships in the tree (Berger *et al*. 2007). Such phenomena have been observed in herbaceous and grass species earlier (Berger *et al.* 2007; Wright *et al*. 1995) and would explain the long-term effects of rust-infection in poplar trees, i.e. reduced biomass production (Wan *et al*. 2013).

Against a stable background of soluble sugars, we identified a sugar alcohol, mannitol, that is dramatically increased in rust-infected leaves compared to uninfected leaves (**manuscript IV**). After infection of another rust species, *Uromyces fabae*, mannitol was shown to accumulate in the apoplast of broad bean leaves as well as in the fungal spores (Voegele *et al.* 2005). The same study identified the biosynthetic enzyme, mannitol dehydrogenase, which catalyzes two reactions, the reduction of fructose to mannitol and the oxidation of mannitol to fructose. Different functions of mannitol have been discussed, such as its action as a carbon storage compound, an osmoregulator or a scavenger of radical oxygen species hence disarming the plant's defense (Voegele *et al*. 2005; Voegele *et al*. 2003; Jennings *et al.* 1998). The overexpression of a mannitol dehydrogenase in tobacco conferred resistance towards a mannitol-secreting pathogen (Jennings *et al*. 2002), demonstrating the importance of mannitol for the virulence of the fungus.

7.2. Multiple attacks in black poplar reduce anti-herbivore defense

Compared to the attack by a biotrophic pathogen, the infestation of a plant with insect herbivores seems to have a much more dramatic impact due to the continuous, long-lasting loss of biomass. But is defense against herbivores always prioritized by plants? Under natural conditions, plants are often attacked by herbivores and other biotic stressors such as pathogens in the same time interval. Therefore, plants have to balance between different defense responses that might be more effective against one attacker than the other. However, our understanding of the signal processing and trade-offs within plants in such multiple attack situations is still in its infancy. I therefore investigated the responses of black poplar trees towards herbivore feeding with a concomitant rust fungus infection.

Similar to the results from herbaceous plants (reviewed in Wasternack & Hause 2013; Koo & Howe 2009), herbivory in black poplar trees induces jasmonic acid (JA) (**manuscript III**; Clavijo McCormick *et al.* 2014). We also showed the induction of allene oxide (**manuscript III**), a gene involved in JA synthesis which is additionally activated by JA *via* positive feedback signaling (Wasternack & Hause 2013). Downstream of JA signaling many different anti-herbivore defenses are activated. I focused on the emission of herbivore-induced plant volatiles (HIPVs) as the dominant component of the indirect defense response against herbivores in black poplar (Clavijo McCormick *et al.* 2014). Feeding by gypsy moth caterpillars strongly induced the emission of all compound classes of HIPVs, i.e. mono-, homo- and sesquiterpenes, aromatic compounds, aliphatic green leaf volatiles and nitrogenous compounds. The increase of terpene emission was also accompanied by the transcriptional activation of terpene synthases, the key enzymes in terpenoid biosynthesis (**manuscript III**). The inducibility of HIPV-biosynthetic enzymes by herbivory or JA and the correlation of biosynthetic enzyme transcripts with the emission of their products has been shown before in poplar trees (Clavijo McCormick *et al.* 2014; Irmisch *et al.* 2014a; Irmisch *et al.* 2014b; Danner *et al.* 2011). These studies documented the sequence from herbivore feeding to phytohormone signaling (induction of JA), subsequent transcription of biosynthetic enzymes (terpene synthases, cytochromes P450), and the emission of the metabolic products (terpenes, nitrogenous compounds) in poplar trees.

As described before, infection with the pathogenic rust fungus also increased the emission of volatiles in black poplar, though to a much lesser extent than herbivory (**manuscript III**). However, when both stressors were applied at the same time, the effects were not additive or

synergistic but antagonistic. Rust infection reduced the emission of HIPVs from herbivore-damaged trees compared to trees with only herbivore damage (**manuscript III**). Factors upstream of HIPV emission were affected as well. The transcription of terpene synthases, allene oxide synthase and JA accumulation were less induced after herbivory in black poplar trees suffering from rust infection compared to herbivory alone (**manuscript III**). Based on the upregulation of SA and SA-responsive genes (**manuscript III**), an antagonistic phytohormone crosstalk seemed to be the underlying mechanism of the repression of HIPVs. This hypothesis was validated by spraying poplar trees with methyl salicylate, an inducer of the SA pathway, which elicited the same inhibitory effects on HIPV emission as rust infection did (**manuscript III**, supplementary data). The detailed molecular mechanism, however, still has to be investigated. According to our results NPR1 (non-expressor of PR genes 1), the key regulator of phytohormone crosstalk in *Arabidopsis thaliana* (Beckers & Spoel 2006; Spoel *et al*. 2003), seems not to be involved in the crosstalk between JA and SA in black poplar. Rather another, NPR1-independent signaling pathway downstream of SA (Xue *et al*. 2013; Blanco *et al*. 2005), mediates the processing of signals from multiple attacks in poplar trees. In general, phytohormone crosstalk has rarely been studied in woody plant species, and the data available draw an inconclusive picture. The infection of black poplar with rust induced both phytohormones JA and SA, even though in different temporal patterns, and based on these results the hypothesis of phytohormone crosstalk in poplar was dismissed (Ullah *et al.* 2018). However, since this study involved only a single attacker species, these results do not provide information on phytohormone crosstalk that results from separate signaling pathways. A study in spruce with artificial hormone and hormone inhibitor treatments concluded that no antagonism exists between JA and SA (Arnerup *et al*. 2013). On the other hand, experiments in eucalyptus suggested an antagonistic crosstalk (Naidoo *et al*. 2013), while a study with ginkgo indicates a complementary relationship between these two phytohormones (Xu *et al.* 2009). It might be that such regulatory mechanisms are species-specific, but it is more likely that other factors, such as time-dependency, environmental conditions or ontogenetic state, influence the outcome of multiple attacks. By surveying the current literature on tripartite interactions in trees some of these factors were discussed in **manuscript I**, for example the identity of the attacker (Ahlholm *et al.* 2002) or the fertilization level of the soil (Eyles *et al*. 2007). But as long as such confounding factors are not identified and characterized, it is difficult to control for them in experiments.

In contrast to phytohormone crosstalk, the mechanisms of volatile emission in woody plant species have been studied in more detail, especially after herbivore treatments. Similar to the results of our study (**manuscript III**) the emission of volatiles is induced by herbivory (Clavijo McCormick *et al.* 2014; Brilli *et al*. 2009; Vuorinen *et al*. 2007; Rodriguez-Saona *et al*. 2006; Arimura *et al.* 2004) or pathogen infection (Patt *et al*. 2018; Toome *et al*. 2010; Vuorinen *et al.* 2007). Vuorinen *et al*. (2007) compared the volatile emission in birch trees after herbivory or pathogen infection, finding qualitative and quantitative differences in the respective blends. However, the simultaneous application of different stressors in woody plants has barely been studied. The additional application of phytohormones after infection with a bacterial pathogen altered volatile emission from orange leaves, but the results were distinct for each hormone and differed among the individual volatile compounds (Patt *et al.* 2018). In herbaceous plant systems, there are a few more examples on the influence of multiple attacks on plant volatile emission, but the results are also inconsistent. Adding pathogen infection to herbivore-infested plants either reduced the emission of HIPVs (Desurmont *et al.* 2016; Rostás *et al*. 2006), had no influence on it (Ponzio *et al*. 2014), increased it (Cardoza *et al*. 2002) or the result was dependent on the virulence of the pathogen (Cardoza *et al.* 2006). Also when herbivores of different feeding guilds – hence also eliciting different defense signaling pathways – were applied, the emission from simultaneously attacked plants was either higher (Delphia *et al.* 2007) or lower (Rodriguez-Saona *et al.* 2003) than from plants infested with a single antagonist.

Studies on multiple attacks investigating indirect defense, i.e. volatile emission, and connecting it with underlying molecular mechanisms are rare (Ponzio *et al.* 2013), and have so far only been conducted in herbaceous plant species (Zhang *et al.* 2013; Runyon *et al.* 2008, Huang *et al.* 2005). **Manuscript III** is therefore the first study focusing on phytohormone crosstalk induced by multiple attacks and its consequence on volatile emission in a tree species.

7.3. Consequences of multiple attacks in black poplar for herbivores

The simultaneous infestation of a plant with different antagonists not only affects the host plant, but also the antagonists themselves. Changes in the host caused by one antagonist, as outlined above (7.1. and 7.2.), that impact another antagonist are called “indirect” or “plant-mediated” effects. In contrast, effects that do not require the participation of the common host are termed “direct”. In **manuscript I** direct and indirect effects of leaf-inhabiting fungi on herbivorous

insects in woody hosts are summarized and discussed. Fungi can cause diseases (pathogens) or live in a symptomless state (endophytes), but both have been shown to influence the preference, performance and abundance of herbivores (**manuscript I**). In the tripartite interaction of black poplar, rust fungus and gypsy moth caterpillars, I demonstrated indirect (**manuscript III**) and direct (**manuscript III + IV**) effects. The repression of HIPV emission of black poplar caused by rust infection is an indirect effect on the herbivore. HIPVs play an important role in the anti-herbivore defense of plants, especially by attracting members of the third trophic level such as parasitoids or predators (Mumm & Dicke 2010; Arimura *et al*. 2005). By reducing the emission of HIPVs, this indirect defense mechanism is impaired and could lead to reduced predation pressure on the herbivores. A similar study on multiple attacks in field mustard supports this hypothesis. Here, plants treated with caterpillars and mildew were less attractive for parasitoids than plants only infested with caterpillars (Desurmont *et al*. 2016). In contrast, infection with a necrotrophic pathogen in maize did not alter the attraction of parasitoids to herbivore-damaged plants, even though HIPV emission was reduced (Rostás *et al*. 2006). In the case of black poplar, the effect of pathogen infection on natural enemies still awaits investigation. Considering previous work that identified nitrogenous compounds as the main volatile cues for the gypsy moth parasitoid *Glyptapanteles liparidis* (Clavijo McCormick *et al.* 2014), rust infection might not impact the attraction of this parasitoid. Nitrogenous compounds were similarly emitted from rust-infected and uninfected trees (**manuscript III**), and therefore this parasitoid species might be able to localize its prey on rust-infected trees as on uninfected trees. However, the altered volatile background consisting of terpenoids, green leaf volatiles and aromatics, still might interfere with the orientation of parasitoids in general, as the mixture of volatiles in blends is often as important as specific individual compounds (Clavijo McCormick *et al.* 2012). Additionally, for many natural enemies, parasitoids, predatory bugs or birds (Unsicker *et al*. 2009), the attractive volatile signals are not identified yet. If a species relies on terpenoids to localize herbivores, it may find its prey less efficiently on a rust-infected tree compared to an uninfected tree.

In addition to changes in HIPV emission, other indirect effects might benefit the herbivores. The reduced activation of JA-signaling in rust-infected black poplar trees (**manuscript III**) most likely has consequences on other JA-dependent defenses that have not been investigated within this thesis. Proteinase-inhibitors (PIs), for example, have been shown to be induced by JA-dependent signaling in poplar (Haruta *et al*. 2001), and a lower amount of PIs would increase the

caterpillar's digestive efficiency and performance (Major & Constabel 2007; Ivashov *et al.* 2001). Furthermore, non-defense-related changes in the plant upon pathogen infection might influence herbivores, such as morphological changes in the leaf matrix (e.g. leaf toughness), altered levels of primary metabolites due to effects on photosynthesis (**manuscript II**) or resource-allocation (Schultz *et al.* 2013). A belowground pathogen infection of oak, for example, increased protein levels in the foliage and this positively influenced the development of leaf-feeding Lepidopteran larvae (Milanovic *et al*. 2015).

Direct effects of fungal plant pathogens on herbivores include the ingestion of fungal cells or compounds during herbivory (**manuscript I**), as well as the utilization of fungal enzymes for herbivore digestion or detoxification (Kukor *et al.* 1988; Martin 1979). However, the nutritional value of fungal and plant tissue differs in some aspects. For example, the absence of condensed tannins in fungal tissue might improve protein digestion (Martin 1979), and assimilation efficiencies in insects are on average higher for fungal mycelia than for tree foliage (Martin & Kukor 1984). Certain micro- and macronutrients also differ in their amounts between fungi and plants. Fungal tissue represents a rich source of choline, unsaturated fatty acids, sterols and B vitamins (Sohn *et al.* 2000; Hatcher 1995; Martin 1979), whereas plant tissue provides high amounts of carbohydrates, including the transport sugar sucrose (Patrick *et al*. 2013; Kozlowski 1992). Furthermore, the nitrogen content of fungi is generally slightly higher than that of plants, although the variability among both fungal and plant species is high (Martin & Kukor 1984). Chemical analysis of black poplar leaves and rust fungus spores revealed approximately 50 % higher nitrogen content in fungal spores compared to tree foliage (**manuscript IV**). Specifically, the content of nitrogen in rust spores reached 3 %, which matches the nitrogen intake target determined for gypsy moth (Stockhoff *et al.* 1993). Additionally, rust spores were almost free of salicinoids (**manuscript IV**), which were previously shown to be detrimental for gypsy moth larvae (Boeckler *et al.* 2014; Lindroth & Hemming 1990). We also observed remarkably high levels of free amino acids in rust spores, including those that are essential for insects (**manuscript IV**). All these chemical factors might be involved in the accelerated larval development that we observed for gypsy moth caterpillars on rust-infected compared to uninfected black poplar trees (**manuscript IV**). The time of larval development is an important parameter of insect fitness, similar to survival or fecundity, as it reduces the exposure to natural enemies, viruses and unfavorable environmental conditions. Positive effects of plant-colonizing fungi on insect fitness

were also observed in other systems, and are reviewed in detail in **manuscript I**. However, only a few studies have tried to unravel the mechanisms behind such performance effects. Johnson *et al.* (2003) found that aphids reared on birch leaves infected with a necrotrophic fungus had a better performance compared to those on uninfected leaves, and suggested increased levels of free amino acids being responsible for this effect. Another study on broad bean demonstrated contrasting effects for a biotrophic and a necrotrophic plant pathogen (Al-Naemi & Hatcher 2013). The authors reported positive effects on the aphids when the host plant was infected with a biotrophic rust fungus, but negative effects when the plants were infected with the necrotrophic grey mold, and both effects correlated with increased and decreased nitrogen content in the plants, respectively.

Knowing that caterpillars profit from feeding on fungal-infected host trees, the question arises whether they have adapted their behavior to this fitness benefit by developing a preference or attraction, or whether they just ingest infected leaves occasionally. In **manuscript III** we could prove that gypsy moth caterpillars are attracted to the smell of rust-infected poplar trees, and even more so to rust spores alone. Additionally, we also observed a strong feeding preference of young gypsy moth larvae for rust-infected compared to uninfected leaves (**manuscript IV**). In search for the trigger of that preference, we identified the sugar alcohol mannitol, which accumulated in infected leaves and even more in fungal spores. Preference assays revealed mannitol to be a feeding attractant for gypsy moth larvae (**manuscript IV**), but did not influence their performance (**manuscript IV, supplementary data**). This shows that gypsy moth use mannitol as an indicator for the presence of fungal infection, even though the compound itself is not beneficial for them. In general, the behavioral responses of insects towards pathogen-infected host plants are diverse, ranging from attraction (McLeod *et al*. 2005) and preference (Mondy *et al*. 1998) to avoidance (Simon *et al*. 2003). In the case of grey mold-infected grape vine, insect preference was dependent on the severity of infection, as they preferred mildly infected but avoided heavily infected leaves (Rizvi *et al*. 2015). In our system, the preference of gypsy moth caterpillars was dependent on the time course of rust infection, switching from avoidance at early stages to strong preference at later stages of infection (**manuscript IV**). At later stages of infection (7 days post-infection) the rust fungus sporulates and orange sporangia become visible on the abaxial leaf surface. Interestingly, gypsy moth larvae started to feed selectively on these sporangia, rather than accidently ingesting them together with the leaf tissue (**manuscript IV**). The same behavior could

be observed with a closely related species to gypsy moth, the rusty tussock moth (*Orgyia antiqua*), which also removed sporangia specifically and preferred infected over uninfected leaves (**manuscript IV**). To address the specificity of the fungal pathogen, we tested the preference of gypsy moth caterpillars towards mildew infection and could observe the same preference and selective feeding patterns as for rust infection. Also in case of mildew infection, the preference was linked to the presence of mannitol (**manuscript IV**). Both results indicate that the preference for infected tissue and feeding on fungal material, i.e. mycophagy, is not limited to one fungal and one insect species. Facultative mycophagy in Lepidoptera has been reported a few times, for example for the navel orangeworm feeding on *Aspergillus flavus* (Ampt *et al*. 2016), the agreeable tiger moth feeding on *Trichaptum biforme* (Moskowitz & Haramaty 2012) or *Anatatha lignea* feeding on shiitake mushrooms (Yoshimatsu & Nakata 2006), but how broadly this phenomenon is distributed among insects still remains elusive. With the limited data available so far it is also difficult to estimate the importance of facultative mycophagy for insect fitness. However, given the prevalence of fungi – pathogens, symbionts and endophytes likewise – in plants, it is possible that insects adapted to plant-inhabiting fungi and might even require fungal colonization for successful development. In contrast to plant-colonizing microbes, insect-colonizing microbes such as gut microbes have received much more attention in the recent decades (Engel & Moran 2013; Dillon & Dillon 2004). Research in the field of insect-microbe associations has demonstrated the importance of microbes for insects, not only by providing essential nutrients (Hansen & Moran 2013), but also in the interaction with the host plant, for example by detoxifying plant metabolites (Berasategui *et al*. 2017; Mason *et al*. 2014). Nevertheless, plant-inhabiting microbes should no longer be neglected in the field of plant-insect interactions, as they most probably also shaped the interaction between plants and insects (Biere & Tack 2013), a phenomenon that could be termed “tripartite co-evolution”.

7.4. Conclusion and Outlook

The results obtained within this thesis provide insights into the tripartite relationship between a deciduous tree species (black poplar, *Populus nigra*) and two of its antagonists, the biotrophic rust fungus *Melampsora larici-populina* and the leaf-chewing gypsy moth caterpillars (*Lymantria dispar*). The response of the host tree to simultaneous attack by both antagonists differed significantly from its response to either antagonist alone. The pathogen repressed anti-herbivore

defenses *via* phytohormone signaling. Moreover, early stages caterpillars fed specifically on fungal tissue that was rich in nitrogen and amino acids, so that caterpillars reared on infected host trees developed faster than conspecifics on control trees. In short, both direct and indirect effects of the pathogenic fungus on the herbivores could be demonstrated. The results emphasize the importance of plant-inhabiting fungi for herbivorous insects and provide a firm basis for further research.

Despite the clear conclusions of this thesis, many aspects of tripartite interactions are still unknown, ranging from molecular mechanisms to ecological consequences. Research under controlled laboratory conditions with simultaneous and subsequent infestations of plants can help to identify temporal patterns and other factors that determine the response of a plant to multiple attacks. To elucidate the underlying molecular mechanisms, genetically modified plants that lack or overexpress regulators of the respective phytohormone signaling pathways can be employed. Future studies might also address the consequences of multiple attack scenarios at the third trophic level, i.e. parasitoids and predators, to reveal the importance of the reduction in volatile emission as observed in **manuscript III**. Defense mechanisms beside HIPV emission, such as proteinase inhibitors or toxins, should also be investigated more thoroughly in the tripartite relationship of plants, pathogens and herbivores, especially with trees. Once more knowledge is available on systems that contain three organisms, more levels of complexity can be added by including a fourth or fifth organism, such as other herbivores, plant-inhabiting microbes, gut microbiota of the insect or hyperparasites of the pathogenic fungus. Furthermore, the prevalence of facultative mycophagy in insect herbivores should be explored by surveying more insect species in various ecosystems. The specificity of such behavior could be investigated by testing insect preference and performance systematically for a larger number of fungal species, ideally of different life styles, and maybe even broaden this study by including bacterial species as well. Last but not least, the usage of sterile plants without any microbial colonization will help to reveal the importance of facultative mycophagy, as plants in natural habitats are commonly colonized by microbes.

The field of tripartite interactions is becoming a focus of plant-insect and plant-microbe research, though almost exclusively in herbaceous model species. Extending this work to woody plants will give us a more comprehensive insight into the chemical ecology of natural ecosystems.

8. SUMMARY

Trees and other woody plant species harbor a huge diversity of microbial, invertebrate and vertebrate species. Due to their large size and long life span, trees can interact with a multitude of beneficial, commensal and detrimental organisms at the same time and so are important in shaping ecosystems. Nevertheless, the interaction of trees and their associated organisms is much less understood than for herbaceous plants. How trees respond to simultaneous multiple attacks, for example by an insect herbivore and a fungal pathogen, is rarely investigated. Even though such multiple attack situations are common in nature, the interaction of plants with herbivores and pathogens has mostly been studied in simple two-way situations.

In my thesis I therefore studied the tripartite interaction of a tree species (black poplar, *Populus nigra*) and two of its antagonists, an insect herbivore (gypsy moth, *Lymantria dispar*) and a fungal pathogen (poplar leaf rust, *Melampsora larici-populina*). Whereas the rust fungus specifically colonizes trees of the genus *Populus*, the gypsy moth is a generalist and feeds on a wide range of host plants from different families, among them Salicaceae to which the genus *Populus* belongs. I studied the tree's response to rust infection alone and together with subsequent feeding by gypsy moth. Further, I investigated the consequences of rust infection for the preference and performance of the insect.

Rust infection of black poplar triggered immediate, long-term physiological changes, i.e. photosynthesis reduction, but did not affect the levels of soluble sugars and phenolic defense compounds (salicinoids) in the leaves. Additionally, infection with rust induced the defense-related phytohormone salicylic acid (SA) and downstream signaling. In a multiple attack situation with gypsy moth feeding, rust infection reduced the jasmonic acid (JA)-dependent anti-herbivore defenses of the tree. This was demonstrated by alterations in phytohormone levels, gene expression and emission of volatile organic compounds. The molecular basis for this pathogen-mediated repression of anti-herbivore defenses likely lies in the antagonistic crosstalk between the induced phytohormones SA and JA, as described in studies with herbaceous plant species.

When gypsy moth larvae fed on rust-infected black poplar trees, they developed faster than individuals that fed on uninfected trees. In the first two instars gypsy moth larvae fed selectively on the sporangia of the rust fungus, which were found to contain high levels of nitrogen, free

amino acids and the sugar alcohol mannitol. The latter acted as a feeding stimulant to gypsy moth larvae, and also accumulated in infected poplar leaves, which were consequently preferred over uninfected leaves by gypsy moths. In addition to their feeding preference, gypsy moth larvae showed attraction to rust-infected trees by olfactory cues.

In this thesis, the importance of a fungal pathogen in tree-insect interactions was demonstrated by assessing the direct and indirect effects of the fungus on the insect herbivore (Figure 2). Further, this study connected phytochemical changes (volatile emission, primary metabolites), molecular mechanisms (phytohormone crosstalk) and ecological consequences (insect behavior and performance) of multiple attacks in a tree species for the first time. To determine the generality of the patterns observed in this project, many more tripartite interactions of different host and attacker species have to be investigated. However, it is already evident that including plant-inhabiting microbes in studies of plant-insect interactions will be essential for understanding relationships in complex natural ecosystems.

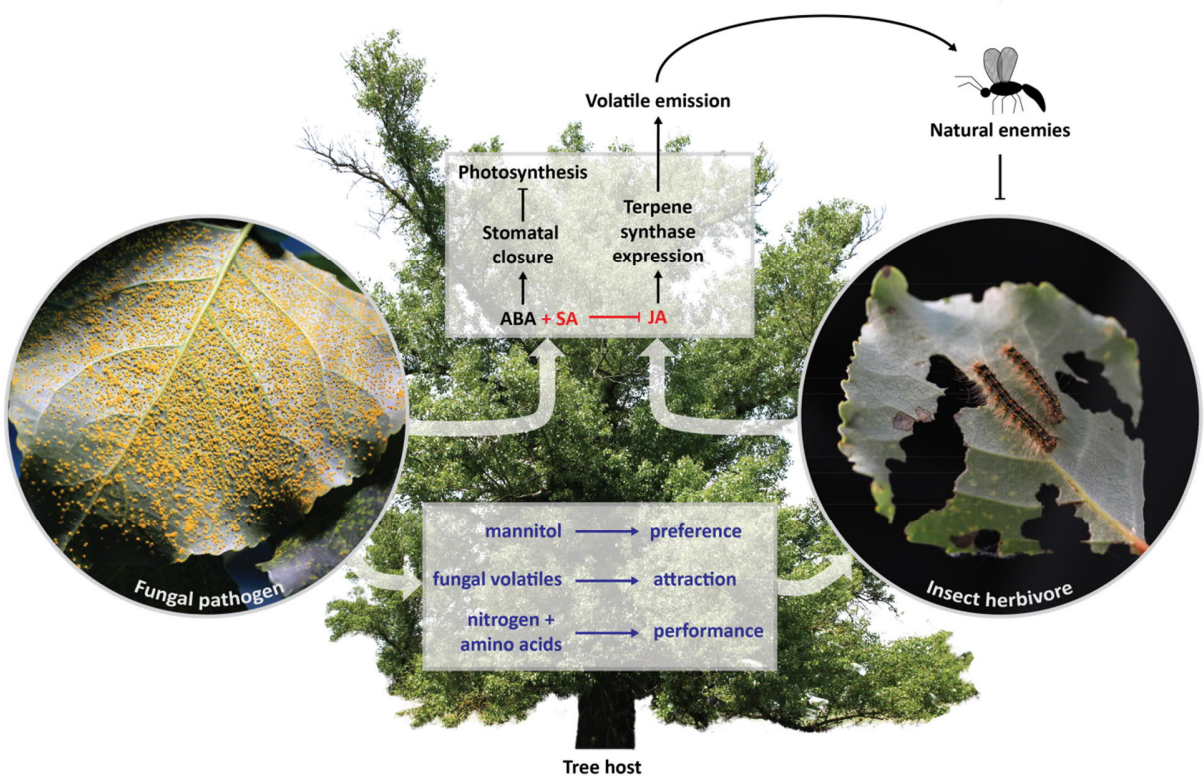

Figure 2. Graphical summary of the direct (blue font, lower inset) and indirect (black font, upper inset) interactions between the fungal pathogen *Melampsora larici-populina* and the insect herbivore *Lymantria dispar* on their common tree host *Populus nigra*. Insect herbivory triggers jasmonic acid (JA) signaling that induces the expression of terpene synthases in the tree. The metabolic products of these and similar enzymes are emitted into the environment (volatile emission) and attract natural enemies of the feeding herbivore. Simultaneous infection with the fungal pathogen induces salicylic acid (SA), which antagonizes JA and its responses (red font). At the same time, pathogen-induced signaling *via* abscisic acid (ABA) leads to a decreased photosynthetic rate due to stomatal closure, which might affect other primary and secondary metabolic processes in the tree. Direct interactions between the fungal pathogen and the insect herbivore are marked by the accumulation of mannitol in infected leaves and fungal spores, which triggers a feeding preference of the herbivores; the emission of fungal volatiles that attract the herbivores; and increased levels of nitrogen and free amino acids in fungal tissue which are presumably responsible for the enhanced fitness of herbivores feeding on pathogen-infected trees.

9. ZUSAMMENFASSUNG

Bäume und andere Gehölzpflanzen werden von einer immensen Vielfalt an Mikroben, Invertebraten und Vertebraten besiedelt und sind so für Ökosysteme von enormer Bedeutung. Ihre große Dimension und Langlebigkeit ermöglicht es Bäumen zeitgleiche oder zeitlich versetzte Interaktionen mit vielen nützlichen, kommensalen und schädlichen Organismen einzugehen. Nichtsdestotrotz ist über die Interaktionen zwischen Bäumen und den mit ihnen assoziierten Lebewesen im Vergleich zu krautigen Pflanzen weitaus weniger bekannt. Wie Bäume auf den gleichzeitigen Befall mit verschiedenen Schädlingen, beispielsweise einem herbivoren Insekt und einem pathogen Pilz, reagieren, wurde bislang kaum untersucht. Obwohl derartiger zeitgleicher Befall mit verschiedenen Schädlingen in der Natur sehr verbreitet ist, werden Interaktionen von Pflanzen mit Herbivoren oder Pathogenen mehrheitlich in Einzelbefallszenarien untersucht.

In meiner Dissertation habe ich daher die dreiseitige Interaktion zwischen einer Baumart (Schwarzpappel, *Populus nigra*) und zwei ihrer Schädlinge, einem herbivoren Insekt (Schwammspinner, *Lymantria dispar*) und einem pathogenen Pilz (Pappelblattrost, *Melampsora larici-populina*), erforscht. Während der Rostpilz spezifisch Arten der Gattung *Populus* befällt, ernähren sich die Raupen des generalistischen Schwammspinners von einer großen Bandbreite an Wirtspflanzen unterschiedlicher Familien, darunter die Salicaceae, zu denen die Gattung *Populus* zählt. Während meiner Arbeit habe ich die Reaktion des Wirtsbaumes auf Rostpilzinfektion allein, als auch in Kombination mit anschließendem Raupenfraß, analysiert und darüber hinaus die Konsequenzen des Pilzbefalls für die Fitness und das Verhalten der Insekten untersucht.

Die Infektion mit Rostpilz löste in der Schwarzpappel unmittelbare und langanhaltende physiologische Veränderungen aus, wie anhand der reduzierten Photosyntheseleistung gezeigt wurde. Im Gegensatz dazu blieb der Blattgehalt an löslichen Zuckern und phenolischen Glykosiden (Salicinoide) unverändert nach Infektion. Die Rostpilzinfektion induzierte zudem eine Akkumulation des verteidigigungsrelevaten Pflanzenhormons Salizylsäure (SA), sowie dessen nachgeschaltete Signalkaskade. Bei gleichzeitigem Befall mit Schwammspinnern reduzierte die Pilzinfektion in der Pappel die Jasmonsäure (JA)-gesteuerte Verteidigungsantwort gegen Herbivoren. Dies konnte auf Ebene der Pflanzenhormonkonzentrationen, der Genexpression sowie der Duftstoffemission nachgewiesen werden. Die durch den pathogenen Pilz ausgelöste Verminderung der Pflanzenverteidigung basiert, molekular betrachtet, vermutlich auf einer

antagonistischen Überlagerung („Crosstalk“) von den induzierten Pflanzenhormonen SA und JA, wie sie aus Studien mit krautigen Pflanzensystemen bekannt ist.

Raupen des Schwammspinners, die auf rostpilzinfizierten Schwarzpappeln gezüchtet wurden, entwickelten sich schneller als solche, die auf nicht infizierten Bäumen aufwuchsen. In den ersten beiden Larvenstadien fraßen Schwammspinnerraupen darüber hinaus selektiv an den Sporangien des Rostpilzes, welche einen hohen Gehalt an Stickstoff, freien Aminosäuren und dem Zuckeralkohol Mannitol aufwiesen. Letzterer zeigte eine fraßstimulierende Wirkung auf die Schwammspinnerlarven und verursachte somit wahrscheinlich auch deren Präferenz für rostpilzinfizierte Pappelblätter, gegenüber nicht infizierten Blättern. Zusätzlich zur Fraßpräferenz wurden Schwammspinnerraupen auch von olfaktorischen Signalen rostpilzinfizierter Bäume angelockt.

Im Rahmen dieser Dissertation konnte die Bedeutung eines pathogenen Pilzes für Baum-Insekten-Interaktionen demonstriert werden, indem direkte und indirekte Effekte vom Pilz auf die herbivoren Insekten evaluiert wurden (Abbildung 2). Erstmals stellt die hier dargelegte Arbeit eine Verknüpfung von phytochemischen Veränderungen (Duftstoffemission, Primärmetaboliten), molekularen Mechanismen (Pflanzenhormon-Crosstalk) und ökologischen Auswirkungen (Verhalten und Fitness der Insekten) in Bäumen dar, die von mehreren Schädlinge befallen wurden. Inwieweit die Ergebnisse dieser Dissertation auf andere Systeme übertragen werden können, ist noch durch zukünftige Studien mit weiteren Arten von Wirtspflanzen und Schädlingen zu belegen. Erwiesen ist jedoch bereits, dass die Integration von pflanzenassoziierten Mikroben in Studien über Pflanzen-Insekten-Interaktionen unerlässlich ist, um die Zusammenhänge in komplexen natürlichen Ökosystemen zu verstehen.

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12. 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.

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13. Curriculum Vitae

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Work experience

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List of Publications

Eberl F, Perreca E, Vogel H, Wright L, Hammerbacher A, Veit D, Gershenzon J, Unsicker SB (2018). Rust infection of black poplar trees reduces photosynthesis but does not affect isoprene biosynthesis or emission. *Frontiers in Plant Science*, 9:1733.

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Eberl F, Hammerbacher A, Gershenzon J, Unsicker SB (2017). Leaf rust infection reduces herbivore-induced volatile emission from black poplar and attracts a generalist herbivore. *New Phytologist* (printed 2018), 220 (3):760-772.

Kissen R, Eberl F, Winge P, Uleberg E, Martinussen I, Bones AM (2016). Effect of growth temperature on glucosinolate profiles in *Arabidopsis thaliana* accessions. *Phytochemistry*, 130:106-118.

List of Conference contributions

Oral presentations

Eberl F (2018). Small size, big effects: a fungal plant pathogen manipulates tree-insect interactions. *Symposium of the MPI for Chemical Ecology*, Jena, DE.

Eberl F (2018). Small size, big effects: how a plant pathogen influences tree-insect interactions. *34th Meeting of the International Society of Chemical Ecology (ISCE)*, Budapest, HU.

13. Curriculum Vitae

Eberl F (2017). Small size, big effects: how a fungal pathogen influences tree-insect interactions. *Seminar of Entomology Laboratory, University of Wageningen*, Wageningen, NL.

Eberl F (2017). Poplar-herbivore-pathogen interaction: Herbivores take advantage of infected host plants. *Gordon Research Seminar: Plant-Herbivore Interactions,* Ventura, CA, US.

Eberl F (2016). Plant-pathogen-herbivore interaction: Combined attack of black poplar trees and its consequences on volatile emission and herbivore behavior. *Seminar on "Chemical Interactions among Plants, Insects and Fungi", University of Pretoria*, Pretoria, ZA .

Eberl F (2016). Trees, insects and pathogens: How a multiple attack influences the host's defense and its attackers. *15th Symposium of the International Max Planck Research School (IMPRS)*, Dornburg, DE.

Poster presentations

Eberl F, Hammerbacher A, Fernandez de Bobadilla M, Gershenzon J, Unsicker SB (2017). Poplar-herbivore-pathogen interaction: Herbivores take advantage of infected host plants. *Gordon Research Conference: Plant-Herbivore Interactions,* Ventura, CA, US.

Eberl F (2017). Poplar-herbivore-pathogen interaction: herbivores take advantage of infected host plants. *16th Symposium of the IMPRS*, Dornburg, DE.

Eberl F, Lackner S, Fabisch T, Unsicker SB, Gershenzon J (2016). Pop(u)lar Science. *Scientific Advisory Board Meeting at the MPI for Chemical Ecology*, Jena, DE.

Eberl F, Hammerbacher A, Gershenzon J, Unsicker SB (2016). How do multiple attacks influence the volatile emission of *Populus nigra* and the behavior of the attacking herbivore? *Gordon Research Conference: Plant Volatiles*, Ventura, CA, US.

Eberl F, Lackner S, Fabisch T, Gershenzon J, Unsicker SB (2015). Pop(u)lar Science. *Symposium of the MPI for Chemical Ecology*, Jena, DE.

Eberl F, Boeckler A, Hammerbacher A, Gershenzon J, Unsicker SB (2015). Poplars, pathogens and herbivores: multi-trophic interactions in a woody plant community. *International Symposium on Communication in Plants and their Responses to the Environment*, Halle (Saale), DE.

Eberl F, Hammerbacher A, Gershenzon J, Unsicker SB (2015). Poplar responses to simultaneous herbivore and pathogen attack. *14th Symposium of the IMPRS*, Dornburg, DE.

Ullah C, Eberl F, Gershenzon J, Unsicker SB, Hammerbacher A (2014). The effect of rust infection on black poplar leaf chemistry and tree responses to insect herbivory. *Symposium of the MPI for Chemical Ecology*, Jena, DE.

Eberl F, Hammerbacher A, Gershenzon J, Unsicker SB (2014). Volatile emission in black poplar (Populus nigra) after combined pathogen and herbivore attack. *15th International Symposium on Insect-Plant Relationships*, Neuchatel, CH.

Hammerbacher A, Gershenzon J, Unsicker S, Eberl F, Ullah C (2014). The effect of rust infestation on black poplar leaf chemistry and tree responses to insect herbivory. *Scientific Advisory Board Meeting at the MPI for Chemical Ecology*, Jena, DE.

Awards

Aug 2018	Student Travel Award, *Meeting of the ISCE*, Budapest, HU.
Feb 2016	IMPRS travel award for the best talk. *15th Symposium of the IMPRS*, Dornburg, DE.
Jul 2008	Karl-von-Frisch Abiturientenpreis, Halle (Saale), DE.

Scientific activities

Feb 2019	Supervision of master student for internship.
Nov 2017	Demonstration at “Long Night of Science”.
Mar – Aug 2016	Supervision of master student for thesis.
Apr 2016	Demonstration at “Forsche-Schüler-Tag”.
Jun 2015	Supervision of bachelor student for internship.
Jul 2014	Supervision of practical course for master students.

Language Skills

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

14. Supplementary Data

14.1. Manuscript I – Supplementary data

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

	Species	**Family**	**Order**	**Phylum**
Tree species	*Acacia dealbata*	Fabaceae	Fabales	Magnoliophyta
	Arbutus unedo	Ericaceae	Ericales	Magnoliophyta
	Betula pendula	Betulaceae	Fagales	Magnoliophyta
	Betula pubescens	Betulaceae	Fagales	Magnoliophyta
	Castanea mollissima	Fagaceae	Fagales	Magnoliophyta
	Cinnamomum yabunikkei	Lauraceae	Laurales	Magnoliophyta
	Cordia alliodora	Boraginaceae	Lamiales	Magnoliophyta
	Embothrium coccineum	Proteaceae	Proteales	Magnoliophyta
	Picea glauca	Pinaceae	Pinales	Coniferophyta
	Picea rubens	Pinaceae	Pinales	Coniferophyta
	Pinus nigra	Pinaceae	Pinales	Coniferophyta
	Populus nigra	Salicaceae	Malpighiales	Magnoliophyta
	Populus spp.	Salicaceae	Malpighiales	Magnoliophyta
	Populus tremula	Salicaceae	Malpighiales	Magnoliophyta

	Species	Family	Order	Phylum
	Pseudotsuga menziesii	Pinaceae	Pinales	Coniferophyta
	Quercus emoryi	Fagaceae	Fagales	Magnoliophyta
	Quercus garrayana	Fagaceae	Fagales	Magnoliophyta
	Quercus robur	Fagaceae	Fagales	Magnoliophyta
	Quercus rubra	Fagaceae	Fagales	Magnoliophyta
	Quercus spp.	Fagaceae	Fagales	Magnoliophyta
	Salix viminalis	Salicaceae	Malpighiales	Magnoliophyta
	Salix x cuspidata	Salicaceae	Malpighiales	Magnoliophyta
Fungal species (pathogens)	*Colletotrichum sp*.	Glomerellaceae	Glomerellales	Ascomycota
	Drepanopeziza populi	Dermateaceae	Helotiales	Ascomycota
	Erysiphe alphitoides	Erysiphaceae	Erysiphales	Ascomycota
	Marssonia betulae	Dermateaceae	Helotiales	Ascomycota
	Melampsora allii-fragilis	Melampsoraceae	Pucciniales	Basidiomycota
	Melampsora epitea	Melampsoraceae	Pucciniales	Basidiomycota
	Melampsora larici-populina	Melampsoraceae	Pucciniales	Basidiomycota
	Melampsoridium betulinum	Pucciniastraceae	Pucciniales	Basidiomycota
	Melanopsichium onumae	Ustilaginaceae	Ustilaginales	Basidiomycota
	Phytophtora plurivora	Pythiaceae	Peronosporales	Oomycota
	Sphaeropsis sapinea	Botryosphaeriaceae	Botryosphaeriales	Ascomycota
	Uromycladium spp.	Pileolariaceae	Pucciniales	Basidiomycota

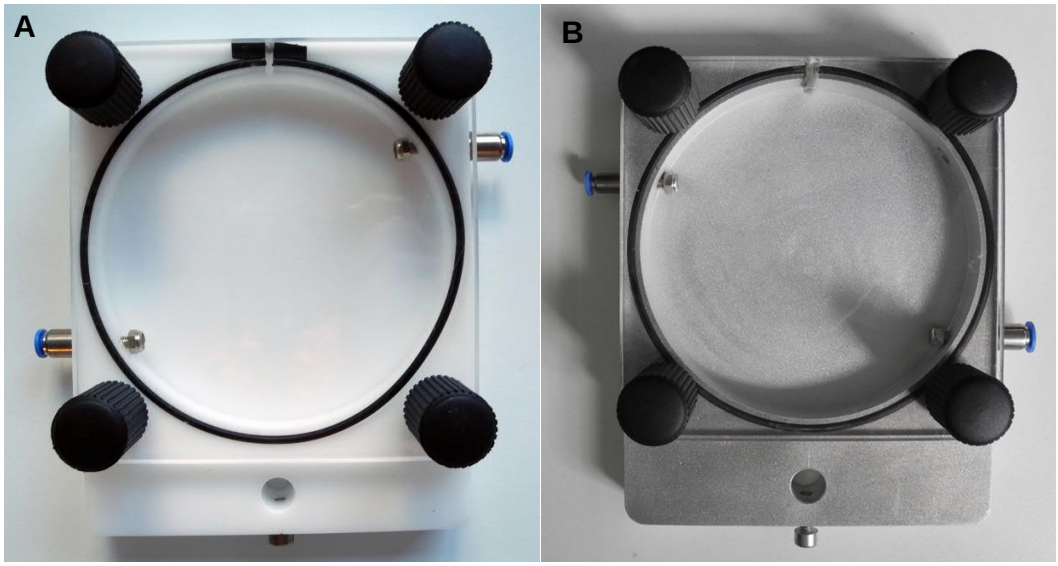
	Species	Family	Order	Phylum
Fungal species (endophytes)	*Asteromella sp.*[1]	Mycosphaerellaceae	Capnoidales	Ascomycota
	Aureobasidium sp.	Dothioraceae	Dothideales	Ascomycota
	Diplodia pinea	Botryosphaeriaceae	Botryosphaeriales	Ascomycota
	Discula quercina	Gnomoniaceae	Gnomoniaceae	Ascomycota
	Fusicladium sp.	Venturiaceae	Pleosporales	Ascomycota
	Melanconium sp.	Melanconidaceae	Diaporthales	Ascomycota
	Phialocephala sp.	Vibrisseaceae	Helotiales	Ascomycota
	Plectophomella sp.[2]	Botryosphaeriaceae	Botryosphaeriales	Ascomycota
	Rhabdocline parkeri	Hemiphacidiaceae	Helotiales	Ascomycota
	Talaromyces pinophilus	Trichocomaceae	Eurotiales	Ascomycota
	Phialocephala scopiformis	Vibrisseaceae	Helotiales	Ascomycota
Insect species	*Acronicta psi*	Noctuidae	Lepidoptera	Arthropoda
	Acyrthosiphon pisum	Aphididae	Hemiptera	Arthropoda
	Arge sp.	Argidae	Hymenoptera	Arthropoda
	Atta colombica	Formicidae	Hymenoptera	Arthropoda
	Bassettia ligni	Cynipidae	Hymenoptera	Arthropoda
	Besbicus mirabilis	Cynipidae	Hymenoptera	Arthropoda
	Cameraria sp.	Gracillariidae	Lepidoptera	Arthropoda
	Choristoneura fumiferana	Tortricidae	Lepidoptera	Arthropoda
	Contarinia spp.	Cecidomyiidae	Diptera	Arthropoda

	Species	Family	Order	Phylum
	Cynipidae	Cynipidae	Hymenoptera	Arthropoda
	Deporaus betulae	Rhynchitinae	Coleoptera	Arthropoda
	Dineura pullior	Tenthredinidae	Hymenoptera	Arthropoda
	Dryocosmus kuriphilus	Cynipidae	Hymenoptera	Arthropoda
	Epirrita autumnata	Geometridae	Lepidoptera	Arthropoda
	Eriophyes rudis	Eriophyidae	Trombidiformes	Arthropoda
	Euceraphis betulae	Aphididae	Hemiptera	Arthropoda
	Lambdina fiscellaria	Geometridae	Lepidoptera	Arthropoda
	Lymantria dispar	Erebidae	Lepidoptera	Arthropoda
	Neodiprion sertifer	Diprionidae	Hymenoptera	Arthropoda
	Phratora vitellinae	Chrysomelidae	Coleoptera	Arthropoda
	Phratora vulgatissima	Chrysomelidae	Coleoptera	Arthropoda
	Plagiodera versicolor	Chrysomelidae	Coleoptera	Arthropoda
	Priophorus pallipes	Tenthredinidae	Hymenoptera	Arthropoda
	Tischeria ekebladella	Tischeriidae	Lepidoptera	Arthropoda
	Zeiraphera canadensis	Tortricidae	Lepidoptera	Arthropoda

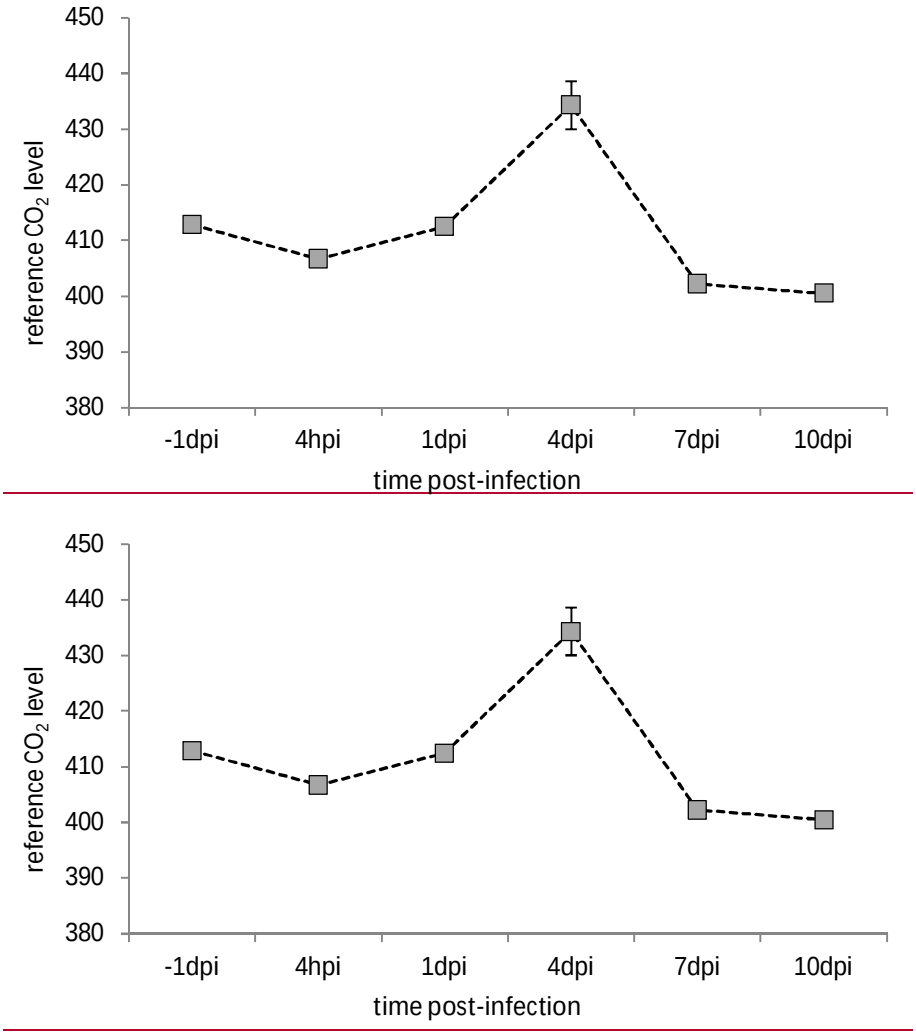
[1] Order and family classification are shown for *Mycosphaerella sp* (synonymous)
[2] Order and family classification are shown for *Dothiorella sp* (synonymous)

14.2. Manuscript II – Supplementary data

Supplementary Figure S3. Second mature leaf from all rust-infected black poplar trees used in the isoprene experiment at 7 dpi, the first occurrence of rust sporangia. Pictures were taken from the abaxial side of the leaves.

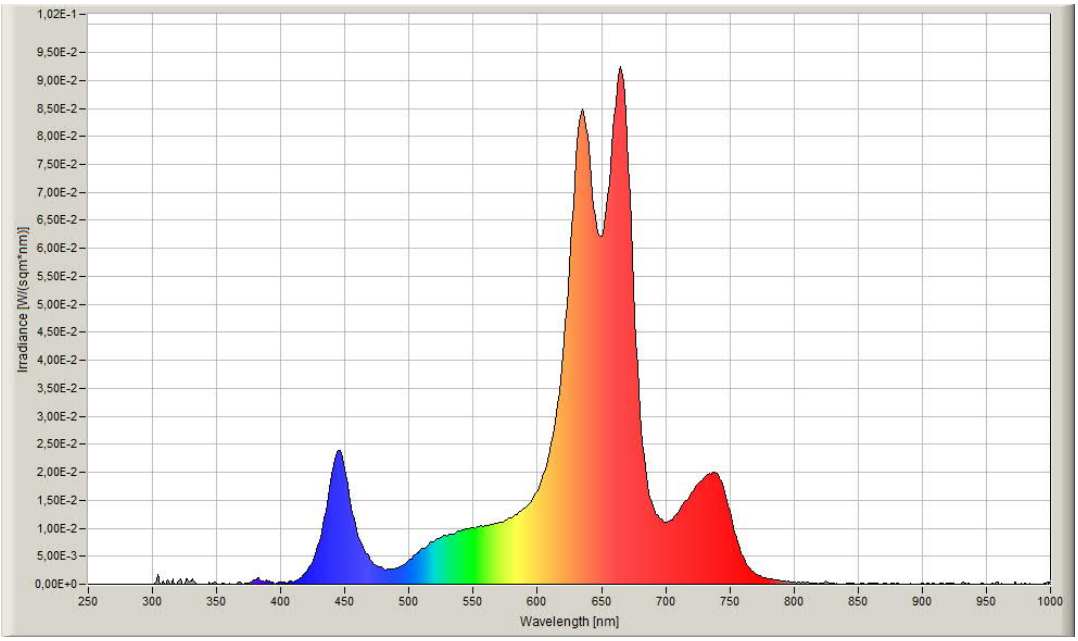


Supplementary Figure S2. Custom-made single leaf chambers (diameter 13 cm) for photosynthesis experiment (A) and isoprene experiment (B). Air enters through Teflon tubing in an arrangement to achieve diagonal air flow (700 µmol s^{-1} in photosynthesis experiment; 1 ml min^{-1} in isoprene measurement). After the leaf is inserted, the chamber can be closed tightly by screws.

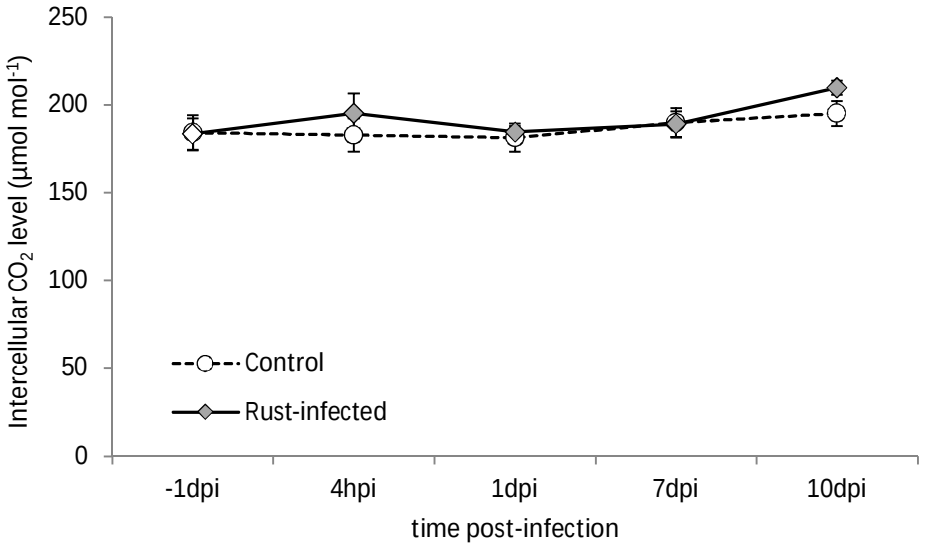


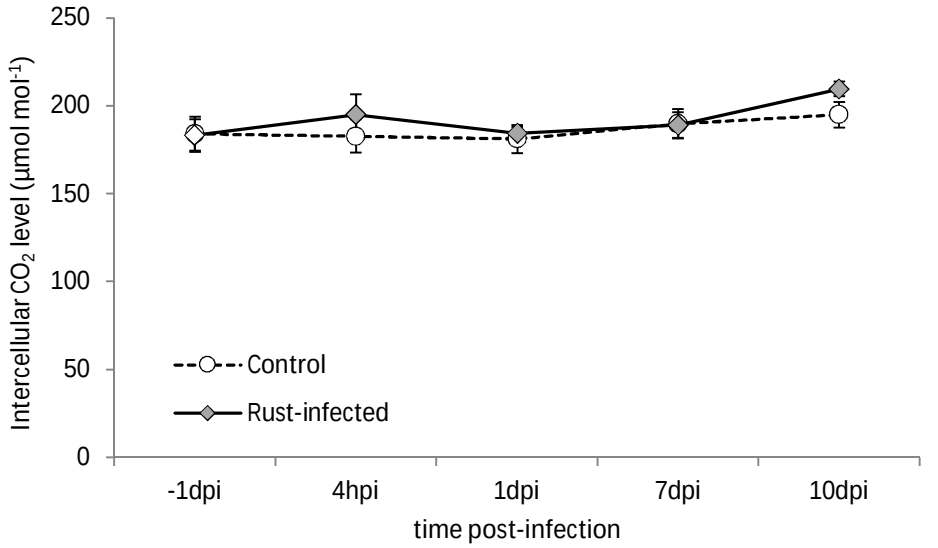
Supplementary Figure S3. Reference CO_2 levels detected during measurement of photosynthetic parameters of rust-infected and uninfected black poplar trees at various hours or days post-infection (hpi/ dpi); -1 dpi refers to 1 day before infection.

14. Supplementary Data



Supplementary Figure S4. Light spectrum of the LED lamp used for photosynthesis measurements and isoprene emission analysis. Light intensity on the analyzed leaf was 850 PAR.





Supplementary Figure S5. Intercellular CO_2 level in leaves of rust-infected black poplar trees (filled symbols) and uninfected controls (open symbols) at different time points after infection (dpi = days post-infection, hpi = hours post-infection; -1 dpi = 1 day before infection). Measurements were made on the second mature leaf counting from the apex. Shown are means ± SEM (n = 6). Repeated measures ANOVA yielded no significant effect of either time, rust infection or the interaction of both.

Supplementary Figure S6. Leaf of a black poplar tree infected with *Melampsora larici-populina* used for the analysis of isoprene emission at 10 dpi. Chlorotic and yellow lesions are visible around the rust pustules, but no necrosis is seen.

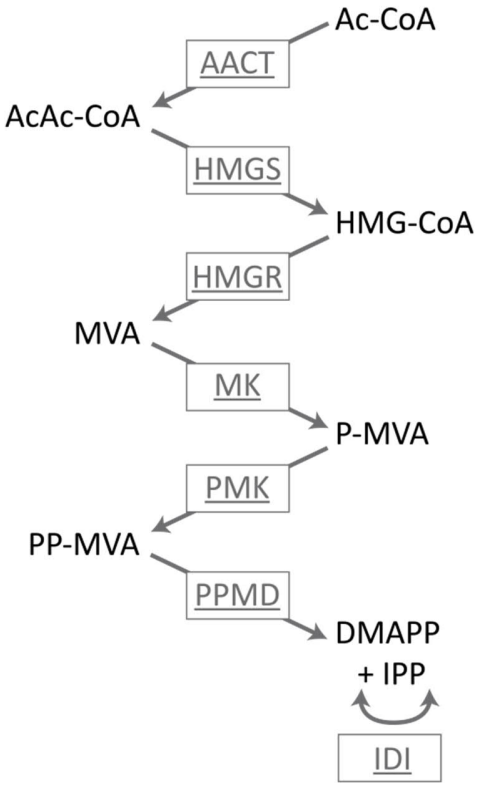
14. Supplementary Data

Supplementary Table S1. Carotenoid levels in uredospores of the rust fungus *Melampsora larici-populina* (picture right side). Spores were carefully collected with a brush and scalpel from artificially infected poplar trees. Extraction and analysis was done as described for plant tissue, except for additional grinding (in aluminum tube with steel balls, 5 min x 3, 900 strokes min^{-1}) prior to extraction. Presented is the mean ± SEM (n = 3 independent spore collections) in mg g^{-1}fresh weight. nd – not detected.

	β-Carotene	Lutein	Neoxanthin	Violaxanthin
Rust fungus spores	2.17 ± 0.38	nd	nd	nd

Supplementary Table S2. Genes encoding enzymes of isoprenoid biosynthesis that were found in the transcriptome of rust-infected black poplar leaves, but were annotated as genes of *Melampsora larici-populina*. In this rust fungus, like all fungi, the mevalonate pathway is the sole pathway of isoprenoid biosynthesis. The mevalonate pathway enzymes are listed by their Enzyme Commission codes (left panel) and abbreviations (for full names, see Figure 5 legend). Shown is mean ± SEM (n = 4) of total raw count of contigs in infected leaves; these contigs were absent in uninfected control leaves.

Contig	Enzyme Codes	Enzyme Abbreviation	Total raw count
C62979	EC 2.3.3.10	HMGS	30.0 ± 6.8
C78714	EC 1.1.1.3	HMGR	13.5 ± 3.1
C78818	EC 2.7.1.36	MK	1.5 ± 1.2
C79234	EC 2.7.1.36	MK	11.3 ± 5.5
C79468	EC 2.7.1.36	MK	6.5 ± 3.7
C79542	EC 2.7.1.36	MK	3.8 ± 0.9
C80675	EC 2.7.4.2	PMK	2.0 ± 0.9
C79192	EC 2.7.4.2	PMK	3.3 ± 1.7
C43752	EC 2.7.4.2	PMK	4.0 ± 2.1
C60670	EC 4.1.1.33	PPMD	12.0 ± 4.4
C79769	EC 5.3.3.2	IDI	6.8 ± 2.3
C79071	EC 5.3.3.2	IDI	5.8 ± 2.0



14.3. Manuscript III – Supplementary data

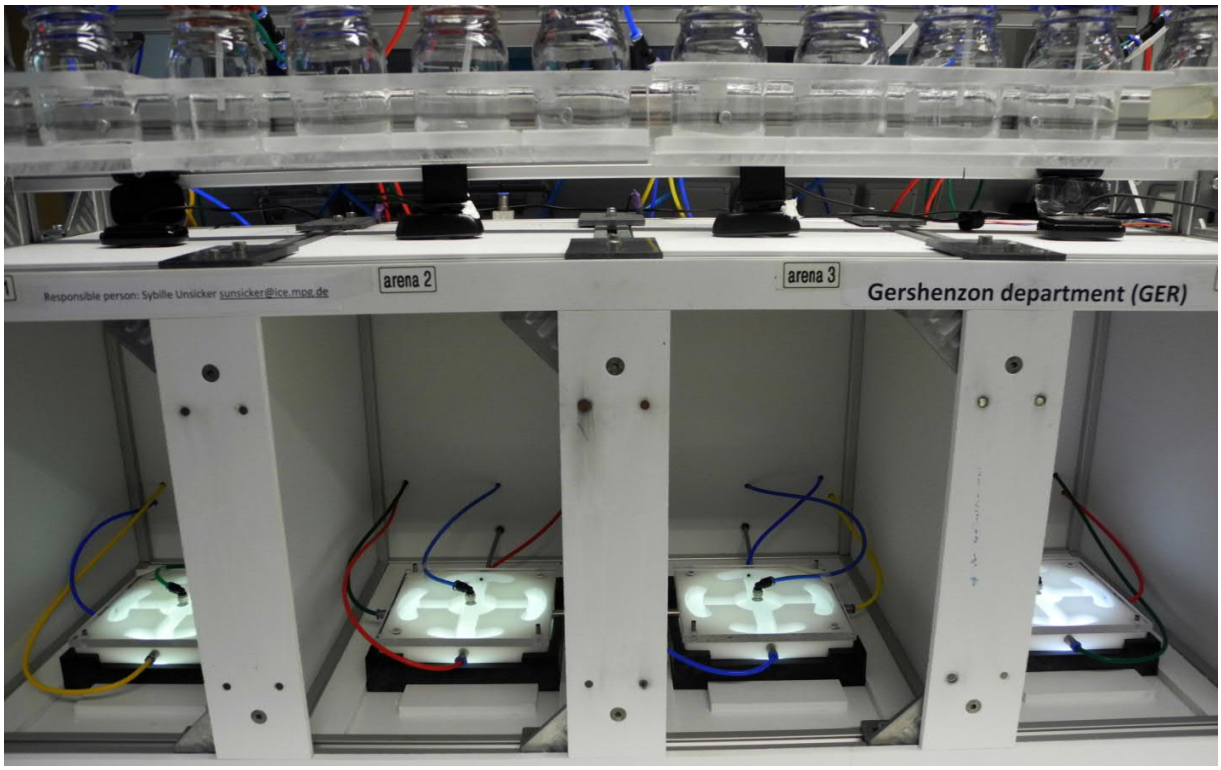


Fig. S1 Arena-setup (with open lids in front) for the olfactometer experiments with *Lymantria dispar*. Active charcoal-filtered air entered each arm of the arena at a flow rate of 0.2 l min^{-1}. At the center of the arena air was sucked out at a flow rate of 1.0 l min^{-1} thus creating a constant flow from each arm towards the middle. During experiments the opposite arms always received the same odor. The bottom of the arena was illuminated to enable video recording from the top with a digital video camera (Logitech, Romanel-sur-morges, Switzerland). For testing individual compounds, dispensers were used consisting of a 1.5 ml glass vial (VWR International) and a 10 µl glass capillary pierced through the septum of the lid. Each compound was dissolved in hexane and 200 µl of the solution used for one dispenser. Each dispenser containing one compound was placed into a glass bottle connected to the arena via Teflon tubes. Charcoal filtered air was pumped into each bottle to be mixed with the compound and then entered one arm of the arena.

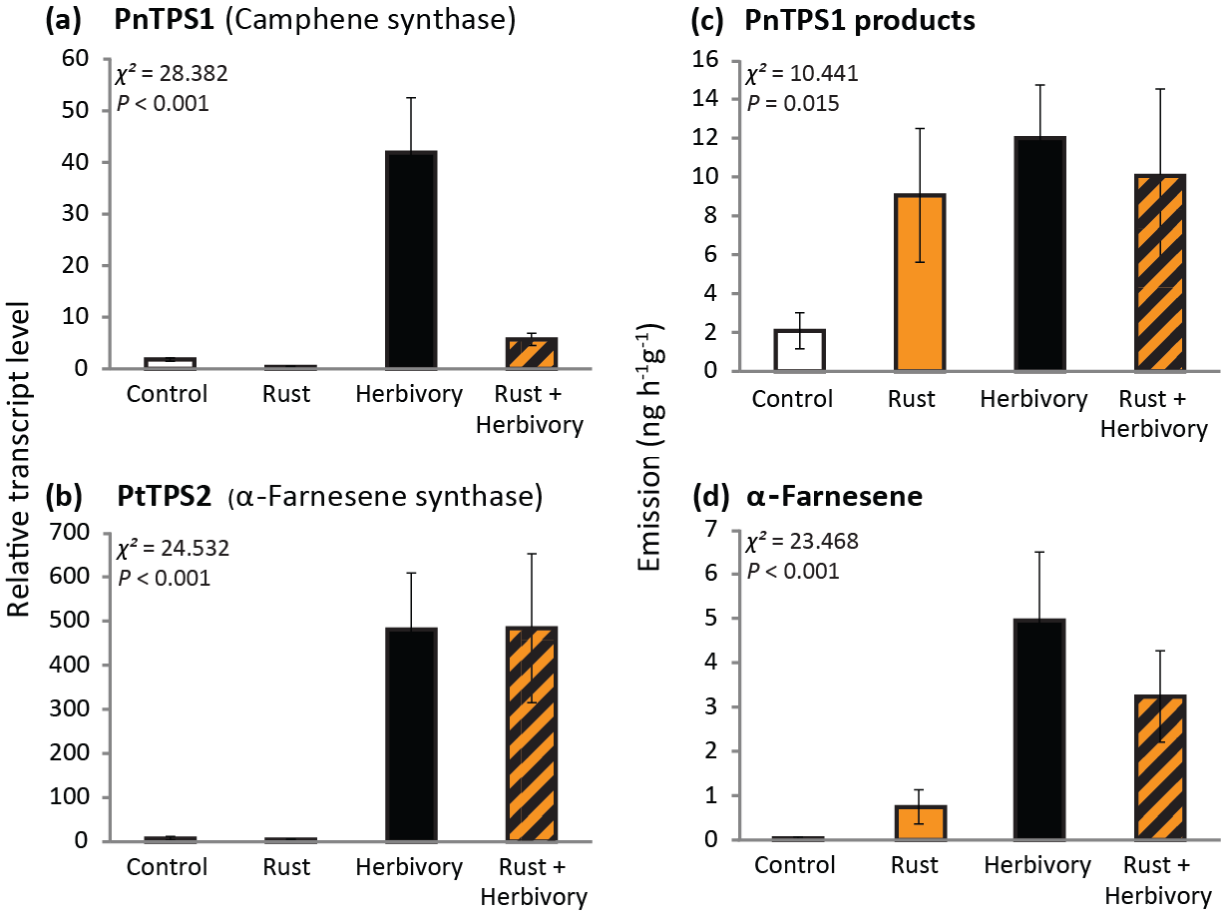


Fig. S2 Transcript levels of terpene synthases and the emission of their products from *Populus nigra* trees that were either non-infested (Control), herbivory infested, rust-infected or infested with both rust and herbivores (Rust+Herbivory). *Lymantria dispar* larvae were allowed to feed for 2 d directly before and during volatile collection. Trees were infected with rust fungus 12 d before insect feeding. (a, b) Transcript levels of terpene synthases previously identified in *P. nigra* (*PnTPS*) or *P. trichocarpa* (*PtTPS*). (c, d) Emissions of products of the corresponding synthases. PnTPS1 (a) is a multiproduct enzyme and the sums of its products is presented (c). Mean ± 1 SE (n = 8 - 9). Results of Kruskal-Wallis test are shown on top left.

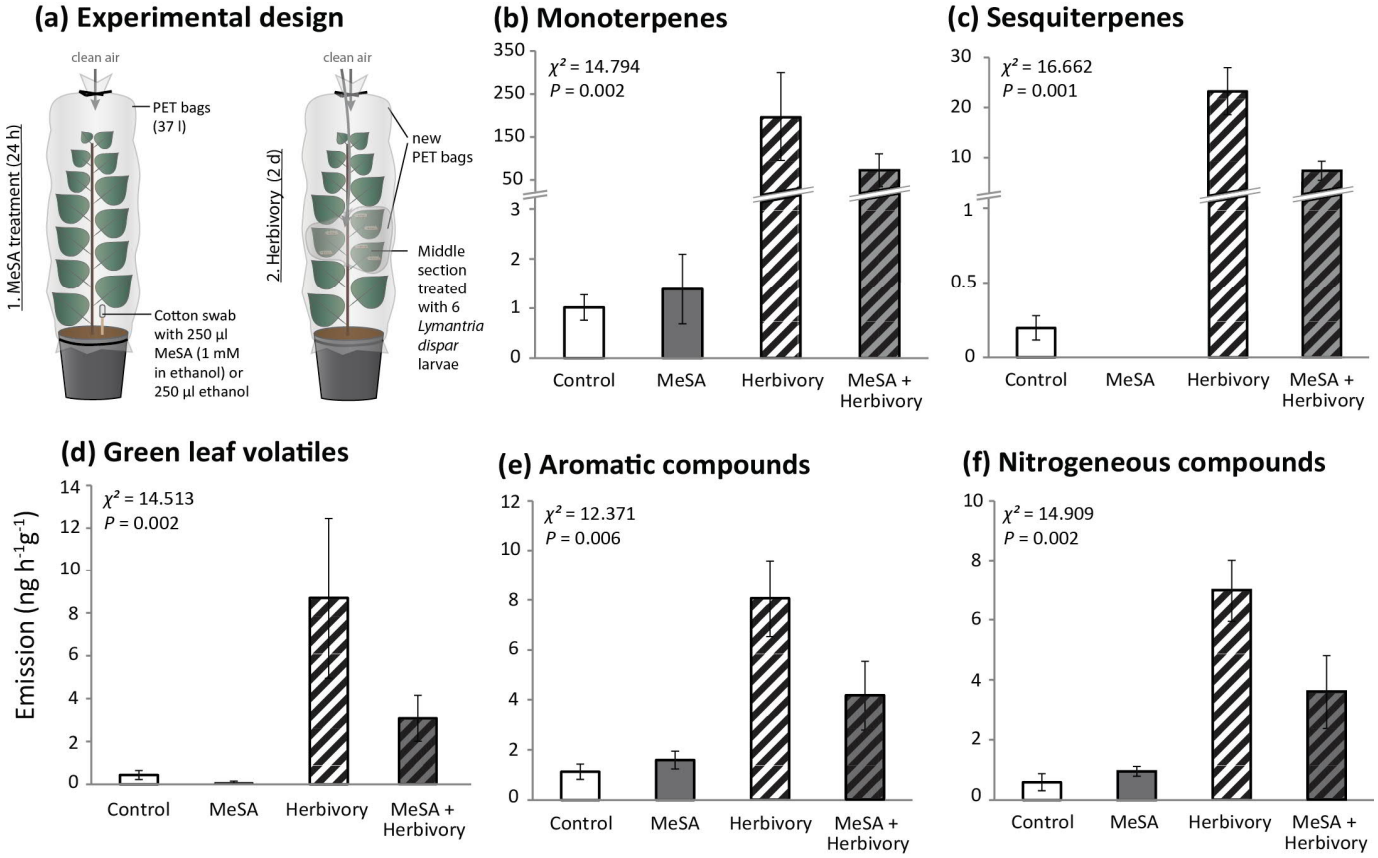


Fig. S3 Effect of artificial methyl salicylate (MeSA) application on the volatile emission of *Populus nigra* trees. Experimental design (a): five month old trees were fumigated with MeSA (250 µl of 1 mM solution in ethanol) or solvent control for 24 h and subsequently damaged by *Lymantria dispar* caterpillars for 2 d (leaf area loss: Herbivory = 3.4 ± 0.9 cm², MeSA + Herbivory = 3.6 ± 0.5 cm²). Herbivory treatment and volatile collection were conducted as described in *Methods and Material*. Volatiles were sorted by compound class (b – f) according to classification in Table S3. Mean ± 1 SE (n = 5), results of Kruskal-Wallis tests are shown on top left.

14. Supplementary Data

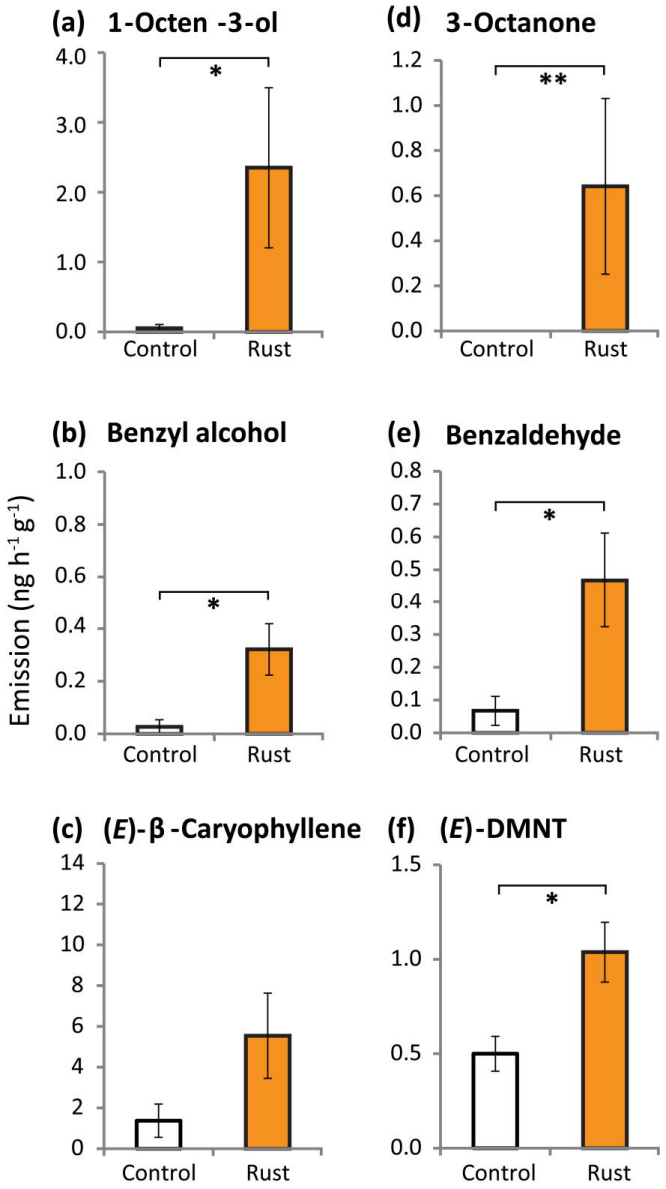


Fig. S4 Emission of volatiles from uninfected controls and rust-infected *Populus nigra* trees. Trees were infected with rust fungus 14 dpi before volatile collection. Shown are volatile compounds used in the behavior assays (Fig. 6). Mean ± 1 SE (n = 8 - 9). Mann-Whitney U-test, significant differences are marked with asterisks (* $P < 0.05$; ** $P < 0.01$).

Table S1 Primer sequences used for qRT-PCR to analyze transcript levels and fungal abundance. Mlp = *Melampsora larici-populina*.

	fw	rev	Reference
PnTPS1	GGCGCTCTGGAAATTATCCC	CAGCATCCAATGGTTTCTCAAG	McCormick *et al.* 2014
PnTPS2	GTCTGTCCTCATAGATCCTAACC	CATTGAGCGTCCCGTAAAGAT	McCormick *et al.* 2014
PtTPS1	GGATACTAGGAGTGTACTTTGAGCC AGAG	GATCTACGGCAGTAATTTCC CACC	Danner *et al.* 2011
PtTPS2	GGGTGACTGAGAAGCTGAGGG	CAGGCCGAGCTTTATGACGC	Danner *et al.* 2011
PtTPS3	GCCTGTGGGATGACTTGGG	CCTGGTTGAGTTGTTTCCACG	Danner *et al.* 2011
PtTPS9	TAGTGGTGGCGCCTATGTC	GTGAACCCTGAGGTGTATAGC	Irmisch *et al.* 2014
NPR1	CTAGATGATGCGCATGCACTCC	GCATTGCAGCGACATGCAGCAC	-
AOS	CATGCCATAATCTTCTCTTCGCCAC	GCATCTGTTCCATACCCCTCATTG	-
WRKY70	CCAGCAAACAGAAAGAAGG	GGCGAACCCACTTGTG	-
WRKY89	AATCCAAGGAGCTACTAC	GTTACCATTGTTGTTGTGG	Jiang et al. 2014
PR-1	TGGGTTGATGAGAAACCAAAGTATG	GCTGCACCTTGCTTTAGCAC	Boyle *et al.* 2010
Mlp ITS	GCCCGTCAAAAGGTTAGCAGTG	CGAGGGGGGTTTCGTGACATTC	-
Actin2	CCCATTGAGCACGGTATTGT	TACGACCACTGGCATACAGG	Ramirez-Carjaval *et al.* 2008

14. Supplementary Data

Table S2. Herbivory damage and pathogen infection on *Populus nigra* trees from different treatments: Control (non-infested), Rust (*Melamspora larici-populina* infection only), Herbivory (*Lymantria dispar* feeding only), Rust + Herbivory (herbivory 12 d after fungal inoculation), Rust + previous Herbivory (herbivory 2 d before fungal inoculation). Damage by herbivory was determined by reconstructing the original leaf area in digital photographs taken of all leaves from the experimental trees with an image editing software (Adobe Photoshop CS5, Adobe, San Jose, CA, USA). Leaf area loss is expressed in cm^2. Fungal abundance was measured relative to the amount of plant tissue by comparing qRT-PCR; the quantity of fungal genomic DNA (gDNA) amplified was normalized to the amount of plant gDNA. Disease severity was assessed as pustule density, i.e. counting of yellow sporangia in pictures of three randomly chosen leaves of each tree. Mean ± 1 SE (n = 7-9). *P*-values of t-tests (Herbivory, Pustule density) or 1-way ANOVA (Fungal DNA) are given. The pictures below the table are examples of rust-infected leaves from different *Populus nigra* genotypes.

	Control	**Rust**	**Herbivory**	**Rust + Herbivory**	**Rust + previous Herbivory***	***P*-value**
Herbivory (cm^2)	-	-	89.0 ± 7.2	91.3 ± 7.1	nd	0.828
Fungal gDNA (rel.)	-	87.3 ± 39.6	-	28.0 ± 27.1	40.0 ± 22.9	0.616
Pustule density (cm^{-2})	-	9.0 ± 2.65	-	8.8 ± 2.07	nd	0.940

nd = not determined

* Data were obtained from trees at the same time as for the other four treatment groups. Details on treatments and harvesting are identical to those described in the Method & Material part except of timing (herbivory being before infection).

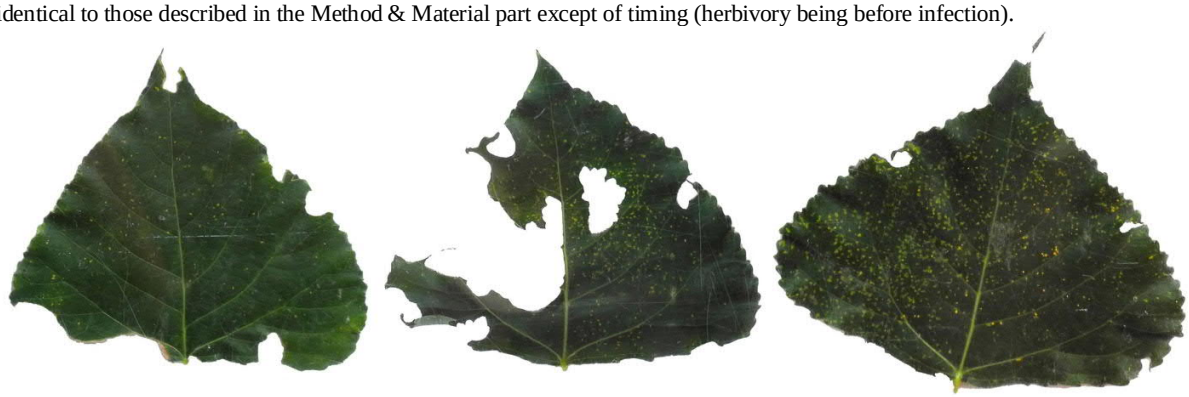


Table S3. Compounds used for olfactory choice assay. Aimed emission was calculated by multiplying measured emission (ng h-1 g-1 fresh weight, in brackets the according treatment for calculation is given) by 20 g fresh weight which is assumed for a young black poplar tree. Measured emission describes the amount that was detected from the dispenser during volatile collections with PoroPak filters.

Compound	Supplier	Aimed emission (ng h^{-1})	Dispenser concentration (µg $µl^{-1}$)	Measured emission (ng h^{-1})
Benzaldehyde	FLUKA	10	0.1	12
Benzyl alcohol	Sigma Aldrich	6	0.2	12
(*E*)-**β**-Caryophyllene	Sigma Aldrich	110	100	108
(*E*)-DMNT	Chemical synthesis	21	1	15
3-Octanone	Sigma Aldrich	13	0.05	12

(*E*)-DMNT = (*E*)- 4,8-dimethyl-1,3,7-nonatriene

Table S4. Volatile compounds emitted by *Populus nigra* trees and classified as shown in Fig. 1. The trees were either uninfested (Control), rust-infected (Rust), herbivory treated (Herbivory) or infested with both the rust and herbivores (Rust + Herbivory). *Lymantria dispar* larvae were allowed to feed for 2 d in herbivory treatments directly before sampling. Trees were infected with rust fungus 12 d before insect feeding. Mean ± 1 SE (*n* = 8-9). Compounds are sorted by chemical class and retention time. The emission rate is given in ng h^{-1} g^{-1} FW.

	Control	**Rust**	**Herbivory**	**Rust + Herbivory**
Monoterpenes	***4.19 ± 1.26***	***15.95 ± 5.50***	***121.62 ± 41.36***	***61.9 ± 23.02***
Tricyclene	0.00 ± 0.00	0.10 ± 0.05	0.08 ± 0.04	0.04 ± 0.04
α-Thujene	0.00 ± 0.00	0.08 ± 0.05	0.32 ± 0.07	0.18 ± 0.09
α-Pinene	0.56 ± 0.22	2.19 ± 0.83	3.18 ± 0.68	2.55 ± 1.15
Camphene	0.85 ± 0.42	3.82 ± 1.43	4.35 ± 1.20	4.01 ± 1.79
Sabinene	0.35 ± 0.12	0.38 ± 0.18	3.34 ± 0.85	1.12 ± 0.54
β-Pinene	0.49 ± 0.23	2.40 ± 0.93	2.83 ± 0.65	2.57 ± 1.14
Myrcene	0.31 ± 0.07	0.30 ± 0.08	1.70 ± 0.33	0.79 ± 0.24
Limonene	0.18 ± 0.06	0.64 ± 0.26	1.63 ± 0.32	0.93 ± 0.38
Eucalyptol	0.32 ± 0.14	0.89 ± 0.69	4.52 ± 1.18	2.22 ± 1.29

14. Supplementary Data

	Control	**Rust**	**Herbivory**	**Rust + Herbivory**
(*Z*)-Ocimene	0.23 ± 0.17	0.85 ± 0.38	4.74 ± 1.40	2.67 ± 0.76
(*E*)-β-Ocimene	0.59 ± 0.14	1.20 ± 0.3	84.04 ± 38.02	41.87 ± 20.95
γ-Terpinene	0.00 ± 0.00	0.05 ± 0.04	0.29 ± 0.09	0.12 ± 0.06
Linalool oxide	0.00 ± 0.00	0.00 ± 0.00	1.15 ± 0.37	0.13 ± 0.09
α-Terpinolene	0.00 ± 0.00	0.02 ± 0.02	0.26 ± 0.08	0.10 ± 0.05
Linalool	0.15 ± 0.15	1.60 ± 1.37	5.49 ± 1.99	0.88 ± 0.26
MT 1	0.00 ± 0.00	0.00 ± 0.00	0.29 ± 0.14	0.14 ± 0.09
Camphor	0.12 ± 0.05	0.98 ± 0.46	1.67 ± 0.71	1.09 ± 0.58
Borneol	0.03 ± 0.03	0.23 ± 0.12	0.58 ± 0.15	0.25 ± 0.11
α-Terpineol	0.00 ± 0.00	0.07 ± 0.05	0.65 ± 0.20	0.10 ± 0.07
MT 2	0.00 ± 0.00	0.15 ± 0.09	0.51 ± 0.05	0.14 ± 0.06
Homoterpenes	**0.50 ± 0.09**	**1.04 ± 0.16**	**129.62 ± 22.77**	**39.37 ± 12.99**
(*Z*)-DMNT	0.00 ± 0.00	0.00 ± 0.00	0.66 ± 0.12	0.29 ± 0.11
(*E*)-DMNT	0.50 ± 0.09	1.04 ± 0.16	128.97 ± 22.65	39.08 ± 12.88
Sesquiterpenes	***3.98 ± 3.01***	***24.22 ± 11.56***	***48.57 ± 8.03***	***19.71 ± 5.20***
α-Cubebene	0.00 ± 0.00	0.05 ± 0.03	0.52 ± 0.10	0.06 ± 0.05
α-Copaene	0.05 ± 0.05	0.39 ± 0.22	0.75 ± 0.15	0.20 ± 0.06
β-Cubebene	0.00 ± 0.00	0.15 ± 0.10	1.47 ± 0.27	0.27 ± 0.14
ST 1	0.00 ± 0.00	0.15 ± 0.15	0.10 ± 0.10	0.02 ± 0.02
(*E*)-β-Caryophyllene	1.37 ± 0.82	5.54 ± 2.09	10.56 ± 2.08	6.12 ± 1.84
ST 2	0.00 ± 0.00	0.09 ± 0.06	0.18 ± 0.04	0.04 ± 0.03
Bergamotene	0.00 ± 0.00	0.55 ± 0.48	0.50 ± 0.34	0.12 ± 0.07
ST 3	0.00 ± 0.00	0.07 ± 0.05	0.07 ± 0.06	0.00 ± 0.00
ST 4	0.00 ± 0.00	0.14 ± 0.09	0.23 ± 0.08	0.07 ± 0.03
α-Humulene	0.90 ± 0.85	3.83 ± 2.19	3.38 ± 0.47	2.29 ± 0.67
ST 5	0.00 ± 0.00	0.07 ± 0.07	0.05 ± 0.05	0.01 ± 0.01
Aromadendrene	0.18 ± 0.15	0.93 ± 0.59	0.50 ± 0.43	0.21 ± 0.11
Bicyclosesquiphellandrene	0.00 ± 0.00	0.22 ± 0.22	0.66 ± 0.16	0.16 ± 0.09
ST 6	0.08 ± 0.08	0.38 ± 0.27	0.42 ± 0.07	0.20 ± 0.08
γ-Curcumene	0.11 ± 0.08	1.54 ± 1.21	1.61 ± 1.19	0.33 ± 0.18
Germacrene D	0.31 ± 0.21	1.8 ± 1.03	14.05 ± 2.25	3.46 ± 1.68
ST 7	0.00 ± 0.00	0.14 ± 0.08	0.10 ± 0.05	0.04 ± 0.03
Germacrene B	0.08 ± 0.08	0.50 ± 0.31	1.04 ± 0.26	0.27 ± 0.09
α-Muurolene	0.15 ± 0.13	1.14 ± 0.66	1.25 ± 0.46	0.45 ± 0.14
α-Farnesene	0.04 ± 0.03	0.74 ± 0.39	4.95 ± 1.54	3.24 ± 1.03
γ-Cadinene	0.22 ± 0.17	1.81 ± 1.06	1.53 ± 0.87	0.52 ± 0.22

	Control	**Rust**	**Herbivory**	**Rust + Herbivory**
δ-Cadinene	0.46 ± 0.35	3.51 ± 2.02	3.33 ± 1.53	1.15 ± 0.42
ST 8	0.00 ± 0.00	0.18 ± 0.12	0.23 ± 0.09	0.09 ± 0.03
ST 9	0.03 ± 0.03	0.31 ± 0.2	0.38 ± 0.12	0.11 ± 0.05
ST-OH	0.00 ± 0.00	0.00 ± 0.00	0.69 ± 0.18	0.29 ± 0.13
Aromatic compounds	***0.37 ± 0.11***	***0.95 ± 0.25***	***17.32 ± 6.32***	***9.43 ± 3.28***
Benzaldehyde	0.07 ± 0.04	0.47 ± 0.14	0.48 ± 0.12	0.52 ± 0.18
Benzyl alcohol	0.03 ± 0.03	0.32 ± 0.10	0.67 ± 0.15	0.61 ± 0.22
Salicylaldehyde	0.00 ± 0.00	0.00 ± 0.00	7.31 ± 3.52	4.10 ± 1.72
2-Phenylethanol	0.00 ± 0.00	0.00 ± 0.00	0.27 ± 0.03	0.06 ± 0.03
Benzyl cyanide	0.00 ± 0.00	0.00 ± 0.00	6.58 ± 2.16	3.49 ± 1.21
4-Ethylacetophenone	0.28 ± 0.08	0.16 ± 0.06	0.20 ± 0.08	0.15 ± 0.05
Indole	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.09	0.00 ± 0.00
Eugenol	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.21	0.32 ± 0.16
3-Hexenol benzoate	0.00 ± 0.00	0.00 ± 0.00	1.13 ± 0.40	0.18 ± 0.08
Nitrogeneous compounds	***0.26 ± 0.09***	***0.15 ± 0.06***	***26.82 ± 7.21***	***16.02 ± 5.17***
(*Z*)-2-Methylbutyraldoxime	0.00 ± 0.00	0.03 ± 0.03	12.78 ± 3.16	8.5 ± 2.72
(*E*)-2-Methylbutyraldoxime	0.00 ± 0.00	0.00 ± 0.00	3.74 ± 1.05	2.35 ± 0.91
(*E*)-3-Methylbutyraldoxime	0.00 ± 0.00	0.00 ± 0.00	2.14 ± 0.64	1.24 ± 0.33
Benzyl cyanide	0.00 ± 0.00	0.00 ± 0.00	6.58 ± 2.16	3.49 ± 1.21
(*E*)-Phenyl acetaldoxime	0.26 ± 0.09	0.12 ± 0.06	0.55 ± 0.15	0.20 ± 0.07
(*Z*)-Phenyl acetaldoxime	0.00 ± 0.00	0.00 ± 0.00	0.16 ± 0.09	0.04 ± 0.03
Indole	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.09	0.00 ± 0.00
2-Phenyl nitroethane	0.00 ± 0.00	0.00 ± 0.00	0.69 ± 0.19	0.19 ± 0.07
Green leaf volatiles	***0.53 ± 0.35***	***0.16 ± 0.06***	***12.13 ± 5.02***	***11.45 ± 5.01***
(*E*)-2-Hexenal	0.00 ± 0.00	0.00 ± 0.00	1.33 ± 0.73	1.98 ± 1.28
(*Z*)-3-Hexenol	0.08 ± 0.06	0.03 ± 0.03	6.10 ± 2.52	4.83 ± 1.81
(*Z*)-3-Hexenylacetate	0.45 ± 0.29	0.11 ± 0.06	3.30 ± 1.50	3.87 ± 2.48
Hexylacetate	0.00 ± 0.00	0.02 ± 0.02	0.24 ± 0.09	0.15 ± 0.09
(*Z*)-3-Hexenylbutyrate	0.00 ± 0.00	0.00 ± 0.00	0.39 ± 0.12	0.13 ± 0.07
(*Z*)-3-Hexenylmethylbutyrate	0.00 ± 0.00	0.00 ± 0.00	0.76 ± 0.28	0.50 ± 0.22
C_8-Compounds	***0.05 ± 0.05***	***2.99 ± 1.49***	***0.04 ± 0.04***	***1.38 ± 0.69***
1-Octen-3-ol	0.05 ± 0.05	2.35 ± 1.15	0.04 ± 0.04	1.17 ± 0.57
3-Octanone	0.00 ± 0.00	0.64 ± 0.39	0.00 ± 0.00	0.21 ± 0.13

14. Supplementary Data

	Control	Rust	Herbivory	Rust + Herbivory
Others	***1.23 ± 0.28***	***1.02 ± 0.18***	***5.30 ± 1.08***	***3.56 ± 0.84***
Isoamylacetate	0.00 ± 0.00	0.00 ± 0.00	0.54 ± 0.18	0.48 ± 0.21
Unidenitified 1	0.00 ± 0.00	0.00 ± 0.00	2.44 ± 0.59	1.54 ± 0.42
Unidenitified 2	0.00 ± 0.00	0.00 ± 0.00	0.78 ± 0.26	0.39 ± 0.1
Nonanal	0.68 ± 0.10	0.75 ± 0.16	0.95 ± 0.13	0.70 ± 0.13
Unidenitified 3	0.08 ± 0.08	0.00 ± 0.00	0.20 ± 0.07	0.09 ± 0.05
Unidenitified 4	0.12 ± 0.05	0.11 ± 0.04	0.10 ± 0.05	0.09 ± 0.04
Unidenitified 5	0.13 ± 0.06	0.04 ± 0.04	0.08 ± 0.06	0.03 ± 0.03
Unidenitified 6	0.23 ± 0.08	0.08 ± 0.06	0.12 ± 0.08	0.11 ± 0.05
Unidenitified 7	0.00 ± 0.00	0.05 ± 0.05	0.10 ± 0.07	0.14 ± 0.08
Total Plant Volatiles	***11.06 ± 4.11***	***43.48 ± 16.48***	***354.62 ± 80.87***	***157.96 ± 52.53***

MT = unidentified monoterpene; ST = unidentified sesquiterpenes; ST-OH = hydroxylated ST
DMNT = 4,8-dimethyl-1,3,7-nonatriene

Table S5 Importance ranking of compounds that are most discriminative for the volatile blends of herbivory-infested and combined infested (rust fungus + herbivory) *Populus nigra* trees, as determined by Random Forest analysis.

Rank	Compound	MDA
1	α-Cubebene	0.026
2	β-Cubebene	0.025
3	Phenylethanol	0.024
4	Germacrene D	0.023
5	MT 2	0.015
6	α-Copaene	0.013
7	Eucalyptol	0.012
8	Sabinene	0.012
9	3-Hexenol benzoate	0.010
10	Germacrene B	0.010

Out-of-bag (OOB) error = 0.111, classification error: 'herbivory' < 0.01 / 'rust+herbivory' = 0.222.
MT = unidentified monoterpene

Table S6 Sugar and amino acid concentrations in leaves of *Populus nigra* from four different treatments: Control (non-infested), Rust (*Melamspora larici-populina* infection), Herbivory (*Lymantria dispar* feeding), Rust + Herbivory (herbivory 12 d after fungal inoculation). Soluble sugars and free amino acids (AA) were measured from methanol extracts (see *Methods*, *Defense hormone analysis*) as described in Madsen *et al*. 2015 (sugars) and Crocoll *et al*. 2016 (AA). Sugar levels are given in mg g^{-1} DW, amino acid levels in nmol g^{-1} DW as mean ± SEM (n = 8-9). Results of two-way ANOVA with factors 'Rust', 'Herbivory' and their interaction are shown.

	Control	**Rust**	**Herbivory**	**Rust + Herbivory**	**'Rust'**		**'Herbivory'**		**'Rust x Herbivory'**	
					F	**P[1]**	**F**	**P[1]**	**F**	**P[1]**
Glucose	4.6 ± 0.4	5 ± 0.7	4.7 ± 0.5	6.7 ± 0.8	3.77	0.061	2.06	0.161	1.80	0.189
Fructose	1.8 ± 0.2	1.9 ± 0.4	1.9 ± 0.2	2.7 ± 0.6	0.86	0.360	1.15	0.292	0.81	0.375
Sucrose	27 ± 1.1	29 ± 2.0	23.2 ± 1.4	26.9 ± 1.9	3.13	0.086	3.35	0.077	0.28	0.603
Total sugars	*33.2 ± 1.1*	*35.9 ± 2.5*	*29.7 ± 1.8*	*36.2 ± 3.1*	3.94	0.056	1.02	0.319	1.07	0.308
Alanine	2.97 ± 0.38	2.35 ± 0.23	2.76 ± 0.2	2.59 ± 0.27	1.88	0.180	0.10	0.749	0.32	0.574
Serine	0.72 ± 0.1	0.93 ± 0.14	0.74 ± 0.09	0.69 ± 0.07	0.60	0.445	1.22	0.278	1.68	0.204
Proline	0.07 ± 0.01	0.09 ± 0.01	0.13 ± 0.01	0.1 ± 0.01	0.00	0.973	19.54	**< 0.001**	7.45	**0.010**
Valine	0.17 ± 0.03	0.21 ± 0.02	0.31 ± 0.03	0.28 ± 0.02	0.96	0.334	15.74	**< 0.001**	2.84	0.102
Threonine	0.46 ± 0.05	0.56 ± 0.04	0.48 ± 0.04	0.54 ± 0.03	4.64	**0.039**	0.01	0.942	0.29	0.595
Isoleucine	0.16 ± 0.03	0.15 ± 0.01	0.24 ± 0.03	0.19 ± 0.01	Kruskal-Wallis test: χ^2 = 6.832; P = 0.077					
Leucine	0.07 ± 0.01	0.11 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.80	0.378	12.20	**0.001**	5.78	**0.022**
Aspartate	2.54 ± 0.38	2.41 ± 0.25	1.91 ± 0.19	1.99 ± 0.22	0.01	0.926	3.63	0.066	0.15	0.697
Glutamate	13.92 ± 1.61	12.37 ± 1.13	10.59 ± 0.95	9.05 ± 0.77	1.77	0.193	8.17	**0.008**	0.00	0.997
Methionine	0.02 ± 0.002	0.019 ± 0.002	0.027 ± 0.002	0.02 ± 0.002	2.64	0.114	4.35	**0.045**	1.17	0.288
Histidine	0.37 ± 0.02	0.41 ± 0.02	0.41 ± 0.03	0.47 ± 0.02	4.28	**0.047**	5.37	**0.027**	0.07	0.795
Phenylalanine	0.13 ± 0.03	0.16 ± 0.02	0.16 ± 0.02	0.13 ± 0.02	0.02	0.894	0.00	0.952	1.84	0.185
Arginine	0.025 ± 0.005	0.024 ± 0.003	0.025 ± 0.004	0.027 ± 0.004	0.28	0.600	0.45	0.508	0.02	0.895
Tyrosine	0.06 ± 0.005	0.08 ± 0.008	0.1 ± 0.008	0.09 ± 0.005	1.08	0.306	15.47	**< 0.001**	7.94	**0.008**

14. Supplementary Data

	Control	Rust	Herbivory	Rust + Herbivory	'Rust'		'Herbivory'		'Rust x Herbivory'	
					F	P[1]	F	P[1]	F	P[1]
Tryptophan	0.23 ± 0.003	0.24 ± 0.005	0.3 ± 0.01	0.29 ± 0.008	0.58	0.450	78.27	< **0.001**	3.08	0.089
Asparagine	0.05 ± 0.01	0.07 ± 0.02	0.07 ± 0.01	0.08 ± 0.01	1.08	0.306	1.03	0.318	0.14	0.715
Glutamine	2.93 ± 1.59	2.12 ± 0.31	3.66 ± 0.4	4.02 ± 0.58	0.42	0.520	11.69	**0.002**	0.14	0.711
Lysine	0.013 ± 0.003	0.015 ± 0.002	0.022 ± 0.003	0.016 ± 0.002	0.85	0.365	4.70	**0.038**	4.45	**0.043**
Total AA	*24.89 ± 3.71*	*22.32 ± 1.68*	*22.08 ± 1.44*	*20.7 ± 1.28*	0.32	0.576	0.39	0.535	0.01	0.922

[1] Significant P-values are written in bold text. Cysteine and Glycine could not be detected.

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14.4. Manuscript IV – Supplementary data

Figure S1. Setup of the performance assay for gypsy moth larvae. Trees were put into a stand (A) on which nine single leaf boxes (B) were mounted. Each box was perforated on top and bottom to enable air exchange and prevent condensation of water. One larva per box was allowed to feed for three days until being transferred onto a new tree. All larvae were feeding on leaves (control or rust-infected) until pupation.

14. Supplementary Data

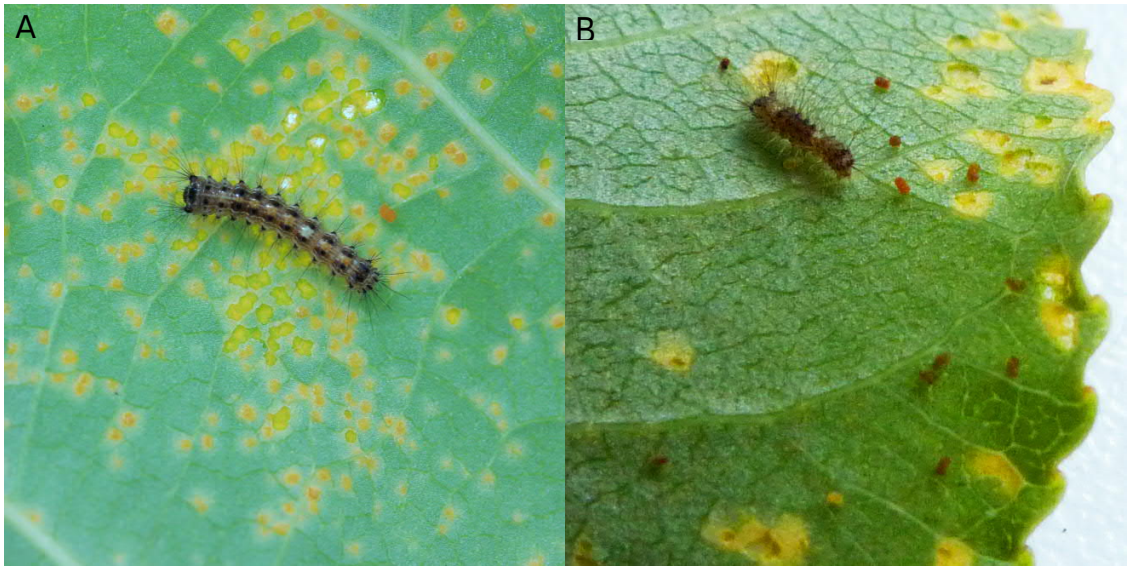

Figure S2. Selective feeding on fungal sporangia by 1st instar larvae. Caterpillars of gypsy moth (*L. dispar*, A) and the related species rusty tussock moth (*O. antiqua*, B) selectively ingested sporangia of the rust fungus growing on the abaxial side of black poplar leaves.

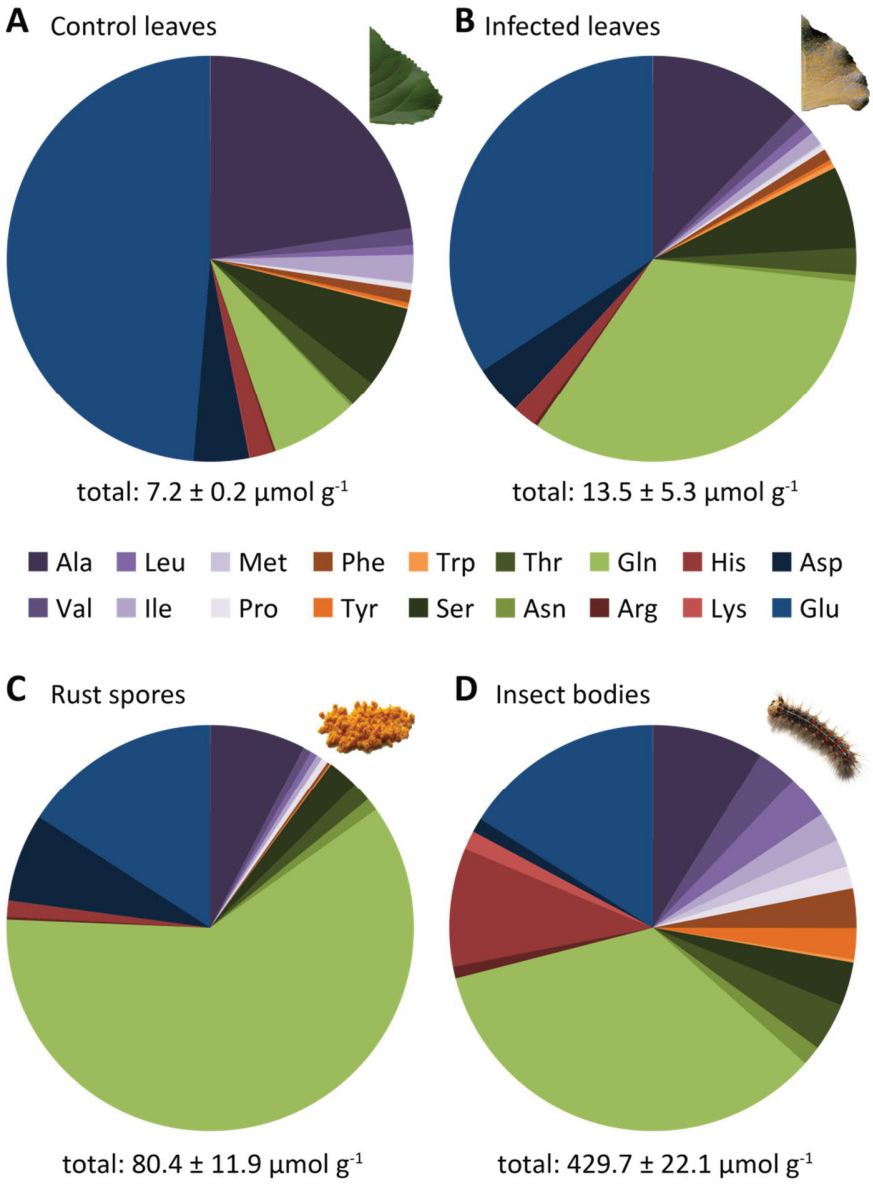

Figure S3. Composition of free amino acids in poplar leaves, fungal spores and caterpillar bodies. Leaves of black poplar were either rust-infected (10 dpi, leaves were already carrying sporangia) or uninfected, for details see *Methods: Chemical analysis*. Uredospores of *Melampsora larici-populus* were separated from leaf material prior analysis. Gypsy moth caterpillar bodies (4th instar) were analyzed after the larvae had fed for 13 d on black poplar leaves. Shown are results for caterpillars that fed on uninfected leaves, which did not differ from those that fed on rust-infected leaves. Total amount of amino acids per dry weight is given below the charts as mean ± SEM (n = 3 for leaves, spores; n = 10 for insects). Amino acids are sorted by type of side chain: aliphatic (purple), aromatic (orange), polar (green), positively charged (red), negatively charged (blue).

14. Supplementary Data

Table S1. Preference of gypsy moth caterpillars in different instars and previous diets. The preference assay with leaf discs of rust-infected *vs.* uninfected black poplar trees, as described in *Methods: Preference assays,* was repeated with 1st, 2nd and 3rd instar larvae. If not stated differently caterpillars were reared on diet. One group of 2nd instar larvae was reared on black poplar leaves prior the preference assay (2nd instar/ poplar). Preference was evaluated as % consumed area of total leaf area (n = 14 for 1st and 3rd instar; n = 20 for 2nd instar, n = 25 for 2nd instar/ poplar), paired t-test (2nd instar) or Wilcoxon signed-rank test (1st instar, 3rd instar, 2nd instar/ poplar).

Larval instar	**Damage control (%)**	**Damage rust-infected (%)**	***P***
1st instar	0.6 ± 0.3	2.1 ± 0.6	0.056
2nd instar	4.9 ± 0.7	11.9 ± 1.1	**< 0.001**
2nd instar/ poplar	9.0 ± 1.1	13.8 ± 0.7	**0.005**
3rd instar	16.8 ± 4.7	17.5 ± 5.0	0.657

Table S2. Performance of gypsy moth larvae under addition of mannitol. Gypsy moth caterpillars were feeding on black poplar (one genotype) leaves coated with plant agar containing mannitol (0.2 mg ml^{-1}; + Mannitol) or not (- Mannitol) as described for mannitol preference assays (*Method: Preference assays*) and were weighted at the beginning of the experiment (d = 1, late 1st /early 2nd instar) and five different time points until reaching the 4th instar (d = 13). Shown is mean ± SEM (n = 20). Repeated measures ANOVA; time: ***P* < 0.001**; treatment: P = ns, treatment x time: P = ns.

Time (d):	**1**	**4**	**6**	**8**	**11**	**13**
- Mannitol	6.3 ± 0.2	13.9 ± 0.1	18.1 ± 0.8	37.3 ± 2.0	58.1 ± 3.0	111.4 ± 6.0
+ Mannitol	6.3 ± 0.2	14.2 ± 0.4	17.8 ± 0.9	37.2 ± 1.8	57.2 ± 3.1	113.7 ± 6.8

Video S1. Feeding behavior of young gypsy moth larvae. 1st instar caterpillars selectively feed on sporangia of the poplar leaf rust fungus on black poplar leaves.