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Volatile emission from *Populus nigra* in response to herbivory and pathogen attack

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Nomenclature

ABA .... Abscisic acid

App. .... Appendix (chapter 6)

CH .... *L. dispar* treated *P. nigra* trees

CTAB .... Cetyltrimethylammonium bromide

CX .... Control group of *P. nigra* trees

DMNT 4,8-Dimethyl-1,3,7-nonatriene

dpi .... Days postinoculation

EDTA .... Ethylenediaminetetraacetic acid

FID .... Flame ionization detector

GC .... Gas chromatography

gDNA .... Genomic deoxyribonucleic acid

HPLC .... High performance liquid chromatography

HT .... Homoterpenes

IS .... Internal Standard

JA-Ile .... Jasmonoyl-isoleucine

JA .... Jasmonic acid

MBA .... Methylbutyraldoxime

MH .... *P. nigra* trees infested with *M. larici-populina* and *L. dispar*

MS .... Mass spectrometry

MT .... Monoterpenes

MX .... *M. larici-populina* infected *P. nigra* trees

NIST .... National Institute of Standards and Technology

OPDA .... *cis*-(+)-12-Oxophytodienoic acid

PH .... Phytohormone

qRT-PCR .... Quantitative real-time polymerase chain reaction

rcf .... Relative centrifugal force

RT .... Room temperature

Rt .... Retention time

SA .... Salicylic acid

SDS .... Sodium dodecyl sulfate

ST .... Sesquiterpenes

TBE .... TRIS/Borate/EDTA buffer

TRIS .... Tris(hydroxymethyl)-aminomethane

VOCs .... Volatile organic compounds

Zusammenfassung

Alltäglich werden Pflanzen von den verschiedensten Schädlingen befallen, zu denen, neben vielen anderen, herbivore Insekten, Bakterien oder Pilze zählen. Meist werden Studien, die sich mit der Verteidigungsreaktion von Pflanzen befassen, an krautigen Pflanzen durchgeführt und die verwendeten Systeme beinhalten oft nur einen biotischen Stressfaktor. Unter natürlichen Bedingungen jedoch werden Pflanzen meist von mehreren Schadorganismen gleichzeitig befallen. Um das weitgehend unerforschte Fachgebiet der multiplen Stressreaktion in Gehölzpflanzen näher zu beleuchten, beschäftigt sich diese Masterarbeit mit der Reaktion der Schwarzpappel *Populus nigra* gegenüber dem simultanen Befall mit dem biotrophen Pathogen *Melampsora larici-populina* und den herbivoren Larven von *Lymantria dispar*. Die Pappeln wurden zuerst mit *M. larici-populina* infiziert und anschließend von Raupen befressen. Hierbei wurde das Pathogenwachstum an drei Zeitpunkten bestimmt und Fraßschaden sowie Gewicht der Herbivoren beobachtet. Weiterhin wurde der Duftstoffausstoß von unbefallenen, pilzinfizierten, befressenen und kombiniert befallenen Bäumen quantitativ und qualitativ analysiert. Die Bestimmung der Phytohormonlevel sollte außerdem Aufschluss über die Regulationsmechanismen der Stressreaktion geben. Die erfolgreiche Infektion durch den Pilz konnte makroskopisch und durch quantitative realtime-PCR verifiziert werden. Raupen, die an inifizierten oder Kontrollpflanzen gefressen hatten, zeigten keinen Unterschied - weder in der Fraßfläche, noch in der Gewichtszunahme oder dem Kotgewicht. In den Bäumen jedoch löste Herbivorenbefall eine erhöhte Konzentration an Jasmonsäurederivaten aus, sowie einen signifikanten Anstieg in der Emission aller Duftstoffklassen, unabhängig vom Pilzbefall. Im Gegensatz dazu war bei *M. larici-populina*-befallenen Bäumen eine Reduktion der Emission zu beobachten, insbesondere unter den Herbivorie-behandelten Gruppen. Darüber hinaus veränderte sich auch die Zusammensetzung des Duftstoffgemisches wenn die Bäume vom Rostpilz befallen waren. Bei einem Langzeitexperiment über 48 h konnte für einige volatile Verbindungen eine Verzögerung der Herbivorie-Induktion festgestellt werden. Als pilzspezifischer Duftstoff wurde 1-Octen-3-ol identifiziert, dessen Emission auch mit dem Wachstum des Pilzes korrelierte. Das Phytohormon Salicylsäure war leicht erhöht in infizierten Bäumen aber unbeeinflusst durch Raupenfraß. Eine deutliche positive oder negative Korrelation zwischen Salicyl- und Jasmonsäure, wie sie häufig in der Literatur beschrieben wird, konnte nicht festgestellt werden. Zusammenfassend kann gesagt werden, dass der Duftstoffausstoß von *P. nigra* von multiplem Befall anders beeinflusst wird als durch einen einzelnen Schadorganismus. Weiterhin scheint die Emission durch den Rostpilz unterdrückt zu werden, was jedoch für die meisten Verbindungen in weiterführenden Projekten statistisch verifiziert werden muss. Die beobachteten Veränderungen in der Komposition des Duftstoffgemisches könnte Einfluss auf biotische Interaktionen zu anderen Organismen haben und sollte daher in Zukunft näher beleuchtet werden. Grundsätzlich kann ein besseres Verständnis der Regulation und der Konsequenzen von simultanem Befall in der Pappel die Schädlingsbekämpfung sowie Züchtung resistenter Hybriden für forstwirtschaftliche Plantagen optimieren.

Abstract

Plants are regularly attacked by different kinds of enemies, including herbivorous insects, fungal or bacterial pathogens. Most studies adressing the defense response in plants focus on herbaceous species and the study systems often include one biotic stress only. In contrast to this, plants are mostly attacked by several pests simultaneously in nature. In order to shed some light on the unexplored field of multiple stress response in woody plants, this Master's thesis studied the effect of a simultaneous infestation of the decidous tree *Populus nigra* with the biotrophic leaf rust pathogen *Melampsora larici-populina* and the leaf feeding caterpillar *Lymantria dispar*. Poplar trees were first infected with *M. larici-populina* and then additionally treated with the herbivorous larvae of *L. dispar* . The pathogen growth was quantified at three time points and the feeding and weight gain of the herbivores was monitored. Further, the emission of volatile organic compounds (VOCs) was measured from control, pathogen infected, herbivory treated plants and plants attacked by both organisms. Phytohormone levels were determined to gain an insight into the regulation mechanism of the stress response. The successful infection of the pathogen was verified macroscopically and by quantitative realtime-PCR. The caterpillars feeding on control and infected leaves did not show any differences in leaf area removal, weight gain or frass weight. However, caterpillar feeding caused an increase in jasmonate derivatives and significantly induced all volatile classes in both control and fungus-infected trees. On the other hand, pathogen infection reduced the amount of emitted volatiles which was especially seen as a strong trend in herbivory-treated plants. Additionally, the composition of the odor blend was changed when the plants were infected with *M. larici-populina*. In a 48 h longterm experiment, the herbivory-induced emission of some compounds was found to be delayed in infected trees. A fungus specific VOC was identified as 1-octen-3-ol and correlated with pathogen growth. Salicylic acid was slightly increased by pathogen attack but was independent from caterpillar feeding. A clear antagonistic or synergistic crosstalk between phytohormones which is often proposed in literature, could not be confirmed in this study. In summary, it can be stated that VOC release from *P. nigra* under multiple attack differed from emissions elicted by a single stress. Moreover, the emission seemed to be repressed or delayed by *M. larici-populina*, which has to be verified statistically with a higher replicate number and contamination free plant material. The observed changes in composition might have consequences for biotrophic interactions in the ecosystem and should be studied in future experiments. Generally, a better understanding of the response to and the consequences of multiple stress in poplar could improve pest management and breeding of more resistant hybrids for silvicultural plantations.

1 Introduction

Plants as sessile organisms are not able to change their location and therefore they face a lot of external dangers every day. These dangers might be abiotic, including changes in temperature, light and nutrient supply or exposure to chemicals. On the other hand, a variety of biotic stressors may additionally attack the plant, e.g. herbivorous insects, mites, mammals, fungi, bacteria, nematodes and viruses all infest plants.

Even if many studies focused on the elucidation of how plants react to all the different stress factors, little is known about the molecular and biochemical mechanisms within a plant when it has to cope with several stressors simultaneously or subsequently. Nevertheless, this scenario is much more realistic and should hence gain more attention in research.

1.1 Plant defense against herbivores and pathogens

During coevolution over 400 million years plants and their enemies have developed fine-tuned, sophisticated mechanisms of defense or circumvention of this defense, respectively [37, 48]. Generally, a defense reaction in plants starts with perception of the attack, leading to the activation of an -often complex- signaling pathway and finally results in effectors or components which inhibit the stressor in one way or another. The defense components can be classified in different manners: they can either be direct or indirect depending on the recipient; consitutive, induced and/or primed with respect to the temporal dynamics; local or systemic when focusing on the spatial pattern; or, grouped by the mode of action, they might be physical, biochemical, chemical or ecological/multitrophic [48]. Further, the defense is strongly dependent on the type of attacker. Best studied and also causing the main part of plant diseases and pests are herbivores and pathogens. These can be further subgrouped into piercing-sucking or chewing herbivores, as well as biotrophic, hemibiotrophic and necrotrophic pathogens. Most surveys dealing with the elucidation of the plant's signaling pathways have focused on only one damaging organism and these studies can be roughly subdivided into plant-herbivore interaction and plant-pathogen interaction themes (Fig. 1).

Plant-Herbivore Interaction

When a plant is attacked by a herbivorous insect it is able to detect so called "herbivore-associated molecular patterns" (HAMPs) [86], e.g. compounds in the oral secretion of caterpillars, such as volicitin [1], or the temporal pattern of tissue damage [87]. Also compounds in oviposition fluids can induce a defense reaction as observed in pea plants responding to bruchins [33]. A very rapid molecular reaction on the local site of damage is a calcium ion (Ca^{2+}) influx and membrane depolarization which is then spreading throughout the entire leaf [83]. Also part of

the early signaling is a production of reactive oxygen species [71] and the activation of protein kinases [138]. Whether these components act down- or upstream to each other is still not clearly understood [137]. The subsequent phytohormone regulation involves jasmonic acid (JA), or rather its amino acid conjugates, and ethylene as keyplayers [109, 95]. JA, a product of the octadecanoid pathway, is converted to jasmonoyl isoleucine (JA-Ile), which releases transcription factors by activating the degradation of the repressor JAZ (jasmonate ZIM-domain protein) [137]. Ethylene is believed to "fine-tune" or potentiate the JA-dependent signaling [95].

The above shortly described induced defense reaction activated upon attack, together with the constantly present constitutive defense strategy, displays the huge variety of plant "weapons" against the herbivores. Beside toxic secondary metabolites, e.g. alkaloids, glucosinolates or terpenoids, there are also proteinase inhibitors to reduce digestibility, as well as morphological characteristics such as waxes, trichomes or laticifiers [48]. Further, several features act in an indirect mechanism: volatile organic compounds (VOCs), extrafloral nectar, food bodies and nesting- or refuging sites [48] attract carnivorous predatores or parasitoids of the herbivore and hence indirectly help the plant to cope with the attackers.

Plant-Pathogen Interaction

Corresponding to the recognized HAMPs in the herbivore-plant interaction "microbial- or pathogen-associated molecular patterns" (MAMPs or PAMPs) are the initial point of the induced defense against bacteria and fungi. The MAMPs or PAMPs are recognized by the plant and induce PAMP-triggered immunity as a result. Some pathogens will be successful in infecting the plant nevertheless and secrete effectors which lead to a suppression of the plant's immunity and so makes the plant susceptible ("effector triggered susceptibility"). If the plant is able to detect these effectors it will initiate a secondary defense response, the "effector triggered immunity", and gain resistance towards the pathogen [19, 65, 9]. This "ping-pong" between plant and pathogen might be continued when the pathogen evolves new effectors and the plant in turn establishes another immune response [65].

The defense strategies of plants to gain resistance towards pathogens include a variety of chemical, biochemical or physical features. Waxes, suberins, lignification or papillae, for example, represent structural and chemical barriers which inhibits penetration by the pathogen [39]. Further, secondary metabolites that are either constantly present in the plant or induced upon infection act directly as toxic compounds, e.g. phenolics, saponins or the phytoalexin camalexin [15]. Beside those secondary metabolites also enzymes such as chitinases degrading the fungal cell wall [14], or inhibitors of pathogenic enzymes, e.g. polygalacturonase-inhibitors [103], are released by the plant. Many antifungal and antimicrobial peptides target the plasma membrane, often by forming pore-like structures that cause an ion flux and hence change the membrane potential [122]. The most important group of defensive proteins are the pathogenesis-related proteins (PR-proteins). PR-proteins are considered to be *de novo* expressed after infection by fungi, bacteria or viruses

instead of being constitutively present [80]. These proteins include proteinase inhibitors (PR-6) and peroxidases (PR-9) [127]. Lastly, the accumulation of reactive oxygen species is reported. These can have a direct toxic effect on the pathogen, lead to oxidative cross-linking of the cell wall or are involved in signaling to induce the hypersensitive response. The latter results in a local programmed cell death in order to block further spreading of the invader [69].

Phytohormone Crosstalk

All these described defense mechanisms are essentially regulated by phytohormones. Mainly involved are JA, ethylene and saliylic acid (SA). However, these regulators do not respond identically to various stressors. The two main signaling pathways are the JA-ET pathway and the SA pathway. The former is known to be induced by necrotrophic pathogens and chewing herbivores, whereas the latter is mediating resistance against biotrophic pathogens and piercing-sucking insects [51, 5, 102, 29]. It is generally accepted that a crosstalk between these two pathways takes place, especially during multiple attack, but the nature of interaction, antagonistic or synergistic, is still under discussion. Even if there are some examples for cooperation between JA and SA, in most studies an antagonism was reported. For example, SA-inducible PR-gene expression is repressed when JA is exogenously applied, and vice versa [94]. Moreover, mutants which were unable to accumulate SA exhibited elevated JA levels and a higher expression of JA-dependent genes [117]. Further, there are several downstream factors which could be shown to regulate SA or JA oppositely. An overexpression of WRKY70 for example leads to an induction of SA-dependent PR-genes but a repression of a JA-responsive gene. Additionally, a higher resistance towards a biotrophic pathogen and, the other way round, reduced resistance towards a necrotroph could be observed on these plants [78, 77]. At the same time, MPK4 could be shown to be a negative regulator for SA-dependent defense but acts positively on JA-signaling [99]. However, there are also studies providing evidence for a cooperation between these phytohormones. The application of a *Phytophthora*-derived elicitor caused an increase in both SA and JA in *Solanum tuberosum* [54]. Additionally, it could be shown in this study [54] that JA is required for the oxidative burst and hypersensitive response that is triggered by SA. Mur *et al.* suggested a dose dependence that determines whether SA and JA act antagonistically or synergistically [90]: low concentrations of exogenously applied SA and JA worked synergistically, whereas this effect disappeared when using higher concentrations or a longer treatment time.

Also other phytohormones such as ethylene or abscisic acid (ABA) are proposed to be involved in these interactions. Ethylene was not only found to be a synergistic partner of JA in the activation of terpenoid biosynthesis, but also to enhance the expression of SA-induced PR-genes [131]. ABA, on the other hand, suppresses the expression of genes downstream of the JA/ET-pathway [2].

The majority of research covering the phytohormone crosstalk was conducted on herbaceous plants and little is known about the interaction in perennial tree species.

Especially the role of SA in biotic stress defense is not well studied and it was even suggested that there is no general conserved function for SA in tree defense at all [49].

1.2 Volatile Organic Compounds

Volatile organic compounds (VOCs) are secondary metabolites emitted by plants from several organs, the most important ones being flowers and leaves. The foliar volatiles can be released constitutively, but the majority is induced by stress, that is they are synthesized *de novo* or enhanced compared to the constitutive level. VOCs elicited upon biotic stress, especially herbivore-induced plant volatiles, have been the focus of many studies including this Master's thesis.

From an ecological point of view there the main function of VOCs is communication. This can be distinguished between plant-insect and plant-plant communication [133, 25]. VOCs mediating inter-species communication are involved in indirect defense, attracting enemies of the attacking herbivores in order to reduce the herbivore pressure on the plant [70, 124]. These tritrophic systems have been described for at least 49 different plant species, ranging from small herbaceous plants to perennial trees [89]. This wide distribution in the plant kingdom indicates that this kind of defense has its origin at an early point in evolution [89]. The enemies recruited by the plants are mainly carnivorous arthropods and parasitoids [133, 124, 4], but also nematodes [128] and insectivorous birds [91] were reported to be attracted by infested plants. Beside the indirect mode of action, VOCs can also directly affect the fitness or behavior of the attackers. A few compounds such as 2-hexenal, hexenyl-acetate and linalool were found to have antimicrobial or antifungal activity [23, 18]. Moreover, also herbivorous insects are influenced by volatiles. The volatiles of infested plants repelled both feeding lepidopteran larvae and aphids [124, 11] and adult, egg-laying females [27, 74]. However, several studies also indicate VOCs to be semiochemicals mediating inter- and/or intraplant communication [67, 16]. Interestingly, the receiving plants upregulated defense genes [36, 6, 38] or gained increased resistance [32] when exposed to volatiles from infested or wounded plants. Additionaly, intraplant communication, the transmission of information within the same individual, might contribute to the systemic response to better defend parts of the plants which are not attacked yet [57].

The variety and specificity of functions is based on the complexity of the bouquets emitted by different plant species in response to distinct stresses. Basis for this complexity is the enormous variety of compounds which can be classified according to their biosynthetic pathways. Compounds released quite rapidly after damage are fatty acid derivatives, which are products of the lipoxygenase pathway [41]. The enzymatic hydrolyzation of fatty acids whithin this pathway shortly takes place after tissue damage and results in C_6 alcohols, aldehydes and esters, also referred to as green leaf volatiles (GLVs) [85]. Furthermore, the predominant group not only of VOCs but of natural products in general are terpenoids

[50]. Within the whole class of terpenoids, only a few subclasses are volatile: hemiterpenes (C_5) such as isoprene, monoterpenes (C_{10}) such as ocimene, linalool or pinene, sesquiterpenes (C_{15}) such as farnesene, germacrene or caryophyllene, and hemiterpenes, e.g. 4,6-dimethyl-1,3,7-nonatriene (DMNT), with an irregular carbon number. The biosynthesis of terpenoids is splitted into two distinct pathways which both yield different compounds: isoprene and monoterpenes are products of the methylerythritol 4-phosphate pathway, whereas sesquiterpenes and DMNT are derived from the mevalonate pathway [106, 89]. Two smaller classes of VOCs are the aromatic as well as the nitrogen- and sulphur-containing compounds. The latter are amino acid derivatives and the best known group within this class are the glucosinolates. These compounds limited to *Brassicales* are precursors of several volatile products, with isothiocyanates being the most abundant [56]. The aromatic compounds originate from the shikimate pathway and can further be differentiated in the phenylalanine-derived phenylpropanoids and benzenoids, the tryptophan-precursor indole, and salicylate, which is either biosynthesized via phenylalanine or from isochorismate [89, 134].

1.3 Study system

The system used in this project to study the plant response to herbivore- and pathogen-stress consists of the deciduous poplar tree *Populus nigra*, the fungal pathogen *Melampsora larici-populina* and the herbivorous insect *Lymantria dispar*.

Populus nigra

The genus *Populus* belongs to the *Salicaceae* family and comprises deciduous, dioecious trees. The native habitat of poplars are riparian ecosystems in the Northern hemisphere [52]. Whereas the model plant for this project, the European black poplar *Populus nigra*, is endangered by now, hybrids of it and of other poplar species are grown worldwide in plantations [63, 52] and gain increasing economic importance [68, 123]. Due to their fast growth [10, 88, 7], poplar trees are very suitable to be grown in short rotation coppices [68]. Main products made out of poplar wood are pulp, paper and plywood [63], but also its use as bioenergy source gets more and more interesting [7, 101, 88] regarding the politics of renewable energy [63]. Beside the applications in the forest industry, poplars are also planted in the environment as shelterbelts, windbreaks and carbon sinks [63]. Both indigenous populations and plantations are frequently damaged by diseases and pests. In addition to defoliation by insects, major biotic damage is caused by rust diseases [101]. Generally, poplar trees harbour a vast amount of arthropods and pathogens. Already 37 families of *Lepidoptera* have been discovered on poplar [105], and diseases are especially caused by fungi which develop rapidly in the soft wood and the moist environment [108]. This diversity makes poplar species interesting for plant-insect and plant-herbivore studies.

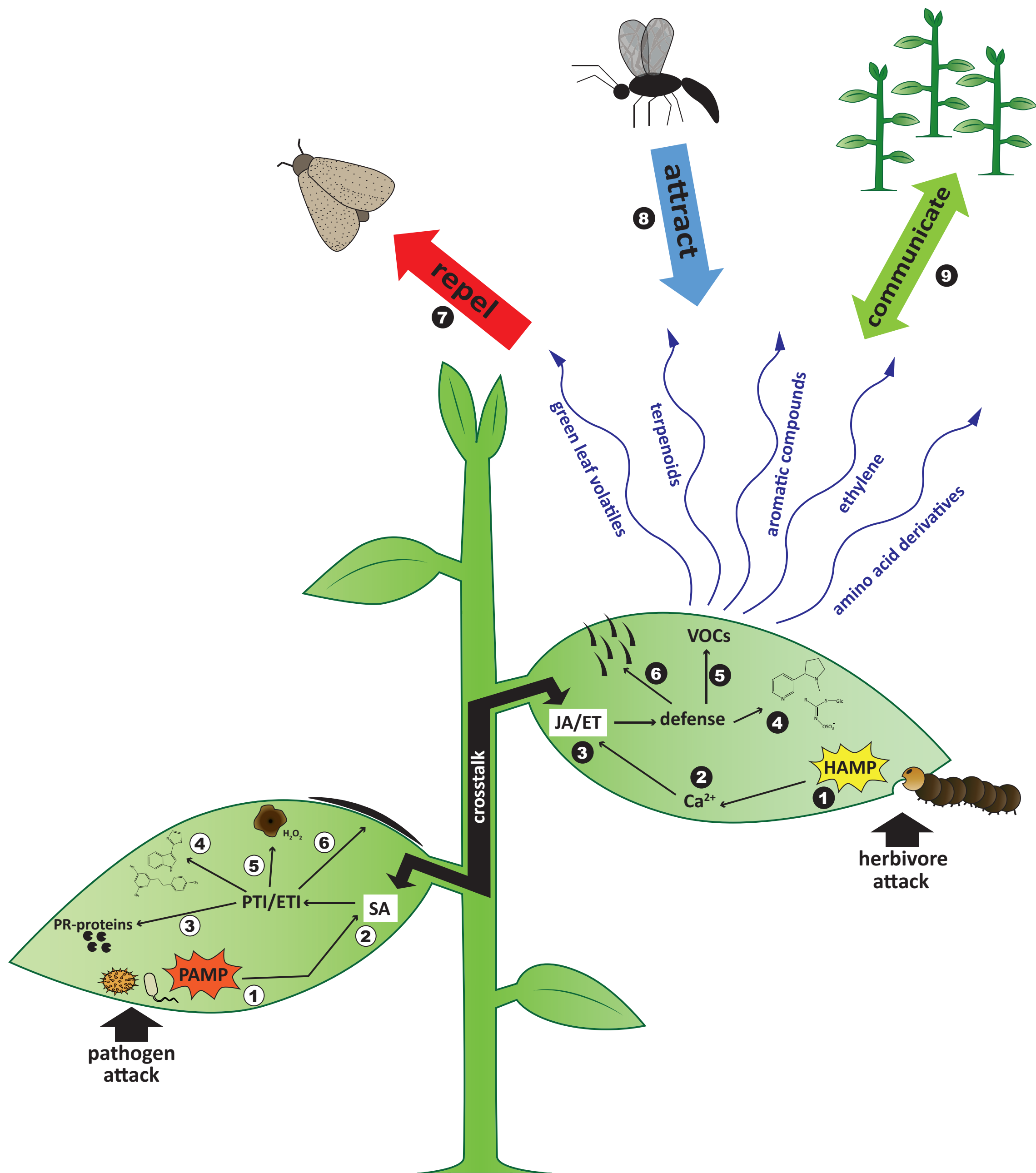


Figure 1: Aboveground plant defense against pathogens and herbivores. **Pathogen attack** (left): 1 - recognition of pathogen-associated molecular patterns (PAMPs), 2 - phytohormone signaling involving salicylic acid (SA) resulting in pathogen or effector triggered immunity (PTI/ETI) 3 - pathogenesis-related proteins (PR-proteins), e.g. chitinases, 4 - phytoalexins, e.g. resveratrol (phenolics), camalexin (alkaloids), 5 - reactive oxygen species, e.g. H_2O_2, and hypersensitive response, 6 - constitutive or induced physical barriers, e.g. waxes, lignification. **Herbivore attack** (right): 1 - recognition of herbivore-associated molecular patterns (HAMPs), 2 - early signaling including calcium ion (Ca^{2+}) influx, 3 - phytohormone signaling involving jasmonic acid (JA) ethylene (ET), 4 - secondary metabolites, e.g. glucosinolates (sulphur and nitrogen containing compounds), nicotine (alkoloids), 5 - emission of volatile organic compounds (VOCs), 6 - other constitutive or induced defenses, e.g. trichomes, 7 - repellence of egg-laying females, 8 - attraction of natural enemies of the herbivores, e.g. parasitoids, 9 - intra- or interplant communication. Phytohormone Crosstalk might be synergistic or antagonistic. See text for references.

The economic importance and the availability of the full genome sequence of (*Populus trichocarpa*, [125]) renders poplar an excellent model species for perennial woody plants.

Melampsora larici-populina

Melampsora larici-populina is a fungal pathogen causing rust disease. It belongs to the order of *Puccinales* and is hence a basidiomycete. Its macrocyclic life cycle includes five different types of spores and is completed on two hosts: *Larix* spp. and *Populus* spp. [130, 53]. The spores of *M. larici-populina* are dispersed by wind, vectors such as insects, or rinsed with rain water and then transported with the water flow [130]. A *Melampsora* infection has severe effects on the plant's energy metabolism. On the site of infection decreased photosynthesis was reported [84], as well as diminished stem diameters and dry matter yield [10], and even increased stool mortality in young stands correlated with the rust disease [75]. Furthermore, a tree once infected with the rust disease is weakened and therefore more susceptible to other diseases and pests. Epidemic outbreaks of several *Melampsora* species are frequently observed in plantations and natural stands, e.g. in South China [132], Argentina, Belgium or Germany [62].

In nature, the biotrophic fungus *M. larici-populina* changes its host from *Populus* species to *Larix* species during the macrocyclic life cycle. However, it is also able to reproduce in a vegetative cycle on poplar only, starting from urediniospores [53]. Due to this possibility of vegetative reproduction and the sequenced genome [34] *M. larici-populina* has been described to be the model pathosystem for forest pathology [35].

Lymantria dispar

The gypsy moth *Lymantria dispar* belongs to the family of *Erebidae*, hence to the large order of *Lepidoptera*, and is endemic to Europe and Northern Africa. Since its introduction to Massachusetts in 1869 it has developed to one of the most notorious pests in North America [82]. It is a univoltine species, that is it reproduces once a year. Larvae hatch from eggs in April or May and then undergo five (males) or six (females) instars; after having completed the last instar the larvae pupate in June to July and will become adults which mate and deposit eggs to produce the next generation [135, 59]. During the larval stage the caterpillars feed on leaves from a wide range of different host plants. Having hosts from ca. 40 distinct families [105], the gypsy moth is a good example of a generalist feeder that feeds on a wide spectrum of different plant species. A severe infestation leads to defoliation which sometimes ($> 50\%$ defoliated) results in the production of a second flush by the host plant [82]. This energy-demanding process weakens the plant and makes it more vulnerable to further biotic or abiotic stress.

1.4 Aim of study

Many studies dealing with chemical ecology focus on two organisms, for example a plant and a herbivorous insect feeding on it. In contrast, plants are more often facing different biotic stress factors at the same time in nature. A model system consisting of more than one pathogen or insect and their host is therefore ecologically more realistic.

The system used in this Master's project is comprised of *P. nigra* as a host, the biotrophic fungus *M. larici-populina* as pathogen and the generalist *L. dispar* as herbivore. The trees were infested with both organisms - singly or in combination - under controlled laboratory conditions in order to get comparable quantitative data. *P. nigra* was chosen as host plant in order to complement what is knwon on induced plant defenses in herbaceous plants and to increase our knowledge on the defense responses in woody species. Since trees could have developed completely different mechanisms due to their long lifespan, the high modularity and extended size, defense responses in these species require more attention. The second organism used in this study, *M. larici-populina*, is a very common and widespread pathogen in indigenous and artificial poplar populations. Interactions between this important poplar disease and herbivorous insects have not been studied yet in poplar as well as the influence of a multiple attack on the host plant itself.

The aim of this study was to determine the effect of these two biotic factors on the volatile emission from the poplar trees. Phytohormone levels were measured to gain an insight into the signaling events in response to multiple attackers. The spreading of the fungus was monitored by molecular biological methods. Furthermore, it was examined whether or not an impairment or improvement of the caterpillar's growth caused by the rust fungus infection takes place. In addition to time point measurements of VOCs once on the day of sampling, a long-term experiment over 48 h was conducted as well.

The resulting data shall be used to provide ideas for future work on this issue and might answer chemical ecological questions such as: Does a pathogen infection have an effect on the feeding behavior of the herbivores? Does the fungus influence the constitutive or herbivore-induced volatile emission from poplars? Which effect can be seen on the phytohormone level and can it be linked to the VOCs? How does the plant react to a combined attack from both organisms? These and related questions served as motivation for this study.

2 Results and Discussion

2.1 Pathogen growth

The ability of *Melampsora larici-populina* to reproduce vegetatively on poplar from urediniospores was used in this project to infect the host plant. 15, 22 and 27 days postinoculation (dpi) leaves from the infected and control trees were harvested and fungal genomic DNA (gDNA) was quantified by qRT-PCR and normalized to plant gDNA of the host *Populus nigra*. 8 dpi the success of the inoculation procedure already was qualitatively seen as orange pustules, the uredinia, on the abaxial side of the leaves. The qRT-PCR analysis revealed a exponential growth ($R^2 = 0.92$ for exponential regression) of the pathogen in the infected plants (Fig. 2).

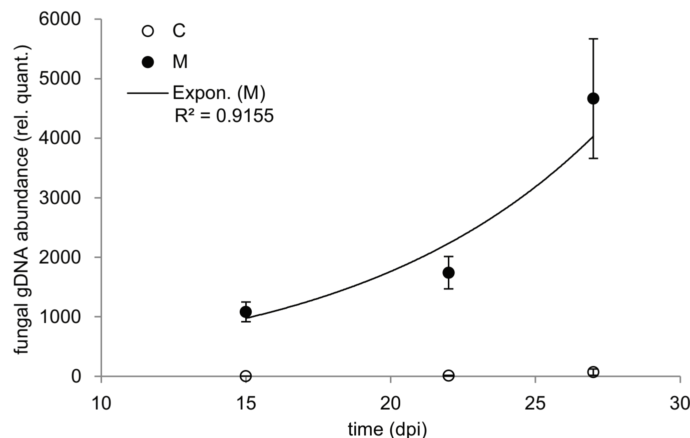


Figure 2: Growth of the biotroph pathogen *M. larici-populina* in leaves of *P. nigra* . Abundance of gDNA of *M. larici-populina* is shown as relative quantity compared to plant gDNA. Tissue was sampled 15, 22 and 27 days postinoculation (dpi) from non-infected control plants (C) and *M. larici-populina* -inoculated plants (M). Data are shown as mean with SEM ($n = 10$, except 22 dpi C, M ($n = 11$) and 27 dpi C ($n = 9$)).

At the late time point (27 dpi) also the control plants showed small positive values in the abundance of fungal gDNA. This is due to contamination with *M. larici-populina* which could also be seen as uredinia on few of the leaves. Even if a spreading of the fungus was obstructed by installing plastic foil on both infected and uninfected trees, spores spread in the ventilated greenhouse. To prevent this kind of contamination in future experiments, separate greenhouse rooms or climate chambers should be used for the two treatment groups.

2.2 Effect of Pathogen Infection on the Herbivores

One of the aims in this study was to investigate the behavior of herbivorous insects towards pathogen infected or non-infected trees. Therefore, the feeding damage as

removed leaf area, the weight gain of the larvae as well as the fresh weight of their frass was determined. Both in feeding damage (Fig. 3) as well as in body weight and frass (Table 1) no significant differences between the two treatment groups were observed.

Only few studies are published handling the effect of a fungal pathogen on herbivorours insects. Nevertheless, mostly a negative impact on the performance of insects is reported. For instance, the infection of rose plants with powdery mildew leads to decreased oviposition, pupal weight, emergence rate and fecundity of of the beet armyworm *Spodoptera exigua*. Further, Simon and Hilker [114, 115] found negative effects using a very similar system of study organisms used in this Master's project: when willow trees (family: *Salicaceae*) were inoculated with the rust fungus *Melampsora allii-fragilis*, adult willow leaf beetles (*Plagiodera versicolor*) avoided feeding and oviposition on infected leaves and larvae were delayed in development and had less weight. Similarly, the leaf beetle *Phratora vulgatissima* was observed to consume less leaf area from *Melampsora epitea* infected willow leaves than from healthy plants [98]. These contradictory observations between feeding behavior of the described herbivores in literature and *L. dispar* in this experiment could be due to the different host preferences of these insects. Whereas *L. dispar* is a generalist feeding on various host species, *Plagiodera versicolor* for instance is a specialist on willow species [58] and might therefore be more sensitive to changes in its host plant's leaf chemistry caused by a pathogen infection.

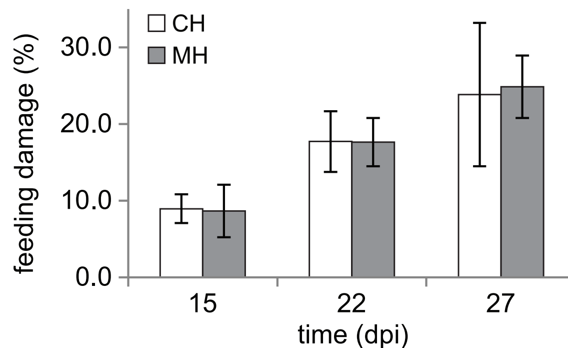


Figure 3: Feeding damage of *L. dispar* larvae on pathogen infected (MH) and control leaves (CH) at different time points. At 15 and 27 dpi eight fourth-instar larvae were used, at 22 dpi nine third-instar plus one fifth-instar larvae. Herbivores were allowed to feed for 48 h. The leaf area removed by the caterpillars was calculated as % of the total leaf area for each tree. Data are shown as mean ± SEM (n = 5). One-way ANOVA (F = 8.499; p = 0.001) revealed significant differences between 15-22 and 15-27 dpi (Tukey's posthoc test) using all data per time point for comparison (n = 10).

Interestingly, all three properties change among the different time points. The feeding damage and frass weight are bigger the later the samples were taken, and at the earliest time point (15 dpi) caterpillars even showed a weight loss after 48 h of feeding (Table 1). Possible reasons for this pattern could be that different instars,

ranging from the third to fifth, of the caterpillars were taken for the experiment. On the other hand, at 15 and 27 dpi the same number of caterpillars from the fourth instar were taken and the feeding damage was different nevertheless. Even if the larvae were reared and fed constantly, there could be a variability within the time period between last feeding with artificial diet and start of the feeding experiment. That this variability leads to such a uniform pattern of increasing feeding damage (Fig. 3) might be unlikely but possible. Another argument for increasing leaf removal at later time points might be the start of leaf senescence. The poplar trees were grown in the greenhouse under natural light conditions and hence were exposed to the decline of light period. In case of senescence the trees reallocate their resources from the leaves to roots and stem [17], which leads to a decrease in nutrient content in the leaves. Due to less nutrients, the caterpillars are forced to feed more in order to get the same energy uptake. In contrast to this hypothesis, the weight gain of the larvae is not constant but raising, at least from 15 to 22 dpi (Table 1). However, the weight gain during the last experiment is not higher than that from the 22 dpi, but between these data also the feeding damage showed no significant differences.

Table 1: Properties of *L. dispar* larvae at different time points. Larvae were allowed to feed for 48 h on control (C) and *M. larici-populina* infected (M) plants. Indicated are weight gain as % of mass before the feeding period and fresh weight of the frass in mg. Data are shown from single time points as well as from the total value of all time points together, as mean ± SEM (n = 5, except of total: n = 15).

time (dpi)	treatment	weight gain (%)	frass (mg)
15	C	-0.34 ± 4.60	175.80 ± 18.79
	M	-5.77 ± 2.88	145.00 ± 30.25
22	C	26.04 ± 5.87	239.00 ± 15.42
	M	30.96 ± 7.14	254.80 ± 31.19
27	C	24.58 ± 8.00	303.80 ± 61.88
	M	28.30 ± 2.60	352.80 ± 35.14
total	C	16.76 ± 4.67	239.53 ± 24.82
	M	17.83 ± 5.12	250.87 ± 28.49

It is also known that poplar leaves infected with rust disease exhibit higher levels in phenolic compounds [64]. Also such a change in secondary metabolites caused by the fungus may influence the feeding behavior of the herbivores. However, feeding stimulation by phenolic glycosides was reported for specialists, whereas generalists such as *L. dispar* usually were deterred [12]. How the content of phenolic compounds changes during infection should be determined in further experiments and correlated to the feeding damage and weight gain. An adaption of the caterpillars to phenolic compounds or other secondary metabolites in poplar leaves can be excluded since all herbivores were reared on artificial diet before starting the feeding experiment.

As a result of these data it is important to keep in mind that the damage which the

plant experiences is not constant. The defense reaction due to this varying damage might therefore also be different and not exclusively a result of the time factor.

2.3 Volatile Emission

Poplar trees are known to emit a huge variety of different VOCs, some of them are constitutively emitted whereas others are induced by biotic or abiotic stress. Even if the same genotype was used for VOC sampling at 15, 22 and 27 dpi the composition of the volatile bouquet varied among these datasets. A list of the VOCs found in each experiment can be seen in the appendix (App., Table 8, 9 and 10).

2.3.1 Total Volatile Emission

The total volatile emission (Fig. 4), that is the sum of all detected volatiles at each single time point, is not constant. The highest volatile emission can be seen at 22 dpi in the herbivore-treated groups, whereas emissions at 15 and 27 dpi are in a lower range. The bigger effect of herbivore feeding on 22 dpi compared to the other time points can be explained by the caterpillars which were used for this treatment: due to limited availability of fourth instar larvae as they were used in the other treatments, in this case nine third instar larvae and one fifth instar caterpillar were used.

It could be shown by McCormick *et al.* [20] that the developmental stage of the herbivore influences the volatile emission of poplars. This was observed especially for nitrogen-containing compounds (N-compounds) but also for mono- and sesquiterpenes. As a result of the smaller but more numerous lesions caused by the small caterpillars the plants emitted higher amounts of certain volatiles and thus the total emission at the 22 dpi time point increased. Using two-way ANOVA, the influence of herbivore stress on volatile emission was found to be significant at 15 dpi ($Fh = 9.595$; $ph = 0.007$) and 22 dpi ($Fh = 43.97$; $ph < 0.001$), but not at 27 dpi. Even if if the significance is not given, there is a trend in the 15 and 22 dpi data that a *M. larici-populina* infection suppresses the induced volatile emission. A reduction of total emission was for example also reported in sqash plants which were infected with the biotrophic powdery mildew [118].

The data obtained from 27 dpi are highly variable and no significant differences were found between the four groups. At this time point many plants were contaminated. Some of the uninoculated control plants were accidently infected by *M. larici-populina* as already discussed earlier and almost half of the plants, mainly control plants (C), were infected with spider mites. Boer et al. [26] showed that a combined attack of caterpillars and mites may have synergistic or antagonistic effects on the volatile emission, depending on the plant species. Which effect a multiple herbivory has on black poplar still has to be elucidated, but an impact of the unwanted spider mites on the VOCs is conceivable. Also an infection with powdery mildew cannot be excluded since neighbouring plants in the same greenhouse showed

symptoms of mildew just some days after the last sampling. Additionally, more than half of the *M. larici-populina* -infected plants had a whitish fuzzy film on the abaxial side of their leaves (Fig. 14).

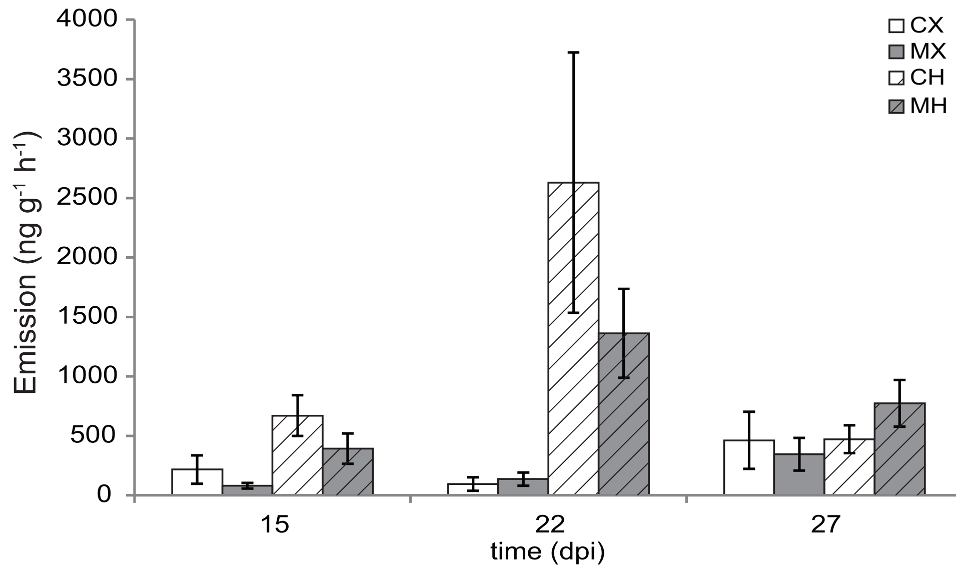


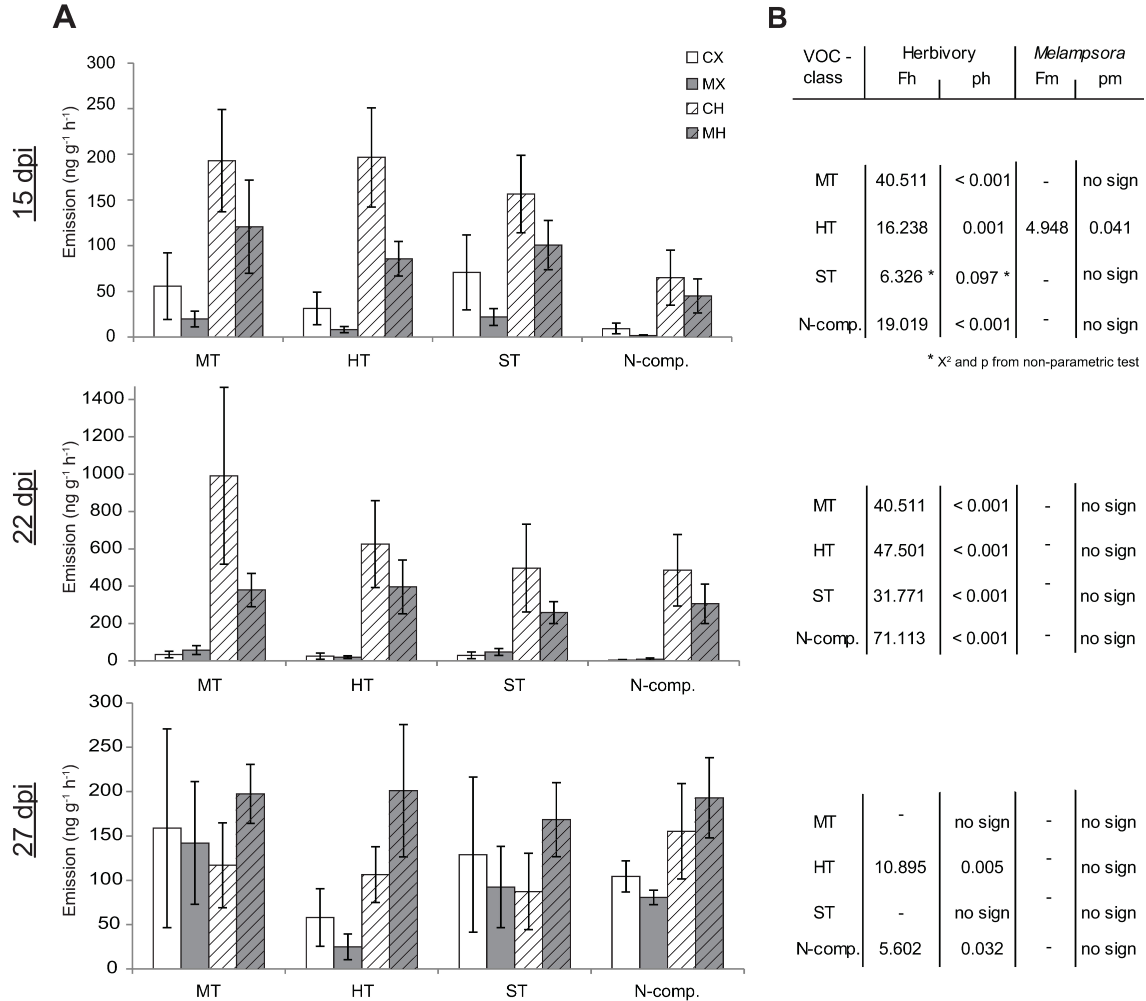
Figure 4: Total volatile emission from *P. nigra* infested with *M. larici-populina* and *L. dispar* alone and in combination. Volatiles were collected 15, 22 and 27 days postinoculation (dpi) and herbivores were allowed to feed for 48 h before the VOC collection. CX - control group, MX - *M. larici-populina* infection, CH - herbivory, MH - combination. Data are shown as mean with SEM (n= 5; except of 22 dpi CX and MX (n= 6) and 27 dpi CX (n= 4)). Significant differences between the treatment-groups were found at 15 dpi (Fh = 9.595; ph = 0.007) and 22 dpi (Fh = 43.970; ph < 0.001; see chapter 3.8 for indices).

The identity of this "film" could not be elucidated, but it seems to be the hyphal network or spores of another fungus, which could parasitize the basidiomycete *Melampsora*. Possible candidates for such a hyperparasitism are *Cladosporium* species which are known to parasitize several rust fungi [113, 93] or *Sphaerellopsis filum* which was also reported to grow on *Melampsora* [79]. Focusing on the plant, it was noticeable that necrosis already started in some of the *M. larici-populina* infected trees. Whether this was caused by an advanced rust infection or hyperparasitism remains unclaer. In any case, this might have influenced the volatile emission from the leaves in a quantitative way since there is less living tissue available for emission, and maybe also in a qualitative manner.

2.3.2 Major Classes of Volatiles and Individual Compounds

The total volatiles can be splitted into distinct compound classes, namely green leaf volatiles (GLVs), monoterpenes (MT), homoterpenes (HT), sesquiterpenes (ST),

nitrogen containing compounds (N-compounds), aromatic compounds and "others" for those which do not fit in either of the groups. In Fig. 5 a selection of the most abundant VOC-classes and how they are emitted after 15, 22 and 27 dpi from *P. nigra* 169-genotype is shown.



VOC - class	Herbivory Fh	ph	*Melampsora* Fm	pm
15 dpi				
MT	40.511	< 0.001	-	no sign
HT	16.238	0.001	4.948	0.041
ST	6.326 *	0.097 *	-	no sign
N-comp.	19.019	< 0.001	-	no sign
22 dpi				
MT	40.511	< 0.001	-	no sign
HT	47.501	< 0.001	-	no sign
ST	31.771	< 0.001	-	no sign
N-comp.	71.113	< 0.001	-	no sign
27 dpi				
MT	-	no sign	-	no sign
HT	10.895	0.005	-	no sign
ST	-	no sign	-	no sign
N-comp.	5.602	0.032	-	no sign

Figure 5: Emission of volatiles from *P. nigra* after single or combined infestation by *M. larici-populina* and *L. dispar* , classified in major compound groups. Volatiles were collected 15, 22 and 27 days postinoculation (dpi) and herbivores were allowed to feed for 48 h before the VOC collection. CX - control group, MX - *M. larici-populina* infection, CH - herbivory, MH - combination. MT - monoterpenes, HT - homoterpenes, ST - sesquiterpenes, N-comp. - nitrogen containing compounds. **A**: values of the compounds belonging to the same class were added for each replicate and from these the mean and SEM (n = 5, except of 22 dpi CX, MX (n = 6), 27 dpi CX (n = 4)) were calculated. **B**: statistical analyses belongig to the graphs in A, using two-way ANOVA (factors 'herbivory' and '*Melampsora* infection') if not indicated differently. no sign - p-value > 0.05.

As already observed in the total emission, plants measured at 22 dpi exhibit the quantitatively highest emission and also the highest diversity in volatile compounds

detected (App., Table 9). Both 15 and 22 dpi data show a clear and significant induction upon herbivore feeding (Fh > 16; ph ≤ 0.001 for all four classes), whereas the effect of herbivory at 27 dpi only was significant for HT (Fh = 10.895; ph = 0.005) and N-compounds (Fh = 5.602; ph = 0.032).

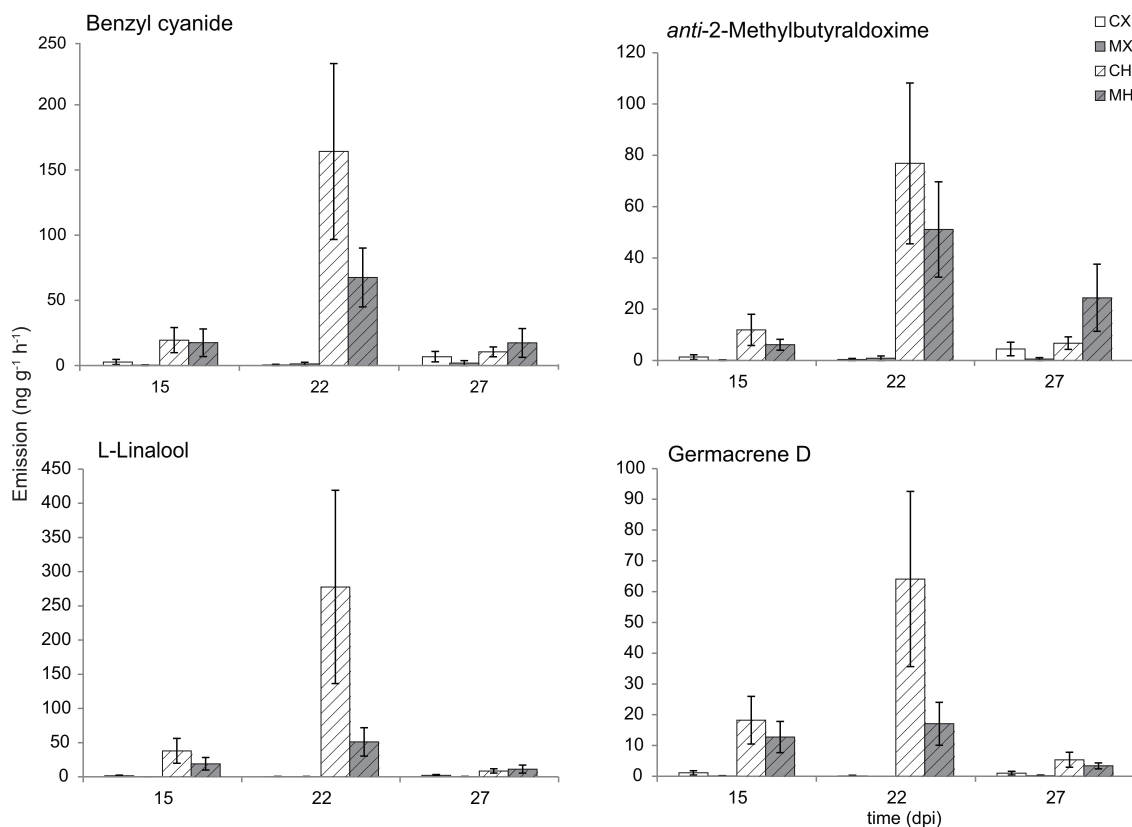


Figure 6: Emission of individual volatile compounds from *P. nigra* infested with *M. larici-populina* and *L. dispar* alone and in combination. Volatiles were collected 15, 22 and 27 days postinoculation (dpi) and herbivores were allowed to feed 48 h before the VOC collection. CX - control group, MX - *M. larici-populina* infection, CH - herbivory, MH - combination. Shown are examples of selected VOC classes: benzyl cyanide (aromatic compounds), *anti*-2-methylbutyraldoxime (N-compounds), L-linalool (MT), germacrene D (ST). Data are shown as mean with SEM ($n = 5$; except of 22 dpi CX, MX: $n = 6$; 27 dpi CX: $n = 4$). Results of the statistical tests for significance are shown in Table 2.

Some individual compounds show a significant herbivory dependence throughout all time points (Table 2), such as benzyl-cyanide, *anti*-2-methylbutyraldoxime, linalool or germacrene D (Fig. 6), which are examples for aromatic compounds, N-compounds, MT and ST, respectively. These four compounds were chosen for visualization and description since they had high ranks in the Random Forest statistical classification (App., Table 6) when all time point-datasets were joined. 4,8-Dimethyl-1,3,7-nonatriene (DMNT) and 1-octen-3-ol, which got the highest ranks, are depicted as HT in Fig. 5 or in Fig. 8, respectively.

Table 2: Statistical analysis on emission of volatile compounds from *P. nigra* trees infested with *M. larici-populina* and *L. dispar* alone and in combination as shown in Fig. 6. For testing significance two-way ANOVA using the factors 'herbivory'(Fh, ph) and '*M. larici-populina* infection' was used if not indicated differently. No significant levels were found for the factor '*M. larici-populina* infection' or interaction of both factors.

	15 dpi		22 dpi		27 dpi	
Compound	Fh	ph	Fh	ph	Fh	ph
Benzyl cyanide	16.804	0.001	87.813	<0.001	7.847	0.013
anti-2-MBA	19.424	<0.001	16.793*	0.001*	9.670	0.007
L-Linalool	10.561*	0.014*	17.160*	0.001*	11.441	0.004
Germacrene D	19.640	<0.001	18.411*	<0.001*	6.863	0.019

* = X^2 and p from non-parametric test

A herbivore induced increase of VOCs, especially MT, but also HT, ST or aromatic compounds, was already reported for a variety of different plant-herbivore systems. Among others linalool, DMNT and germacrene were found to be induced in hybrid poplar trees infested by the forest tent caterpillar [3]. An augmented emission of terpenoids was also found in poplars attacked by the gypsy moth *L. dispar* [45], as it was used in this experiment as well. The nitrogenous methylbutyraldoximes were shown to be induced in Manchurian ash upon herbivory or application of methyl jasmonate [107]. However, indole emission was shown to be enhanced in maize [44] or rice [141], but the effects in perennial species are not studied in detail. While there is relatively much known about the terpenoids, little is reported about the minor group of N-compounds. Nevertheless, especially these not so abundant volatiles might play an important role in the information content of a volatile blend [21] and should therefore be studied further.

In the first two time point experiments there is additionally a trend for repression of the herbivore-induced emission by the pathogen infection, as already described in the total volatile emission. For HT, which are exclusively represented by DMNT, this trend was found to be significant (Fm = 4.948; pm = 0.041). With a higher replicate number and less contamination of the plants (also at 22 dpi few spider mites were already observed) the variability in the data might be reduced and a pattern concerning the influence of rust infection will be more clear.

A similar trend of reduced emission due to the pathogen can be seen in the constitutive (not herbivore induced) volatiles at 15 dpi (Fig. 5). A clear pattern concerning this repression show plants which were measured at 8 dpi (Fig. 7A), that is time of the first occurence of the orange coloured uredinia. Before joining these data (from 15 and 8 dpi) to a conclusion it has to be mentioned that poplars measured at 8 dpi belong to another genotype (41, instead of 169) and hence differ in volatile composition. Additionally, it is not known whether or not there is a difference in susceptibility to the pathogen, which might explain a stronger effect of the *M. larici-populina* infection. Beside the differences between the plants, the

clear reduction pattern could also be the result of the earlier time point of sampling. On willow, a very specific temporal dynamic pattern was found for volatile emission after inoculation with *Melampsora epitea*: fatty acid derivatives (mainly GLVs) as well as ocimene and ST had an emission peak at the first occurence of urediniospores (6-7 dpi) and the onset of necrosis (12 dpi) [121]. Even though this pattern is not consistent with that observed in this project, it shows that the temporal progress of infection causes differences in volatile emission from the plant.

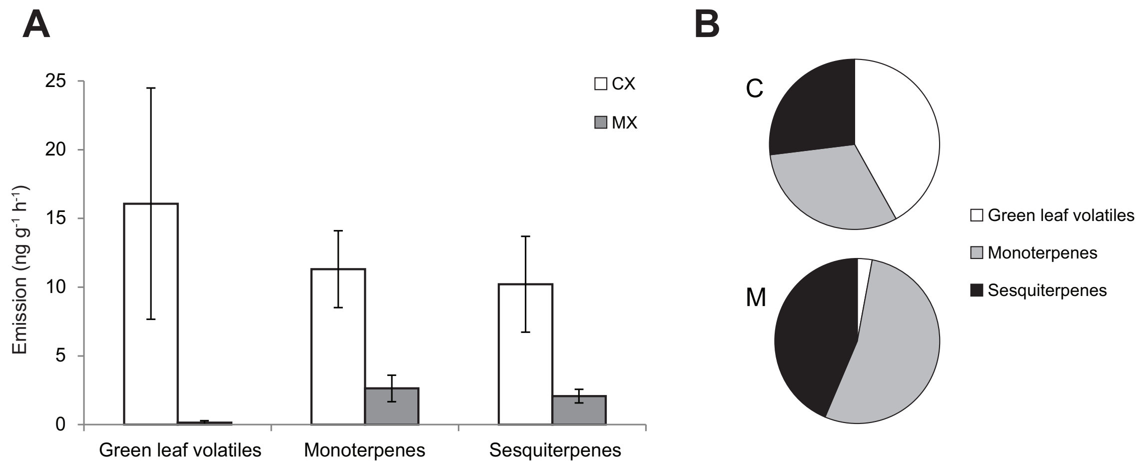


Figure 7: Emission of volatiles from *M. larici-populina* infected *P. nigra* trees compared to uninfected controls, classified in different compound groups. Volatiles were collected 8 dpi from genotype 41 poplar trees. C - control group, M - *M. larici-populina* infection. **A**: Values of the compounds belonging to the same class were added for each replicate and from these sums the mean and SEM (n = 6) were calculated. One-way ANOVA revealed significant differences for all VOC classes: green leaf volatiles (F = 7.843; p = 0.019), monoterpenes (F = 12.622; p = 0.005), sesquiterpenes (F = 9.931; p = 0.010). **B**: Composition of the consitutive volatile blends from poplar trees with (M) and without (C) *M. larici-populina* infection.

Since the drop in emission is not equal in all classes, the composition of the volatile blend also changes (Fig. 7B). Such a difference in composition may have an impact on other organisms interacting with the poplar tree. Even if it could be shown that individual compounds have effects on herbivores as well as on their enemies [30], [50], there are many indications that insects can obtain more information out of a volatile bouquet with a certain composition [66, 129, 22]. Considering this fact and the observation that induced as well as constitutive volatile emission is impaired by the rust disease, the consequences of a *M. larici-populina* infection might be more complex than the simpe fact that the fungus absorbs nutrients from the plant. With taking into account that the volatile blend, that is emitted by a plant and affected by pathogen infestation, is necessary for interaction with herbivores, parasitoids or carnivorous predators, this topic reaches another trophic level.

2.3.3 Fungus Specific Compounds

The comparison of the VOC-bouquet of *M. larici-populina* infected and control plants also showed a qualitative difference. One volatile compound with a retention time of ca. 7.8 min during gas chromatography was exclusively present in the samples where the fungus was present. Small amounts of it that were found in the control group at the late time point (27 dpi) are considered to originate from the *M. larici-populina* contaminations described in chapter 2.1 . Analysis by mass spectrometry and matching this spectrum to data bases revealed this compound to be oct-1-en-3-ol. As the emission of 1-octen-3-ol is, in contrast to the other volatiles mentioned yet, constantly increased with time and correlates with the pathogen abundance (Figure 8) it is assumed that this compound is produced by the fungus itself.

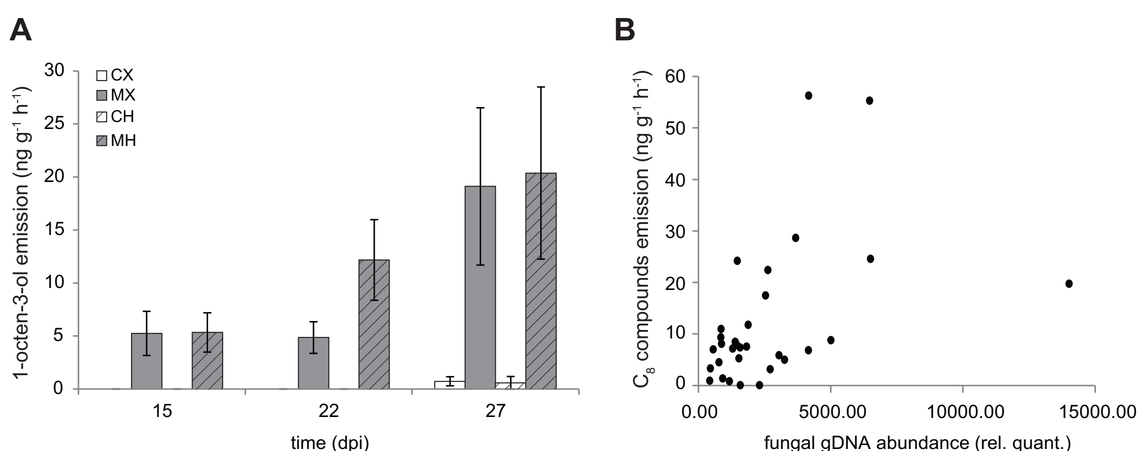


Figure 8: Pathogen specific volatiles emitted from *M. larici-populina* infected *P. nigra* trees. **A**: emission of 1,3-Octenol at different time points after inoculation (dpi - days postinoculation). CX - control group, MX - *M. larici-populina* infection, CH - herbivory, MH - combination. Data are shown as mean with SEM (n = 5; except of 22 dpi CX, MX: n = 6; 27 dpi CX: n = 4). **B**: correlation of emission of the putative fungal volatiles (C_8 compounds) 1-octen-3-ol and 3-octanone (only abundant at 27 dpi) with pathogen abundance: R (Pearson) = 46.2%, p = 0.009; data were obtained from the MX and MH groups (n = 31).

Another C_8 compound, also identified by mass spectrum data base search, is 3-octanone. This ketone was only present in samples taken at 27 dpi from *M. larici-populina* infected plants and could therefore probably derive from the whitish "film" that was discussed to be a hyperparasite of *Melampsora*.

Both C_8 derivatives are commonly present in the volatile bouquet of fungi [43] and especially 1-octen-3-ol contributes most to the typical "mushroom odor [43, 119]. It could be shown that insects are able to react to 1-octen-3-ol: the mycophagous ladybird beetle *Psyllobora vigintimaculata* is attracted by this volatile [118] as well as some generalist beetles which also feed from fungi [47]. How non-mycophagous

insects behave towards 1-octen-3-ol is not studied so far but is necessary to know for better understanding the interaction between a fungal plant pathogen, its host and herbivores feeding on that host. Future work could therefore use choice-experiments instead of forcing the caterpillars to feed on an infested plant.

1-Octen-3-ol was also reported to affect the plant's fitness. It could be shown by Splivallo *et al.* [116] that it leads to inhibited root growth as well as bleaching in *Arabidopsis* seedlings. Additionally, they found increasing reactive oxygen species in the plants. If also poplar trees would exhibit oxidative stress due to the fungus-derived volatile, this would lead to the activation of several antioxidative processes, inlcuding isoprene emission [111]. A depletion of the isoprene pool would hence lead to less substrates for the biosynthesis of terpenoids. This could explain the drop in terpenoid emission in the infected plant compared to the control plants.

2.3.4 Temporal Dynamics of Volatile Emission

Plants are known to respond to the circadian rhythm of light and dark and/or the varying temperature. Prominent examples for processes regulated by diurnal rhythm are leaf movement, stomatal or flower opening [92]. However, also volatile emission is affected by the rhythm of day and night [27] and temporal changes may be important for biotic interactions. Volatiles induced by herbivory have a higher information content ('herbivore presence') to the herbivore's enemies than constitutively emitted ones. Additionally, VOCs which are released independently from the circadian rhythm are able to attract both diurnal and nocturnal herbivore-predators. The temporal dynamics of *M. larici-populina* -infected and uninfected *P. nigra* trees (genotype 41) was investigated in a small experiment using only 3 replicates per group. Due to the small number of replicates, no detailed statistical analysis such as ANOVA was done.

The volatiles of poplars infected with *M. larici-populina* as well as uninfested control plants were collected 22 dpi over a timeperiod of 48 h. These 48 h were splitted into 24 h of constitutive emission (no caterpillars) and 24 h of herbivory (caterpillar feeding). The filters were changed every 4 h (four times during the light- and two times during the dark period) and so it was possible to determine the diurnal rhythm of volatile emission.

It is important to mention that the peak areas of 3-(Z)-hexenol and *syn*-2-methylbutyraldoxime overlapped in the chromatograms. Thus, a quantification of single compounds was not possible and it is assumed that the given quantity reflects a mixture of both. But still it remains unclear which proportions these compounds have within the depicted amount.

The constitutive emission was mainly restricted to cyclic monoterpenes such as camphene (Fig. 13), but also two GLVs and benzaldehyde, an aromatic compound, were present in the volatile blends of the first 24 h period. Nearly all of the 33 identified plant volatiles were induced upon caterpillar feeding. Some of them, especially GLVs (3-(Z)-hexenol, 3-(Z)-hexenyl acetate) and 2-methylbutyraldoximes

(Fig.9), but also the cyclic monoterpenes limonene and camphor, were increased immediately after initiation of the herbivory. On the other hand, most of the measured volatiles such as terpenoids, the aromatic compounds benzyl cyanide and indole were induced much later, 20 h or more after the onset of feeding.

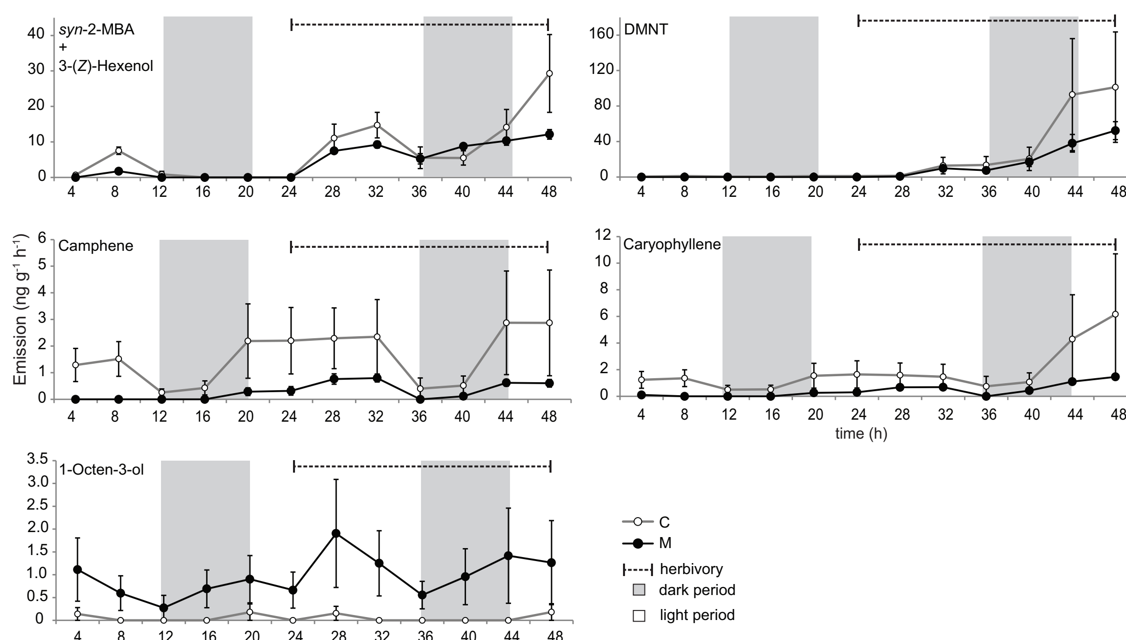


Figure 9: Volatile emission from *P. nigra* trees over 48 h. Volatiles were collected every 4 h, starting with 10 a.m. = 4 h after beginning of the light period. C - control group, M - *M. larici-populina* infection. The first 24 h plants were not treated, but caterpillars were added after 24 h to induce herbivore stress. Examples of different VOC classes are shown: *syn*-2-methylbutyraldoxime (*syn*-2-MBA; N-compound) + ((*Z*)-3-hexenol (green leaf volatile), 4,8-dimethyl-1,3,7-nonatriene (DMNT; homoterpene), Camphene (monoterpene), caryophyllene (sesquiterpene) and 1-octen-3-ol (putative fungal compound). Data are shown as mean with SEM (n = 3).

These differences mainly derive from the biosynthesis and release of the VOCs. Whereas GLVs are products of a rapid enzymatic reaction, the cleavage of membrane lipids [4], [41], the biosynthesis of terpenoids consists of longer pathways including enzymes which are not constitutively present in the plant [24]. As a result, GLVs are also expected to be emitted much earlier than terpenoids. The emission of the remaining groups, namely aromatic and N-compounds, is not studied in so much detail yet. The presence of *M. larici-populina* in the inoculated plants could be verified macroscopically by occurence of the orange-colored uredinia on their leaves. Also the volatile blends show qualitative and quantitative differences as they were already seen in the time point experiments on *P. nigra* (genotype 169) plants. The fungal compound 1-octen-3-ol was emitted from the pathogen infested group but not

from the control plants. Its emission does not seem to follow any specific circadian rhythm but still is varying throughout the measured time period.

Volatiles emitted from the plants are either not affected by the fungus or, and that holds true for most of them, are tendentially repressed. To be more precise, this can be (1) reduction in both constitutive and herbivore-induced emission (e.g. *syn*-2-MBA + (*Z*)-3-hexenol, β-pinene), (2) complete absence of the compound in the constitutive blend (e.g. camphene, α-pinene, myrcene), (3) delayed onset of the induced emission (e.g. linalool, germacrene D, *anti*-2-methylbutyraldoxime) or (4) absence of the compound during the whole measured time period (borneol, indole).

These results have to be verified by repeating the same experiment with more replicates in order to get more statistical power for the evaluation of emission patterns. Moreover, the repression of volatiles and the delayed onset of induced emission could have an impact on the herbivores and their enemies using the volatile blends to find their host plant or prey, respectively. Field data illuminating the issue of bi- and tritrophic interaction would help to answer the question which influence a rust disease has in an ecosystem.

2.4 Phytohormones

Many processes in plants including growth, development or reaction to biotic and abiotic stress are regulated by phytohormones. Plants produce a variety of compounds acting as phytohormones, namely auxins, gibberellins, abscisic acid (ABA), cytokinins, salicylic acid (SA), ethylene, jasmonic acid (JA) and its derivatives, brassinosteroids, peptides and strigolactones [9]. In this study, only some of the mentioned compounds were analyzed by UPLC/MS/MS: SA, ABA, the JA-precursor *cis*-(+)-12-oxophytodienoic acid (OPDA), JA and its conjugates (+)-7-*iso*-jasmonoyl-isoleucine, (-)-jasmonoyl-isoleucine, as well as their degradation products hydroxyjasmonic acid, hydroxyjasmonoyl-isoleucin and carboxyjasmonoyl-isoleucine. Since the time point of sampling is just a snapshot, catabolic products can give hints to previous regulation process and broaden the picture on signaling pathways. Therefore, all jasmonates were summed up and are further referred to as "JA derivatives“.

OPDA did not show any significant differences between the four treatment groups CX, MX, CH and MH. In contrast, ABA exhibited a clear induction by herbivory at 15 dpi ($F_h = 4.667$; $p_h = 0.046$; Fig. 15). The same trend can be seen at 22 dpi, but not significantly verified as a pattern. However, ABA was not influenced by the fungus.

The defense signaling in plants that are attacked by herbivores or pathogens is still not elucidated in detail. Nevertheless, SA and JA, as well as ethylene, are identified to be key players in biotic stress signaling [9]. In this study, SA only showed significant changes in the dataset obtained from 27 dpi ($X^2 = 8.97$; $p = 0.03$), where an increase in fungus infected plants can be seen. In contrast, uninfected control

plants show a lower level (Fig. 10). A non-significant trend for induction can also be observed at 22 dpi between the not herbivore treated groups CX and MX, and at 15 dpi between the herbivore treated groups CH and MH.

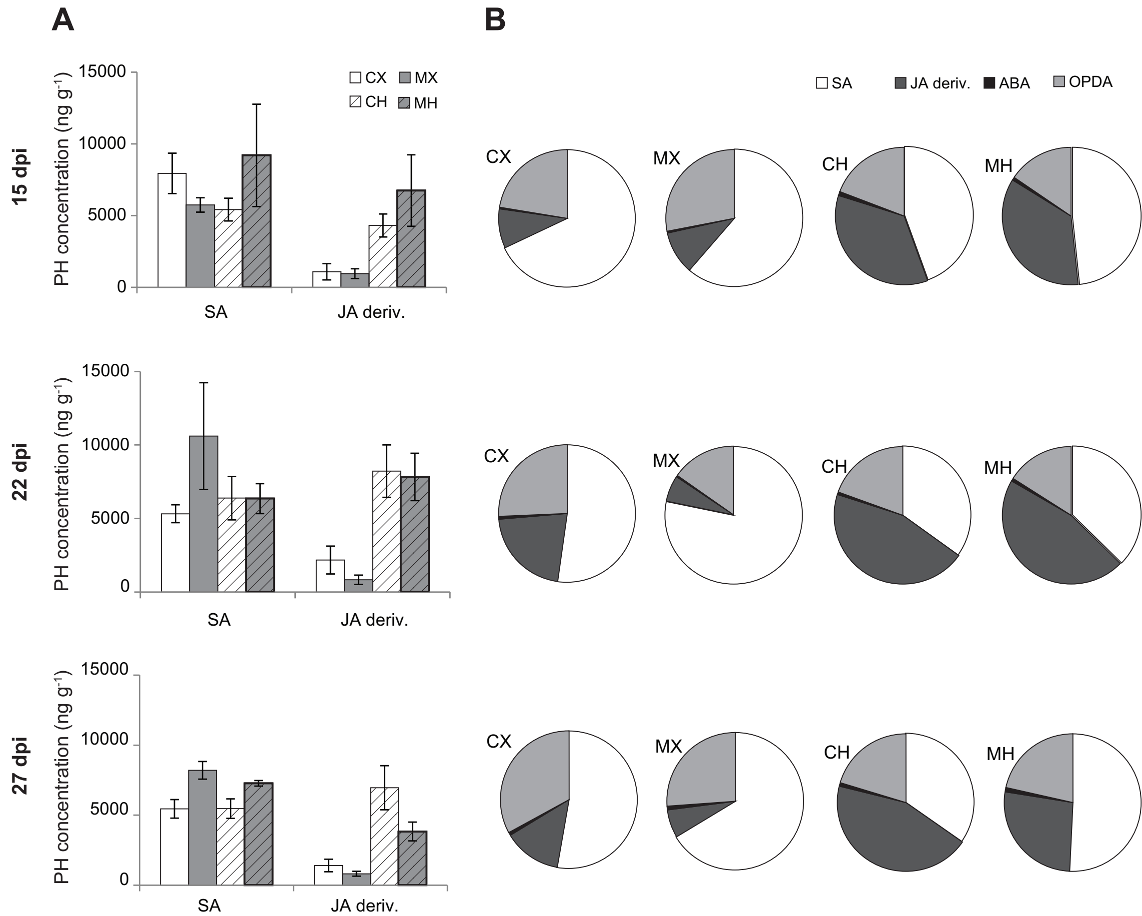


Figure 10: Phytohormone concentrations in *P. nigra* leaves infected with *M. larici-populina* and *L. dispar* alone and in combination. Leaves were sampled 15, 22 and 27 dpi immediately after VOC collection. CX - control group, MX - *M. larici-populina* infection, CH - herbivory, MH - combination. **A**: Concentration of salicylic acid (SA) and all JA derivatives (see text for details). Data are shown as mean with SEM ($n = 5$, except of 22 dpi CX, MX ($n = 6$), 27 dpi CX ($n = 4$)). Significant differences could be found 15 dpi: JA-deriv. ($X^2 = 11.617$; p = 0.009); 22 dpi: JA-deriv (Fh = 15.137; ph = 0.001); 27 dpi: SA ($X^2 = 8.970$; p = 0.030), JA-deriv. (Fh = 19.477; ph = 0.001). **B**: Proportion of the different phytohormones; SA - salicylic acid, JA-deriv. - jasmonic acid + derivatives, ABA - abscisic acid, OPDA - *cis*(+)-12-oxophytodienoic acid.

More obvious is the effect of herbivory on JA derivatives. A statistically verified induction of these phytohormones was found at all three time points 15 dpi ($X^2 = 11.617$; p = 0.009), 22 dpi (Fh = 15.137; ph = 0.001) and 27 dpi (Fh = 19.477; ph = 0.001). The biggest difference between caterpillar-infested and not infested plants could be seen 22 dpi, where JA derivatives are about four to nine times higher

among control (CX vs CH) or *M. larici-populina* -inoculated plants (MX vs MH), respectively.

The observed enhancement of JA derivatives is in accordance with many reports in the literature and underlines its importance as defense regulator against herbivores [137, 5, 48, 72]. JA is known to be upregulated upon wounding [60] and/ or feeding of arthropod insects [72] and to mediate the subsequent response. Part of this response reaction in poplars is the emission of volatiles in order to repel herbivores or to attract enemies of the herbivores [100]. A direct link between JA signaling and VOC emission was shown for many annual plants such as corn [110], rice [81], lima beans [31] or tobacco [136]. However, much less is known about JA signaling and its effects on volatile emission in perennial woody plants. An increase of VOCs in response to exogenously applied JA or methyl jasmonate was shown in *Quercus ilex* [42, 112], *Pinus sylvestris* [112], *Populus trichocarpa* [40] and *P. nigra* [21]. Furthermore, an augmented level of JA and JA-Ile [13] or induced expression of genes regulating the biosynthesis of or being responsive to JA was already reported for *Populus* species in response to *L. dispar* feeding or wounding [76]. During this Master's project the correlation between JA derivatives and volatile emission was statistically assessed showing significant correlations for terpenoids and GLVs (Table 3). These findings support the hypothesis, that in our model system the caterpillar feeding causes an increase in JA derivatives, which in turn activates and/ or enhances volatile emission.

Table 3: Correlation between jasmonate derivatives (JA-deriv.) and volatile emission from *P. nigra* (genotype 169) trees, taken together controls and *M. larici-populina* - and *L. dispar* - and combined infested plants. Volatiles are grouped into major classes and total emission. Correlation analysis was done using a two-tailed Pearson test (SPSS), $n = 61$.

Volatiles correlated with JA-deriv.	**Correlation coefficient**	**Significance level**
Total	0.466	< 0.001
Terpenoids	0.494	< 0.001
Monoterpenes	0.431	< 0.001
Sesquiterpenes	0.478	< 0.001
Homoterpenes	0.555	< 0.001
Green leaf volatiles	0.298	0.020
Aromatic compounds	- 0.230	0.8581
N-compounds	0.246	0.056
Fungus specific compounds	0.127	0.331

How the molecular signaling cascade from jasmonate to final emission is build up still needs further research. Fäldt *et al.* showed an up-regulation of the monoterpene synthase AtTPS03 in *Arabidopsis* in response to JA treatment [46]. Also further experiments in *Populus* could focus on expression of VOC biosynthesis genes and use exogenously applicated jasmonates in order to get a more precise picture of the

regulation of volatile emission by this phytohormone.

The unchanged or slightly decreased (27 dpi) levels of JA in *M. larici-populina* -infected plants compared to controls observed during this study are in contrast to the observations of Azaiez *et al.* [8]. They found in *Melampsora*-infected hybrid poplars an upregulation of genes involved in jasmonate and ethylene biosynthesis and genes responsive to JA. This might be due to a discrepancy between mRNA levels and metabolites or due to the fact that different species of poplars were used. Another reasonable explanation are the different time points (6 dpi in [8] vs 15, 22 or 27 dpi) which lead to contrasting results due to the different developmental stages of the fungus.

Another important phytohormone analyzed in this project is SA. It is known for its crucial role in plant defense against pathogens. So SA was reported to be induced by and mediate resistance against biotrophic or hemibiotrophic pathogens in several herbaceous species, e.g. *Arabidopsis* [28, 117], potato [55] or tobacco [61, 97]. In *Salix* SA was enhanced upon attack by a gall midge [96], indicating a role in the defense against herbivores in trees. However, no consistent or even significant pattern corresponding to caterpillar feeding could be seen in the data obtained throughout all three time points in poplars.

Further, only weak correlations could be seen between SA and volatile emission: the only significant changes in SA levels at all were observed at 27 dpi, which is in contrast the only dataset in which no clear pattern in VOCs could be found (Fig. 5). This contradicts the proposed connection between SA induction and enhanced volatile emissions in tobacco [61], and might therefore once more highlight the differences between herbaceous and woody plants. Germain and Segun [49] speculate that the role of SA in poplars, or trees in general, might not have any universal and conserved role in defense reactions.

Keeping in mind the often proposed - negative or positive - crosstalk (e.g. [29, 9, 117]), the ratio of SA and JA derivatives in the tissue might be of importance (Fig. 10B). A greater portion of SA in pathogen infested plants can be seen in MX at 22 dpi and in MX as well as MH at 27 dpi compared to their respective controls. Nevertheless, the correlation between the concentration of SA and JA derivatives is significant, but very weak ($R = -0.350$; $p = 0.006$).

However, the phytohormone levels itself are just a small part of the network controlling defense against pathogen or herbivores. In order to get a better knowledge on the regulation during a multiple attack, further experiments have to be carried out. These should include evaluation of the expression of SA- and JA-responsive genes, analysis of the phytohormone ethylene which is the third key player in plant resistance, and the order of pathogen and herbivore treatments might be reversed.

3 Methods and Material

3.1 Study Organisms

3.1.1 Plant Material

All stem cuttings used for growing the trees for this project were obtained from the MPI-CE-field station in Isserstedt, Jena. Poplar trees in this stand are genotypes derived from a natural black poplar population in North-Eastern Germany (Küstrin-Kietz Island, 52°34'1"N, 14°38'3"E). For the experiments where samples were taken at different time points *Populus nigra* (*P. nigra*) trees (female, clone 169, 40-80 cm tall, 1.5 yr old) from stem cuttings were grown under natural light conditions in the greenhouse (ca. 14 h photoperiod at time point of inoculation, 22°C, watered 2 x/d, humidity 60% ± 5%) in a 1:1 mixture of sand and soil (Klasmann potting substrate). 3 d before starting the herbivory treatment, plants were transfered in terms of acclimation to the growth chamber (16 h/8 h light/dark, 20°C/16°C day/night, 75 % humidity) where the VOC collection took place. Plants used for the 8 dpi-measurements and the 48h measurements (*P. nigra* , female, clone 41, 40-80 cm tall, 0.5 yr old) were grown in the climate chamber from cuttings under identical conditions than the previous batch of plants.

The time points indicated in the text refer to the day of sampling and VOC-collection. In the case of "8 dpi" there were two batches of plants whose data were pooled in the end. Both batches were VOC-collected at the day when urediniospores occured on the inoculated leaves (8 dpi and 9 dpi, for the first and second batch, respectively), but are all referred to as "8 dpi" in the text.

3.1.2 Pathogen

Urediospores of *Melampsora larici-populina* were harvested from infested *P. nigra* trees, located in the field station in Isserstedt, 4 weeks before use. They were dried over silica under vacuum overnight and stored at RT in the desiccator.

3.1.3 Herbivores

To induce herbivore stress larvae of the European gypsy moth *Lymantria dispar dispar* were used. Egg batches were kindly provided by Melody Keena (USDA Forest Service, Hamden, USA) and the caterpillars were reared on an artificial diet (Gypsy moth diet, MP Biomedicals LLC, Illkirch, France) until the feeding experiments.

Caterpillars of the third, fourth and fifth instar were allowed to feed on four to 14 leaves per tree (500 cm^2 ± 150 cm^2 leaf area), using the same number of caterpillars per instar for each tree. For the treatment either eight larvae of the fourth instar (15 dpi-,27 dpi-, 48 h-measurements) or nine + one larvae of the third + fifth instar, respectively (22 dpi), were used per plant. A gauze was installed around these

leaves with cable ties to prevent spreading of the caterpillars. Control plants also received a gauze to exclude effects caused by different light conditions. After one (48 h experiment) or two days (time point measurements) of feeding the larvae were removed and their frass collected and weighed. The insects were weighed before and after feeding in order to determine weight gain or -loss. The feeding damage was analyzed from pictures of the treated leaves (see Chapter 3.4).

3.2 Experimental Design

The poplar trees were divided into four treatment groups (Figure 11): (1) control group that was not infected with rust disease and not herbivory treated ("CX"); (2) rust disease group that was infected with *M. larici-populina* but not infested by insects ("MX"); (3) herbivore group that was not inoculated with rust fungus spores but treated with the herbivorous *L. dispar* larvae ("CH"); (4) rust-herbivore group that was infested by both rust fungus and herbivores ("MH"). All plants were analyzed for VOCs, phytohormones and the fungal infection was quantified by the amount of genomic DNA (gDNA) in the 15, 22 and 27 dpi samples.

Two experiments were conducted: a time point experiment and a long-term experiment. For the time point measurements, herbivore stress was induced directly before VOC measurements and sampling, except for the 8 dpi measurements where no herbivore stress was induced. In the long-term experiment VOCs were collected from plants which were not herbivore-treated for 24 h and subsequently stress with caterpillars the following 24 h.

3.3 Inoculation Procedure

To determine the amount of germinating spores, 1 mg of spores was dissolved in 1 ml H_2O-agar (0.1 g l^{-1}) and 200 µl of different dilutions (1:2 to 1:512) were plated onto agar (16 g l^{-1}). After incubation at RT in the dark for two days, total and germinating spores were counted under a light microscope (10 x zoom, Figure 12).

An inoculum with ca. 95,000 spores (19,000 germinating) ml^{-1} was prepared by dissolving 430 mg spores in 400 ml distilled H_2O. All leaves were spray inoculated on their abaxial side using a spray flask (commercially available, without filter); control plants were sprayed with H_2O only. After inoculation, each plant was kept in a closed plastic bag ("Bratschlauch", Toppits, Minden, Germany; 100 x 30 cm) which was opened on the upper side after two days. Plants were sprayed with water and monitored daily until occurence of urediniospores.

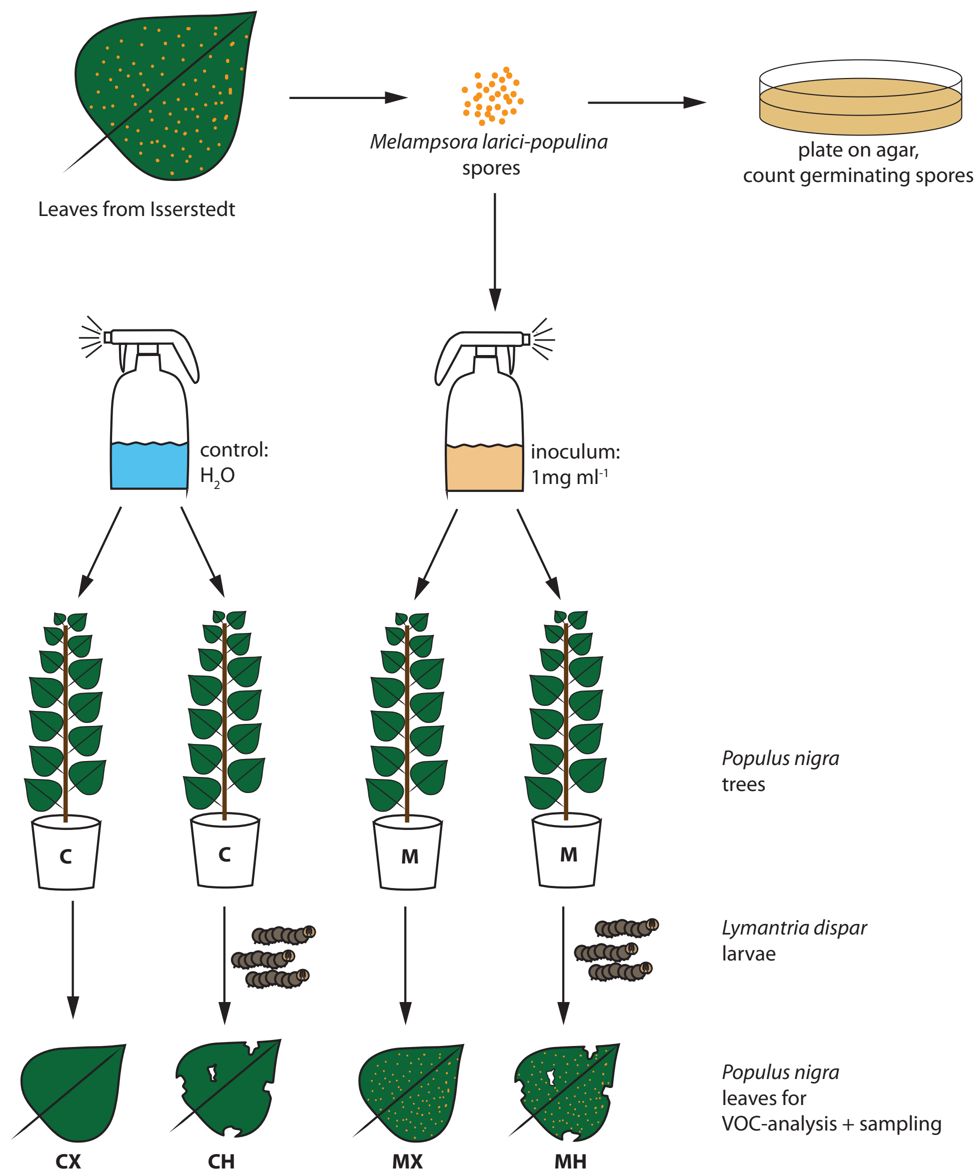


Figure 11: Experimental setup. *M. larici-populina* spores were collected from infested trees from the field station in Isserstedt. The dried spores were plated onto agar plates in order to count the germinating spores. The inoculum was prepared by dissolving spores in H_2O (ca. 19,000 germinable spores ml^{-1}) and was sprayed onto the abaxial side of each leaf of *P. nigra* trees (M). For controls (C), H_2O only was used. Each group was subdivided into two subgroups which were either treated with herbivores (H) or not (X). Leaves were analyzed for VOCs, fungal gDNA and phytohormones.

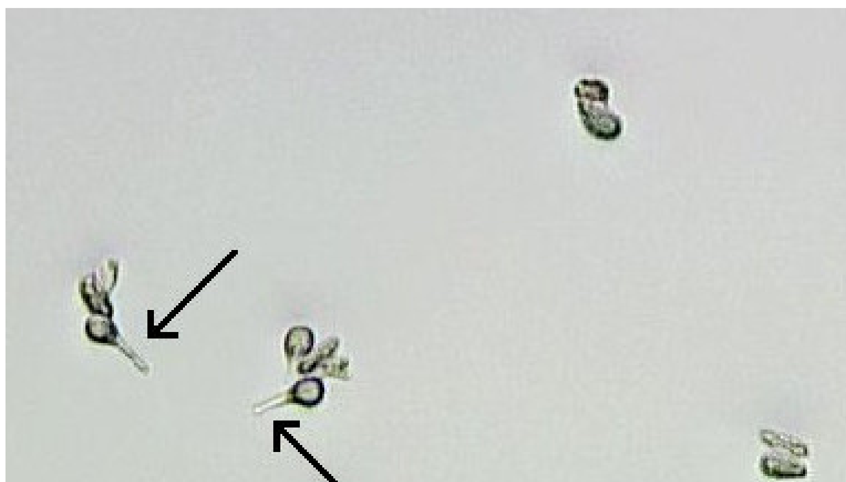


Figure 12: Urediniospores of *M. larici-populina* on agar after incubation at RT in dark for two days (zoom 10x, 1:4 dilution of spore solution 1 mg ml^{-1}). Arrows indicate germinating spores.

3.4 Sampling

After VOC collection, the treated leaves were removed and a picture was taken on a white plate containing a 4 cm^2 normalization field. Using “GNU Image Manipulation Program (GIMP 2.8.2, free license) the feeding damage was determined by reconstructing the leaf and converting the pixel number into cm^2 by making use of the 4 cm^2 field in the picture.

Subsequently, damaged and undamaged leaves were sampled separately. In order to do this, the midrib of each leaf was removed and two aliquots consisting of the alternating left or right part of the leaves were frozen in liquid nitrogen. One of the aliquots was stored at -80°C for potential further molecular biological analyses, whereas the other one was lyophilized and then ground using a paint shaker (Skandex SO-10m Shaker, Fluid Management Europe, Sassenheim, The Netherlands) using 10 steel beads per sample.

3.5 Quantification of Fungus Infestation by qRT-PCR

Fungus infection was determined by quantitative real-time polymerase chain reaction (qRT-PCR) using primers to amplify the internal transcribed spacer region between the 18S rRNA and the 5.8rRNA of *M. larici-populina* (ITS-MLP; GenBank accession AY375268) as marker for *M. larici-populina* genomic DNA (gDNA). Actin (GenBank accession GQ339771) from *P. nigra* gDNA was used as internal standard to express relative quantity of *M. larici-populina* in *P. nigra* leaves. The specifity of the primers was verified using melting curve analysis and agarose gel electrophoresis (2% agarose gel + ethidium bromide (0.08 µl ml^{-1}), 100 V, 30 min).

DNA Extraction

About 50 mg freeze-dried ground tissue per sample was mixed with 500 µl extraction buffer (100 mM TRIS pH = 8.0, 10 mM EDTA, 2% (w/v) SDS) and 100 µl Proteinase K (from *Tritirachium album* (Sigma-Aldrich, USA); 1 µg µl^{-1}) and incubated for 1 h at 60°C with constant agitation. 180 µl NaCl (5M) and 80 µl CTAB (10% (w/v)) were added to the reaction mixture and incubated for 10 min at 65°C. After adding 860 µl chloroform, the tubes were vortexed and kept on ice for 30 min. Afterwards, the mixture was centrifuged for 10 min at RT with maximum speed (21130 rcf, 5424R centrifuge, Eppendorf AG, Hamburg, Germany) and 700 µl of the aqueous phase were transfered into a new 1.5 ml tube. Thereafter, 385 µl isopropanol (ice-cold) was added, samples were incubated for 1 h at -20°C and subsequently centrifuged for 20 min at 4°C with maximum speed. The pellet was washed with 750 µl ethanol (70% in H_2O), centrifuged for 10 min at RT at maximum speed, dried at RT and dissolved in 50 µl MilliQ-H_2O overnight.

DNA was quantified using "NanoDrop2000c Spectrophotometer" (peqlab Biotechnology GmbH, Erlangen, Germany). The DNA solution was stored at -20°C until further use. Impure DNA (260/230 nm or 260/280 nm < 1.9) was further purified by making use of the DNeasy Plant Mini Kit (Quiagen, Hilden, Germany), following the manufacterer's instruction manual from step 2.0.

qRT-PCR

For qRT-PCR analysis the DNA concentration was adjusted to 100 ng µl^{-1}. *Actin2* specific primers [104] were used for normalization and *ITS-MLP* specific primers designed from genomic *M. larici-populina* DNA were used to quantify fungal DNA. The reaction mixture (Table 4) contained "Brilliant III Ultra-Fast SYBR Green QPCR Master Mix" (Agilent, Santa Clara, USA) with ROX as reference dye (Agilent, Santa Clara, USA). The PCR was performed using the Mx3000P Real Time PCR System (Agilent, Santa Clara, USA) using the same program throughout all experiments (Table 5).

Table 4: Reaction mixture for one qRT-PCR reaction, using "Brilliant III Ultra-Fast SYBR Green QPCR Master Mix" (Agilent , Santa Clara, USA).

Reagent	**Volume (µl)**
MilliQ-H_2O	7.2
SYBR Green Master Mix	10
ROX dye (1:500 in MilliQ-H_2O	0.3
Primer fwd (0.1 mM)	0.75
Primer rev (0.1 mM)	0.75
DNA (100 ng µl^{-1})	1
Total volume	*20*

The analysis of the qRT-PCR data was done with the "MxPro" Software (Agilent, Santa Clara, USA) using the "Comparative qRT-PCR" mode. As calibrator for all plates 1 DNA sample from an uninoculated plant was used and data were normalized to that calibrator in order to gain relative quantity values.

Table 5: Programme for qRT-PCR using Mx3000P qRT-PCR cycler (Stratagene).

Step	Number of iterations	Temperature	Time
Denaturation	1	95 °C	3 min
Amplification	45	95 °C	15 s
		60 °C	60 s
Denaturation	1	95 °C	60 s
Melting curve	1	53 °C	30 s
	(gradient)	95 °C	15 s

3.6 Volatile Analysis

For volatile collection in the time-point experiment plastic bags ("Bratschlauch", Toppits, Minden, Germany; 60 x 30 cm) were wrapped around the herbivory-induced leaves or the respective parts of the control plants directly after removal of the insects. During the 48h long-term experiment both a gauze and a Bratschlauch (60 x 30 cm) were installed on each plant (Figure 13) and the filters were changed every 4h, starting at 2.00 p.m.

A push-pull system as described by [120] was used for 4.0h (long-term experiment) or 3.5 h volatile collection (10.30 - 14.00 h in time-point experiment): charcoal filtered air was pumped in with 1 l min^{-1} through Teflon tubes and 0.6 l min^{-1} of the headspace was withdrawn through a volatile trap (filter packed with 20 mg SuperQ material; ARS Inc., Gainsville, USA). The filter was eluted 2 x with 100 µl dichlormethane containing the internal standard (IS) nonyl acetate (10 ng µl^{-1}).

VOCs were then qualitatively and quantitatively analyzed by gas chromatography (GC; injection: 1 µl splitless, flow: 2 ml min^{-1} constant, temperature: 45°C to 180°C with 6°C min^{-1} and to 300°C with 100°C min^{-1}) coupled to a flame ionization detector (FID; operated at 300°C) or mass spectrometer (interface temperature: 270°C, quadrupole temperature: 150°C, electron energy: 70 eV, scan mode with 4.49 scans/s, 33 - 350 m/z), respectively. The volatile blend was seperated with a DB-5MS column (30 m x 0.25 mm x 0.25 µm; Agilent, Santa Clara, USA) and 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 data bases (Wiley275, NIST98, Adams2205) using the PBM Quick Search in the ChemStation Software. Some compounds were identified in previous experiments

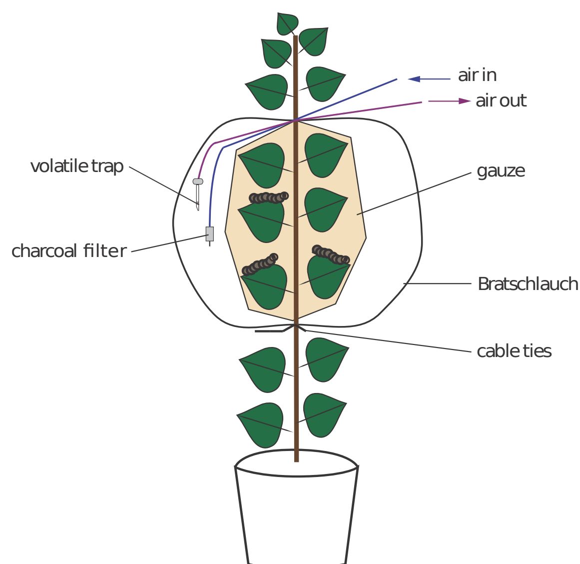


Figure 13: Volatile collection during the longterm experiment. Filters used as volatile traps were changed every 4 h. The gauze was installed at the beginning of the experiment to exclude effects due to changing light conditions. After 24 h caterpillars were added to the plants and the volatiles were collected for another 24 h.

based on authentic standards [24]. The amount of each compound was determined from the GC-FID data based on the peak area in relation to the IS-peak area considering the relative response factor taken from [24]. Then, the emission was calculated by dividing by the freshweight of the measured leaves (g) and the time period of VOC collection (h).

3.7 Phytohormone Analysis

For phytohormone (PH) analysis 10 mg freeze-dried ground tissue was mixed with 1 ml methanol containing PH-IS (D_6-ABA, D_2-9,10-dihydro-JA, D_4-SA (10 µg ml^{-1} each; Santa Cruz Biotechnology, Texas, USA) and ^{13}C-JA-Ile (2 µg ml^{-1}, synthesized as described in [73] using ^{13}C-Ile, Sigma Aldrich, St. Louis, USA) by shaking 30 s on a paint shaker (Skandex SO-10m Shaker, Fluid Management Europe, Sassenheim, The Netherlands). After centrifugation for 2 min with 4300 rpm (4220rcf; Avanti J-20XP centrifuge, Beckman Coulter GmbH, Krefeld, Germany), 400 µl of the supernatant were transfered to a new tube and the pellet was extracted again with 1 ml pure methanol. Both extracts were pooled and kept at -20°C until further use.

Quantification of the PHs was done by an API 3200 UPLC/MS/MS system (API Sciex, Darmstadt, Germany) with scheduled Multiple Reaction Monitoring and parameters were set as described in [126]. Compounds were quantified based on the peak area compared to the area of their corresponding IS. For quantification of *cis*-OPDA, D_2-9,10-dihydro-JA was used as IS.

3.8 Statistical Analyses

All data shown in figures and tables are means and standard errors derived from different plants within one treatment group. The means were statistically compared by two-way ANOVA (using 2 factors: Melampsora infection (C, M), herbivory (X, H)) or Kruskal-Wallis test (using four factors: CX, CH, MX, MH) in case of homoscedasticity, if not indicated differently. Two-way ANOVA examined the influence of herbivory treatment (indicated as Fh and ph), *M. larici-populina* infection (Fm, pm) and interaction of both, whereas Kruskal-Wallis only shows a significant different between the four groups and is indicated as X^2 and the respective p-value (p). Correlations were assessed using a two-tailed Pearson test resulting in the coefficient coefficient (R) and significance level (p). All significance analyses were conducted using the software SPSS 22.0 (IBM Corporation, New York, USA).

Exponential regression (Fig. 2) was done with the software excel from Microsoft office 2010 (Microsoft Corporation, Redmond, USA).

To determine the importance of single compounds emittet by the plants in response to the treatments, a Random Forest analysis was conducted using MetaboAnalyst 2.0 [140, 139]. For this, 10 000 iterations and x predictors to try for each node (x being square root of number of variables) were used.

4 Conclusion and Outlook

Plant-pathogen or plant-herbivore interactions have been intensively studied in predominantly herbaceous plants such as *Arabidopsis*, rice or tobacco. However, studies about multiple attack on perennial plants are very rare. In two studies negative effects on the herbivore feedig and/or oviposition behavior caused by a previous rust infection were observed [98, 115], but consequences of a multiple attack for the tree have not been studied so far. Therefore, this Master's thesis focuses on the effect of single and combined infestation by a biotrophic fungus and a herbivorous insect on the volatile emission and phytohormone levels of the host plant *Populus nigra*. Beyond that, the effect of pathogen infestation on certain traits of the caterpillars were also monitored.

First of all it could be shown by qRT-PCR that the fungus *Melampsora larici-populina* successfully infected its host and grew exponentially. Further, in contrast to the literature, no effects could be observed on the herbivorous *Lymantria dispar* larvae: weight gain, frass weight and consumed leaf area did not differ between the control and the *M. larici-populina* infested plants. It is suggested that *L. dispar* as a generalist feeder is not as sensitive to changes in the provided nutrients as specialists. Therefore, comparing these data to another feeding experiment using a poplar specialist, e.g. *Laothoe populi*, would be very interesting. Further, other traits such as mortality, fecundity, and behavior of larvae or egg-laying females in choice-experiments, could be tested. Choice experiments could also be used to survey the behavior towards the fungus specific compound 1-octen-3-ol which could be identified within this project.

This C_8 compound which is presumably emitted by the fungus was shown to affect root growth and induce oxidative stress in *Arabidopsis* [116]. Whether or not 1-octen-3-ol has similar effects on poplar still has to be explored. Moreover, an oxidative burst as described in [116] could lead to isoprene emission and this in turn affects the isoprene pool needed for terpenoid production. Further studies should therefore examine the effects of purified 1-octen-3-ol on reactive oxygen species (ROS) levels and ROS scavenging enzymes, isoprene emission and a potential linkage to terpenoid biosynthesis in *Populus*.

The emission of volatile organic compounds (VOCs) at 15 and 22 days postinoculation (dpi) was found to be highly induced by herbivore feeding which corresponds to previous studies described in the literature. This induction can further be linked to the levels of jasmonic acid (JA) and its derivatives. This phytohormone was described to induce volatile emission in tree species when applicated exogenously [42, 40, 112, 21]. The correlation of volatile emission and *in planta* jasmonate levels supports the hypothesis of a direct regulation by phytohormones in trees. The influence of the fungal infection on the *P. nigra* genotype 169 volatiles was only significant for the homoterpene DMNT showing a repression of emission. This negative effect could also be observed for other compound classes in induced emission, but could not be verified statistically.

A problem during the late time points was contamination by other pests. For further experiments a contamination-free place should be chosen for growing the trees. Moreover, control and *M. larici-populina* -infested plants should be located in two separate rooms to avoid spreading of the fungal spores. However, the repression of volatile emission by the fungus was not only seen as trend (or pattern in case of DMNT) in genotype 169 but also as significant effect in genotype 41 at an earlier timepoint and during the longterm experiment 22 dpi. This could indicate a difference in pathogen susceptibility between the two genotypes with genotype 41 responding stronger to the fungus. Hence, genotype 41 might be more suitable to investigate plant-pathogen-herbivore interactions in future experiments. The phytohormone levels of fungus-infected plants did not show significant differences for jasmonates, indicating that JA is not involved in poplar's response to pathogen attack. Salicylic acid (SA), which was often described to mediate resistance towards biotrophs, did not show a constant pattern throughout the different time points. It was significantly induced at 27 dpi where no volatile patterns could be observed, but showed only trends of increase in the earlier timepoints. The (negative) correlation between the SA and JA derivatives was only weak and so neither a clear antagonism nor a synergism of both phytohormones could be confirmed. Before concluding from these data that phytohormones are not involved in or interact during response to multiple attacks in poplar, earlier time points should be evaluated concerning phytohormone levels and defense traits. Especially the first eight days after inoculation, when the pathogen develops haustoria, a hyphal network and urediniospores [53], might be more important to elucidate the host's response. Furthermore, quantification of the transcript levels of JA- or SA-responsive genes will provide information about another step between phytohormone levels and final output in terms of volatile emission. The last experiment in which the volatile emission over a period of 48 h was measured, revealed also a repression in emission by the fungus in different kinds: reduction in the VOC release from caterpillar-treated (induced) and non-treated plants (constitutive), abscence of a VOC during constitutive emission, delayed onset of emission after herbivory treatment, and abscence of a VOC at all. In order to verify these findings the experiment has to be repeated with a higher number of replicates to have more statistical power. Prolonging the measuring time could answer the question whether some herbivore-responsive volatiles were suppressed completely or just delayed due to the fungal infection.

Measurements eight dpi have revealed a change in composition of the volatile blends when the tree was infected with the fungus. This could have considerable effects on other organisms, either herbivorous species or natural enemies of the herbivores. These effects have to be explored to get an insight in the ecological consequences of the rust disease. This could include behavioral assays with herbivorous caterpillars, oviposition choice of female moths or attraction to parasitoids and carnivores as natural enemies of the herbivores.

When focusing on ecological consequences it should further be considered that the natural occurence of the two biotic stressors used in this project is reversed in nature.

While in this project *L. dispar* larvae were applied on the trees after rust infection, they would be the first pest to cope with for poplars in the field. Urediniospores of *M. larici-populina* instead occur in the summer and infect the host (*Populus*) until autumn, when the gypsy moth already undergoes pupation or the adult stage.

Further research conducted under controlled laboratory conditions as well as field experiments will help to get a better understanding of how plants tackle simultaneous attacks of pathogens and herbivores. Additionally, investigations on the effect on other trophic levels could give an insight into the ecological impacts of multiple infestations. From an economic point of view, research results regarding this study system could help to optimize pest management using pesiticides effectively against both biotic stressors, or provide molecular tools for breeding of more resistent poplar species.

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Table 6: Results of Random Forest Classification. All analyzed volatiles were ranked with their variable importance determined by Random Forest Classification using MetaboAnalyst and the first six compounds are shown. Importance was evaluated from all three datasets joined together (all time points) or singly for each time point (15, 22, 27 dpi). VOC - volatile organic compound, MDA - MeanDecreaseAccuracy, class. error - classification error, DMNT - 4,8-dimethyl-1,3,7-nonatriene, MBA - methylbutyraldoxime.

	all time points		**15 dpi**		**22 dpi**		**27 dpi**	
rank order	VOC	MDA	VOC	MDA	VOC	MDA	VOC	MDA
1	1-Octen-3-ol	0.1065	1-Octen-3-ol	0.1069	1-Octen-3-ol	0.0608	1-Octen-3-ol	0.0568
2	DMNT	0.0440	DMNT	0.0407	Benzaldehyde	0.0190	Benzaldehyde	0.0326
3	L-Linalool	0.0405	Germacrene D	0.0329	L-Linalool	0.0187	Octanone+Myrcene	0.0252
4	Germacrene D	0.0393	Benzyl cyanide	0.0170	*anti*-3-MBA	0.0177	*anti*-3-MBA	0.0153
5	*anti*-2-MBA	0.0289	*anti*-2-MBA	0.0159	d-Cadinene	0.0165	*anti*-2-MBA	0.0132
6	Benzyl cyanide	0.0288	L-Linalool	0.0155	Germacrene D	0.0165	L-Linalool	0.0115
class. error	CX	0.38	CX	0.20	CX	0.33	CX	0.75
	MX	0.18	MX	0.00	MX	0.33	MX	0.20
	CH	0.27	CH	0.40	CH	0.40	CH	1.00
	MH	0.33	MH	0.40	MH	0.60	MH	0.20

Table 7: Emission of volatile organic compounds 8 dpi from *Populus nigra* plants (genotype 41) grouped into compound classes. CX - control group, MX - *M. larici-populina* infection. Given is the emission in ng g^{-1} h^{-1} as mean ± SEM (n = 6).

	CX	MX
Monoterpenes		
Camphene	2.67 ± 0.97	0.74 ± 0.27
Camphor	2.92 ± 0.57	0.59 ± 0.28
Eucalyptol	1.93 ± 0.45	0.81 ± 0.32
Limonene	0.91 ± 0.31	0.09 ± 0.09
(*Z*)-β-Ocimene	2.76 ± 1.08	0.00 ± 0.00
α-Pinene	1.75 ± 0.62	0.50 ± 0.18
β-Pinene	1.72 ± 0.65	0.48 ± 0.17
Sabinene	1.12 ± 0.50	0.42 ± 0.14
Sesquiterpenes		
β-Caryophyllene	4.58 ± 0.53	1.94 ± 0.56
(*E,E*)-α-Farnesene	10.03 ± 4.54	1.10 ± 0.59
Green leaf volatiles		
3-(*Z*)-Hexenol	16.96 ± 9.89	0.20 ± 0.20
3-(*E*)-Hexenyl-acetate	8.55 ± 4.51	0.00 ± 0.00

Table 8: Emission of volatile organic compounds, grouped into compound classes, 15 dpi from *P. nigra* plants (genotype 169) infested with *M. larici-populina* and *L. dispar* alone or in combination. CX - control group, MX - *M. larici-populina* infection, CH - herbivory, MH - combination. Given is the emission in ng g^{-1} h^{-1} as mean ± SEM (n = 5). DMNT - 4,8-dimethyl-1,3,7-nonatriene, MBA - methylbutyraldoxime.

	CX	MX	CH	MH
Monoterpenes				
L-Linalool	1.44 ± 1.05	0.11 ± 0.11	37.93 ± 18.18	19.13 ± 9.26
(*E*)-β-Ocimene	11.10 ± 6.91	3.52 ± 1.40	25.93 ± 6.58	17.09 ± 6.42
(*Z*)-β-Ocimene	43.09 ± 28.76	16.02 ± 7.14	129.22 ± 35.09	84.43 ± 35.55
Homoterpenes				
DMNT	31.18 ± 17.89	8.02 ± 3.34	196.57 ± 54.28	85.61 ± 18.93
Sesquiterpenes				
β-Caryophyllene	8.71 ± 4.64	5.63 ± 1.88	40.85 ± 11.43	19.84 ± 6.06
(*E,E*)-α-Farnesene	59.41 ± 35.38	15.22 ± 7.16	90.59 ± 26.15	64.52 ± 16.51
Germacrene D	1.13 ± 0.69	0.15 ± 0.10	18.23 ± 7.71	12.78 ± 5.08
α-Humulene	1.49 ± 0.62	0.78 ± 0.27	6.77 ± 1.80	3.48 ± 1.15
Aromatic compounds				
Benzyl cyanide	2.67 ± 1.96	0.23 ± 0.23	19.44 ± 9.66	17.44 ± 10.53
Ethylhexyl salicylate	15.05 ± 5.90	10.43 ± 5.02	10.37 ± 3.33	7.78 ± 3.80
Homomenthyl salicylate	30.61 ± 12.19	19.33 ± 8.55	28.46 ± 9.69	14.81 ± 6.42
Methyl salicylate	2.64 ± 1.80	0.33 ± 0.21	1.44 ± 0.49	1.36 ± 0.64
N-compounds				
anti-2-MBA	1.34 ± 0.93	0.13 ± 0.13	11.95 ± 6.10	6.17 ± 2.13
anti-3-MBA	0.47 ± 0.29	0.00 ± 0.00	4.43 ± 1.85	3.01 ± 0.92
syn-2-MBA	5.21 ± 3.06	1.12 ± 0.59	33.62 ± 14.43	21.29 ± 7.13
Others				
1-Octen-3-ol	0.00 ± 0.00	5.25 ± 2.08	0.00 ± 0.00	5.34 ± 1.85

Table 9: Emission of volatile organic compounds, grouped into compound classes, 22 dpi from *P. nigra* plants (genotype 169) infested with *M. larici-populina* and *L. dispar* alone or in combination. CX - control group, MX - *M. larici-populina* infection, CH - herbivory, MH - combination. Given is the emission in ng g^{-1} h^{-1} as mean ± SEM ($n = 5$ for CH, MH and $n = 6$ for CX, MX). DMNT - 4,8-dimethyl-1,3,7-nonatriene, MBA - methylbutyraldoxime.

	CX	MX	CH	MH
Monoterpenes				
Borneol	0.00 ± 0.00	0.00 ± 0.00	5.76 ± 3.5	0.74 ± 0.35
Camphene	0.57 ± 0.21	0.28 ± 0.15	7.13 ± 2.82	1.88 ± 0.53
Camphor	0.00 ± 0.00	0.00 ± 0.00	7.24 ± 3.22	5.45 ± 2.06
Eucalyptol	0.51 ± 0.25	0.39 ± 0.25	17.4 ± 8.23	6.37 ± 1.90
Limonene	0.4 ± 0.13	0.25 ± 0.16	6.3 ± 2.6	1.72 ± 0.57
L-Linalool	0.37 ± 0.18	0.32 ± 0.32	277.59 ± 141.30	51.09 ± 20.77
Linalool oxide	0.00 ± 0.00	0.00 ± 0.00	25.83 ± 15.3	4.53 ± 2.05
Alloocimeme	0.18 ± 0.18	0.27 ± 0.21	4.75 ± 2.73	2.02 ± 0.45
(*E*)-β-Ocimene	6.17 ± 3.85	10.74 ± 4.56	104.36 ± 55.57	54.13 ± 13.18
(*Z*)-β-Ocimene	24.61 ± 13.24	43.71 ± 17.23	513.86 ± 302.51	243.62 ± 59.14
α-Pinene	0.38 ± 0.18	0.26 ± 0.17	5.88 ± 2.47	1.76 ± 0.48
β-Pinene	0.56 ± 0.18	1.59 ± 0.45	8.3 ± 3.61	3.94 ± 0.77
Sabinene	0.41 ± 0.19	0.30 ± 0.19	6.83 ± 3.08	2.45 ± 0.81
Homoterpenes				
DMNT	25.63 ± 16.95	20.13 ± 7.31	625.35 ± 232.48	396.1 ± 143.67
Sesquiterpenes				
α-Bergamottene	2.11 ± 1.34	2.35 ± 0.99	19.74 ± 11	11.04 ± 2.20
α-Cadinene	0.4 ± 0.27	0.00 ± 0.00	5.25 ± 2.15	2.08 ± 0.50
δ-Cadinene	0.07 ± 0.07	0.00 ± 0.00	9.82 ± 4.27	2.83 ± 1.08
γ-Cadinene	0.00 ± 0.00	0.00 ± 0.00	2.60 ± 1.20	0.45 ± 0.28
Calarene	0.00 ± 0.00	0.00 ± 0.00	5.26 ± 2.03	1.57 ± 0.97
β-Caryophyllene	7.13 ± 2.5	6.57 ± 2.76	92.83 ± 39.68	40.59 ± 11.00
α-Copaene	0.00 ± 0.00	0.00 ± 0.00	25.47 ± 10.51	5.17 ± 3.25
β-Cubebene	0.00 ± 0.00	0.00 ± 0.00	6.47 ± 2.91	1.96 ± 0.84
(*E*,*E*)-α-Farnesene	18.87 ± 14.43	38.14 ± 14.67	235.13 ± 155.94	166.15 ± 34.61
Germacrene D	0.16 ± 0.16	0.00 ± 0.00	64.1 ± 28.45	17.08 ± 6.97
α-Humulene	0.86 ± 0.36	0.83 ± 0.33	18.51 ± 7.38	7.14 ± 2.06
Nerolidol	0.47 ± 0.3	0.00 ± 0.00	11.68 ± 5.17	2.69 ± 1.72
Aromatic compounds				
Benzaldehyde	0.00 ± 0.00	0.00 ± 0.00	4.29 ± 1.84	1.86 ± 0.39
Benzyl cyanide	0.43 ± 0.43	1.23 ± 1.23	164.22 ± 67.48	67.51 ± 22.64
Methyl salicylate	0.00 ± 0.00	0.00 ± 0.00	5.39 ± 2.62	2.92 ± 1.81
Hexyl benzoate	0.00 ± 0.00	0.00 ± 0.00	6.03 ± 4.05	4.22 ± 2.79
N compounds				
Indole	0.00 ± 0.00	0.00 ± 0.00	8.27 ± 3.86	3.43 ± 1.37
anti-2-MBA	0.39 ± 0.39	0.89 ± 0.89	76.86 ± 31.33	51.1 ± 18.59
anti-3-MBA	0.07 ± 0.07	0.00 ± 0.00	27.67 ± 9.54	20.64 ± 7.13
syn-2-MBA	3.39 ± 2.09	6.98 ± 4.84	210.11 ± 81.25	163.96 ± 57.06
Phenyl nitroethane	0.00 ± 0.00	0.00.00 ± 0.00.00	6.98 ± 2.49	2.93 ± 0.93
Green leaf volatiles				
3-(*E*)-Hexenyl-acetate	0.00 ± 0.00	0.00 ± 0.00	5.29 ± 3.91	3.37 ± 1.43
Others				
*iso*amyl *iso*butyrate	0.92 ± 0.65	1.49 ± 0.85	9.41 ± 2.07	9.29 ± 2.55
1-Octen-3-ol	0.00 ± 0.00	4.86 ± 1.49	0.00 ± 0.00	12.18 ± 3.80

Table 10: Emission of volatile organic compounds, grouped into compound classes, 27 dpi from *P. nigra* plants (genotype 169) infested with *M. larici-populina* and *L. dispar* alone or in combination. CX - control group, MX - *M. larici-populina* infection, CH - herbivory, MH - combination. Given is the emission in ng g^{-1} h^{-1} as mean ± SEM ($n = 5$, except of CX: $n = 4$). DMNT - 4,8-dimethyl-1,3,7-nonatriene, MBA - methylbutyraldoxime.

	CX	MX	CH	MH
Monoterpenes				
Camphene	0.51 ± 0.31	0.45 ± 0.18	0.94 ± 0.59	0.83 ± 0.25
Eucalyptol	0.52 ± 0.52	0.48 ± 0.35	2.47 ± 1.03	2.56 ± 0.46
Limonene	0.33 ± 0.33	0.22 ± 0.22	0.65 ± 0.37	0.65 ± 0.23
L-Linalool	2.13 ± 1.25	0.3 ± 0.3	8.61 ± 3.12	11.32 ± 5.79
Myrcene	1.26 ± 0.75	0.00 ± 0.00	0.58 ± 1.01	0.34 ± 0.56
Alloocimene	1.07 ± 0.8	0.56 ± 0.37	0.54 ± 0.34	0.81 ± 0.25
(*E*)-β-Ocimene	29.02 ± 18.73	19.71 ± 11	16.53 ± 6.99	32.04 ± 6.86
(*Z*)-β-Ocimene	123.17 ± 88.99	119.68 ± 56.27	84.28 ± 35.62	147.05 ± 19.96
α-Pinene	0.31 ± 0.31	0.23 ± 0.23	1.17 ± 0.59	0.72 ± 0.22
Sabinene	0.41 ± 0.41	0.44 ± 0.33	0.66 ± 0.51	0.88 ± 0.31
Homoterpenes				
DMNT	58.17 ± 32.41	25 ± 14.48	106.36 ± 31.45	201.05 ± 74.67
Sesquiterpenes				
α-Bergamottene	3.53 ± 2.36	2.21 ± 1.09	3.21 ± 1.55	3.88 ± 1.07
δ-Cadinene	0.2 ± 0.2	0.16 ± 0.16	1.13 ± 0.47	0.58 ± 0.24
β-Caryophyllene	10.53 ± 5.89	15.01 ± 7.83	15.12 ± 3.75	18.59 ± 5.55
β-Cubebene	0.1 ± 0.1	0.11 ± 0.11	0.63 ± 0.32	0.39 ± 0.16
(*E,E*)-α-Farnesene	111.14 ± 76.49	72.3 ± 35.18	75.77 ± 34.17	138.79 ± 33.69
Farnesol	1.19 ± 1.19	1.06 ± 0.77	0.43 ± 0.35	0.32 ± 0.32
Germacrene D	0.99 ± 0.6	0.21 ± 0.21	5.39 ± 2.44	3.39 ± 0.97
α-Humulene	1.34 ± 0.79	1.36 ± 0.65	2.62 ± 0.79	2.39 ± 0.80
Aromatic compounds				
Benzylaldehyde	0.00 ± 0.00	1.11 ± 0.22	0.1 ± 0.1	0.7 ± 0.68
Benzyl cyanide	6.75 ± 4.06	1.83 ± 1.83	10.49 ± 3.74	17.3 ± 11.08
Methyl salicylate	9.09 ± 9.09	0.82 ± 0.82	0.78 ± 0.78	0.61 ± 0.90
N compounds				
Indole	0.23 ± 0.23	0.00 ± 0.00	0.00 ± 0.00	0.34 ± 1.29
anti-2-MBA	4.50 ± 2.67	0.56 ± 0.56	6.76 ± 2.45	24.48 ± 13.11
syn-2-MBA + 3-(*Z*)-Hexenol	91.99 ± 17.54	78.28 ± 6.94	136.88 ± 49.32	128.50 ± 19.38
anti-3-MBA	0.94 ± 0.94	0.11 ± 0.11	1.1 ± 0.73	11.78 ± 6.71
Green leaf volatiles				
3-(*Z*)-Hexenyl-acetate	0.13 ± 0.13	0.16 ± 0.16	1.69 ± 1.47	2.77 ± 2.15
syn-2-MBA + 3-(*Z*)-Hexenol	91.99 ± 17.54	78.28 ± 6.94	136.88 ± 49.32	128.50 ± 19.38
Others				
Isoamyl isobutyrate	2.24 ± 1.3	1.21 ± 0.76	2.42 ± 0.85	7.44 ± 2.14
1-Octa-3-diol	0.33 ± 0.19	2.3 ± 0.98	0.87 ± 0.6	0.86 ± 0.44
1-Octen-3-ol	0.73 ± 0.43	19.12 ± 7.42	0.59 ± 0.59	20.37 ± 8.12
3-Octanone	0.00 ± 0.00	4.69 ± 1.42	0.00 ± 0.00	2.93 ± 1.20

Table 11: Emission of volatiles from *M. larici-populina* infected *P. nigra* trees (genotype 41) ca. 22 dpi. Volatiles were collected over 48 h every 4 h. The first 24 h (no caterpillars , "X") are shown here, the last 24 h (herbivory, "H") are shown in Table 12. C - control group, M - *M. larici-populina* infection, numbers indicate time of VOC collection. Given is the emission in ng g^{-1} h^{-1} as mean ± SEM ($n = 3$.). DMNT - 4,5-dimethyl-1,3,7-nonatriene.

	CX 10-14	MX 10-14	CX 14-18	MX 14-18	CX 18-22	MX 18-22	CX 22-02	MX 22-02
Monoterpenes								
Borneol	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.30	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Camphene	1.29 ± 0.62	0.00 ± 0.00	1.52 ± 0.37	0.00 ± 0.00	0.26 ± 0.13	0.00 ± 0.00	0.43 ± 0.26	0.00 ± 0.00
Camphor	0.81 ± 0.27	0.00 ± 0.00	1.01 ± 0.64	0.00 ± 0.00	0.21 ± 0.21	0.00 ± 0.00	0.20 ± 0.20	0.00 ± 0.00
Eucalyptol	0.91 ± 0.36	0.00 ± 0.00	0.71 ± 0.24	0.00 ± 0.00	0.22 ± 0.22	0.00 ± 0.00	0.20 ± 0.20	0.00 ± 0.00
Limonene	0.30 ± 0.30	0.00 ± 0.00	0.39 ± 1.11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Myrcene	0.47 ± 0.31	0.00 ± 0.00	0.45 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Z-β-Ocimene	0.14 ± 0.14	0.00 ± 0.00	0.12 ± 0.26	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	
α-Pinene	0.77 ± 0.39	0.00 ± 0.00	0.86 ± 1.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.17	0.00 ± 0.00
β-Pinene	1.35 ± 0.81	0.77 ± 0.40	1.20 ± 0.47	0.64 ± 0.16	0.18 ± 0.18	0.00 ± 0.00	0.22 ± 0.22	0.00 ± 0.00
Sabinene	0.88 ± 0.49	0.22 ± 0.22	0.47 ± 0.16	0.15 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Homoterpenes								
DMNT	0.56 ± 0.40	0.00 ± 0.00	1.23 ± 0.99	0.00 ± 0.00	0.53 ± 0.33	0.00 ± 0.00	0.60 ± 0.40	0.00 ± 0.00
Sesquiterpenes								
β-Caryophyllene	1.24 ± 0.63	0.11 ± 0.11	1.36 ± 0.84	0.00 ± 0.00	0.50 ± 0.33	0.00 ± 0.00	0.52 ± 0.32	0.00 ± 0.00
(*E*,*E*)-α-Farnesene	0.83 ± 0.65	0.18 ± 0.18	0.36 ± 0.63	0.00 ± 0.00	0.21 ± 0.21	0.00 ± 0.00	0.24 ± 0.24	0.00 ± 0.00
Aromatic compounds								
Benzaldehyde	0.55 ± 0.16	0.33 ± 0.17	0.16 ± 0.65	0.00 ± 0.00	0.15 ± 0.15	0.00 ± 0.00	0.16 ± 0.16	0.00 ± 0.00
Green leaf volatiles								
3-(*Z*)-Hexenol	0.70 ± 0.13	0.00 ± 0.00	7.52 ± 1.03	1.77 ± 0.54	0.86 ± 0.86	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3-(*Z*)-Hexenyl acetate	0.66 ± 0.08	0.00 ± 0.00	5.14 ± 0.17	0.81 ± 0.22	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3-(*Z*)-Hexenyl valerate	0.32 ± 0.16	0.00 ± 0.00	1.2 ± 0.3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Others								
Nonanal	4.66 ± 1.73	2.09 ± 0.53	3.35 ± 0.12	1.2 ± 0.16	2.13 ± 1.07	0.61 ± 0.39	2.22 ± 0.94	0.73 ± 0.07
1-Octen-3-ol	0.14 ± 0.14	1.11 ± 0.69	0.00 ± 0.78	0.60 ± 0.38	0.00 ± 0.00	0.27 ± 0.27	0.00 ± 0.00	0.69 ± 0.41

Table 11:continued.

	CX 02-06	MX 02-06	CX 06-10	MX 06-10
Monoterpenes				
Borneol	0.32 ± 0.32	0.00 ± 0.00	0.32 ± 0.32	0.00 ± 0.00
Camphene	2.19 ± 1.40	0.29 ± 0.14	2.20 ± 1.25	0.32 ± 0.16
Camphor	0.75 ± 0.52	0.00 ± 0.00	1.09 ± 0.50	0.00 ± 0.00
Eucalyptol	0.87 ± 0.64	0.00 ± 0.00	1.51 ± 0.91	0.17 ± 0.17
Limonene	0.65 ± 0.49	0.00 ± 0.00	0.69 ± 0.51	0.00 ± 0.00
Myrcene	0.66 ± 0.52	0.00 ± 0.00	0.52 ± 0.52	0.00 ± 0.00
Z-β-Ocimene	0.43 ± 0.29	0.00 ± 0.00	0.72 ± 0.54	0.46 ± 0.26
α-Pinene	1.26 ± 0.96	0.00 ± 0.00	1.44 ± 0.88	0.00 ± 0.00
β-Pinene	1.69 ± 1.17	0.51 ± 0.12	2.46 ± 1.86	0.57 ± 0.15
Sabinene	0.79 ± 0.79	0.37 ± 0.19	1.62 ± 1.62	0.17 ± 0.17
Homoterpenes				
DMNT	1.23 ± 0.84	0.00 ± 0.00	1.11 ± 0.8	0.00 ± 0.00
Sesquiterpenes				
β-Caryophyllene	1.55 ± 0.93	0.26 ± 0.13	1.65 ± 1.02	0.3 ± 0.15
(*E*,*E*)-α-Farnesene	0.72 ± 0.56	0.00 ± 0.00	1.13 ± 0.92	0.55 ± 0.32
Aromatic compounds				
Benzaldehyde	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Green leaf volatiles				
3-(*Z*)-Hexenol	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3-(*Z*)-Hexenyl acetate	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3-(*Z*)-Hexenyl valerate	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Others				
Nonanal	1.94 ± 0.55	0.97 ± 0.33	1.41 ± 0.51	0.56 ± 0.04
1-Octen-3-ol	0.18 ± 0.18	0.90 ± 0.52	0.00 ± 0.00	0.66 ± 0.40

Table 12: Volatile emission from *M. larici-populina* infected and herbivory treated *P. nigra* trees (genotype 41) ca. 22 dpi. See Table 11 for details. MBA - methylbutyraldoxime.

	CH 10-14	MH 10-14	CH 14-18	MH 14-18	CH 18-22	MH 18-22	CH 22-02	MH 22-02
Monoterpenes								
Borneol	0.21 ± 0.21	0.00 ± 0.00	0.23 ± 0.23	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Camphene	2.29 ± 1.14	0.76 ± 0.19	2.35 ± 1.40	0.80 ± 0.14	0.40 ± 0.40	0.00 ± 0.00	0.52 ± 0.36	0.12 ± 0.12
Eucalyptol	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Limonene	1.24 ± 0.63	0.50 ± 0.28	0.90 ± 0.61	0.26 ± 0.26	0.31 ± 0.31	0.00 ± 0.00	0.25 ± 0.25	0.00 ± 0.00
L-Linalool	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Myrcene	0.80 ± 0.47	0.38 ± 0.20	0.71 ± 0.56	0.25 ± 0.13	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Z-β-Ocimene	0.53 ± 0.37	0.67 ± 0.42	0.77 ± 0.61	0.63 ± 0.17	0.41 ± 0.41	0.17 ± 0.17	1.08 ± 0.91	0.74 ± 0.38
α-Pinene	1.46 ± 0.78	0.42 ± 0.22	1.36 ± 0.85	0.37 ± 0.19	0.2 ± 0.2	0.00 ± 0.00	0.22 ± 0.22	0.00 ± 0.00
β-Pinene	1.50 ± 0.92	0.83 ± 0.43	1.29 ± 0.82	0.69 ± 0.16	0.19 ± 0.19	0.00 ± 0.00	0.22 ± 0.22	0.00 ± 0.00
Sabinene	0.97 ± 0.56	0.24 ± 0.24	0.49 ± 0.49	0.15 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Homoterpenes								
DMNT	1.46 ± 1.02	0.76 ± 0.26	12.91 ± 9.31	9.88 ± 3.5	13.62 ± 9.61	7.49 ± 0.87	20.54 ± 13.19	17.17 ± 5.17
Sesquiterpenes								
α-Bergamottene	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
β-Caryophyllene	1.59 ± 0.91	0.68 ± 0.12	1.47 ± 0.94	0.69 ± 0.1	0.75 ± 0.75	0.00 ± 0.00	1.08 ± 0.69	0.44 ± 0.05
(*E*,*E*)-α-Farnesene	0.96 ± 0.68	0.68 ± 0.34	0.72 ± 0.55	0.58 ± 0.3	0.69 ± 0.69	0.22 ± 0.22	1.11 ± 0.84	0.97 ± 0.5
Germacrene D	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
N compounds								
Benzyl cyanide	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
syn-2-MBA + 3-(*Z*)-Hexenol	11.11 ± 3.89	7.49 ± 0.66	14.73 ± 3.59	9.25 ± 0.97	5.56 ± 3.07	5.27 ± 1.46	5.50 ± 2.01	8.79 ± 0.53
anti-2-MBA	0.48 ± 0.26	0.00 ± 0.00	0.34 ± 0.34	0.22 ± 0.22	0.38 ± 0.38	0.00 ± 0.00	0.60 ± 0.39	0.55 ± 0.34
anti-3-MBA	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.16 ± 0.16	0.00 ± 0.00	0.19 ± 0.19	0.19 ± 0.19
Indole	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Aromatic compounds								
Benzaldehyde	0.00 ± 0.00	0.51 ± 0.3	0.27 ± 0.27	0.54 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Salicylaldehyde	0.00 ± 0.00	0.35 ± 0.35	0.30 ± 0.3	0.54 ± 0.54	0.23 ± 0.23	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Green leaf volatiles								
syn-2-MBA + 3-(*Z*)-Hexenol	11.11 ± 3.89	7.49 ± 0.66	14.73 ± 3.59	9.25 ± 0.97	5.56 ± 3.07	5.27 ± 1.46	5.50 ± 2.01	8.79 ± 0.53
3-(*Z*)-Hexenyl acetate	3.59 ± 0.91	1.71 ± 0.68	5.67 ± 0.82	2.37 ± 0.46	0.00 ± 0.00	0.00 ± 0.00	0.60 ± 0.32	0.29 ± 0.29
3-(*Z*)-Hexenyl-valerate	0.59 ± 0.29	0.62 ± 0.05	1.18 ± 0.34	0.8 ± 0.14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Others								
iso-amyl *iso*-butyrate	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.19 ± 0.19	0.00 ± 0.00
Nonanal	2.06 ± 0.62	1.68 ± 0.27	2.62 ± 1.37	1.54 ± 0.44	1.5 ± 1.18	0.88 ± 0.56	1.53 ± 0.91	1.07 ± 0.32
1-Octen-3-ol	0.15 ± 0.15	1.9 ± 1.18	0.00 ± 0.00	1.25 ± 0.71	0.00 ± 0.00	0.55 ± 0.3	0.00 ± 0.00	0.96 ± 0.61

Table 12: continued.

	CH 02-06	MH 02-06	CH 06-10	MH 06-10
Monoterpenes				
Borneol	0.30 ± 0.30	0.00 ± 0.00	0.43 ± 0.43	0.00 ± 0.00
Camphene	2.88 ± 1.95	0.62 ± 0.13	2.87 ± 1.99	0.60 ± 0.14
Eucalyptol	0.45 ± 0.45	0.31 ± 0.17	1.86 ± 1.32	0.51 ± 0.26
Limonene	1.00 ± 0.72	0.16 ± 0.16	2.00 ± 1.46	0.00 ± 0.00
L-Linalool	0.75 ± 0.48	0.00 ± 0.00	2.76 ± 1.57	0.68 ± 0.35
Myrcene	0.80 ± 0.65	0.28 ± 0.14	1.10 ± 0.94	0.13 ± 0.13
(*Z*)-β-Ocimene	14.19 ± 10.26	5.56 ± 2.29	47.76 ± 31.47	17.72 ± 6.45
α-Pinene	1.7 ± 1.16	0.15 ± 0.15	1.87 ± 1.32	0.26 ± 0.15
β-Pinene	1.62 ± 1.15	0.56 ± 0.22	1.97 ± 1.48	0.54 ± 0.20
Sabinene	0.88 ± 0.72	0.00 ± 0.00	1.58 ± 1.14	0.00 ± 0.00
Homoterpenes				
DMNT	92.89 ± 63.16	38.10 ± 9.89	101.25 ± 62.19	52.35 ± 10.12
Sesquiterpenes				
α-Bergamottene	0.25 ± 0.25	0.00 ± 0.00	0.73 ± 0.54	0.00 ± 0.00
β-Caryophyllene	4.30 ± 3.33	1.11 ± 0.19	6.16 ± 4.53	1.46 ± 0.22
(*E*,*E*)-α-Farnesene	10.79 ± 8.22	3.29 ± 1.30	30.68 ± 16.03	16.34 ± 6.61
Germacrene D	1.07 ± 1.07	0.00 ± 0.00	3.92 ± 3.49	0.12 ± 0.12
N compounds				
Benzyl cyanide	1.28 ± 0.90	0.17 ± 0.17	3.86 ± 2.18	0.83 ± 0.42
syn-2-MBA + 3-(*Z*)-Hexenol	2.99 ± 1.81	1.00 ± 0.25	5.86 ± 0.6	2.42 ± 1.05
anti-3-MBA	1.00 ± 0.52	0.22 ± 0.22	3.44 ± 0.15	1.17 ± 0.69
Indole	0.21 ± 0.21	0.00 ± 0.00	1.19 ± 0.79	0.00 ± 0.00
Aromatic compounds				
Benzaldehyde	0.35 ± 0.18	0.22 ± 0.22	0.46 ± 0.23	0.18 ± 0.18
Salicylaldehyde	0.39 ± 0.39	0.00 ± 0.00	1.07 ± 0.20	0.00 ± 0.00
Green leaf volatiles				
syn-2-MBA + 3-(*Z*)-Hexenol	14.12 ± 5.03	10.34 ± 0.87	29.3 ± 10.95	12.15 ± 1.34
3-(*Z*)-Hexenyl acetate	1.98 ± 0.46	1.16 ± 0.28	3.19 ± 0.98	0.92 ± 0.10
3-(*Z*)-Hexenyl-valerate	0.26 ± 0.26	0.00 ± 0.00	0.87 ± 0.52	0.00 ± 0.00
Others				
iso-amyl *iso*-butyrate	0.92 ± 0.92	0.46 ± 0.24	1.36 ± 0.88	0.88 ± 0.15
Nonanal	1.54 ± 0.65	1.15 ± 0.38	1.96 ± 0.75	0.78 ± 0.28
1-Octen-3-ol	0.00 ± 0.00	1.42 ± 1.04	0.18 ± 0.18	1.27 ± 0.92

Table 13: Phytohormone concentrations in the leaves of *P. nigra* infested with *M. larici-populina* and *L. dispar* alone or in combination. Leaves were sampled after 48 h of insect feeding and at the indicated day after inoculation (dpi). CX - control group, CH - herbivory, MX - *Melampsora* infection, MH - combination. Phytohormone concentrations are given in ng/g dryweight as mean with SEM ($n = 5$; except of 22 dpi CX, MX: $n = 6$; 27 dpi CX: $n = 4$). SA - salicylic acid, JA - jasmonic acid, ABA - abscisic acid, JA-Ile - jasmonoyl isoleucine (two isomeres: (+)/(-)-*iso*), OPDA - *cis*-(+)-12-Oxophytodienoic acid, OH-JA-Ile - hydroxylated JA-Ile, COOH-JA-Ile - carboxylated JA-Ile, OH-JA - hydroxylated JA, JA-deriv. - sum of all JA derivatives.

time (dpi)	Treatment	SA	JA	ABA	(-)-JA-Ile	(+)-7-*iso*-JA-Ile	OPDA
15	CX	6948.64 ± 1462.88	689.98 ± 275.01	33.06 ± 13.14	4.51 ± 1.59	24 ± 8.84	2181.85 ± 576.4
	CH	5910.4 ± 775.55	1261.31 ± 432.76	84.01 ± 22.62	6.89 ± 2.19	45.55 ± 24.24	2483.84 ± 664.58
	MX	10259.88 ± 3286.08	1395.57 ± 541.79	105.17 ± 44.49	13.86 ± 9.33	49.84 ± 25.13	2642.14 ± 585.03
	MH	5521.85 ± 1027.53	1212.12 ± 623.19	110.68 ± 17.15	12.72 ± 6.93	44.11 ± 23.54	2358.1 ± 186.02
22	CX	7276.93 ± 1077.44	1030.32 ± 423.94	79.75 ± 31.27	29.74 ± 27.35	74.07 ± 57.15	3454.36 ± 436.48
	CH	4497.65 ± 485.31	1394.09 ± 446.82	154.93 ± 24.75	10.87 ± 4.21	46.04 ± 20.11	3873.38 ± 574.22
	MX	12012.88 ± 3432.53	496.42 ± 139.33	88.23 ± 26.33	7.88 ± 3.77	25.46 ± 12.67	2603.81 ± 570.37
	MH	5592.55 ± 1045.14	1510.18 ± 453.56	98.07 ± 16.13	16.01 ± 7.26	58.62 ± 21.63	2839.89 ± 433.65
27	CX	6459.54 ± 871.87	1020.64 ± 286.66	138.76 ± 46.1	9.23 ± 1.85	53.02 ± 19.34	3309.47 ± 629.53
	CH	5376.54 ± 731.4	509.05 ± 194.52	34.92 ± 11.62	2.34 ± 0.70	15.24 ± 7.73	2155.5 ± 209.7
	MX	7522.01 ± 627.18	517.76 ± 176.64	83.08 ± 15.35	2.57 ± 0.51	10.16 ± 3.05	3260.77 ± 287.60
	MH	5550.41 ± 552.3	779.89 ± 163.79	128.09 ± 40.99	7.3 ± 2.72	23.71 ± 6	2982.1 ± 221.44

Tabel 13: continued.

time (dpi)	Treatment	OH-JA-Ile	COOH-JA-Ile	OH-JA	JA-deriv.
15	CX	70.53 ± 34.56	17.44 ± 10.47	1703.93 ± 962.98	1079.9 ± 568.27
	CH	69.26 ± 40.17	16.74 ± 9.05	1690.55 ± 825.71	4313.68 ± 800.95
	MX	95.61 ± 53.97	28.02 ± 18.64	3441.31 ± 1956.53	951.38 ± 345.08
	MH	106.95 ± 59.99	31.05 ± 19.72	2367.9 ± 1234.07	6749.26 ± 2492.57
22	CX	158.99 ± 86.41	35.84 ± 18.57	3686.07 ± 1606.09	2182.87 ± 1022.53
	CH	175.34 ± 76.66	49.21 ± 30.8	4376.43 ± 1680.29	8228.69 ± 1780.42
	MX	40.69 ± 24.58	9.6 ± 4.25	1530.68 ± 1051.76	834.69 ± 146.71
	MH	126.36 ± 43.83	35.49 ± 12.41	4696.64 ± 1669.36	7825.18 ± 1607.98
27	CX	115.39 ± 24.33	27.75 ± 9.99	4299.53 ± 1069.43	1403.36 ± 419.77
	CH	14.97 ± 7.66	2.9 ± 1.57	405.09 ± 234.11	5857.71 ± 1572.13
	MX	24.11 ± 8.7	6.16 ± 1.25	730.89 ± 259.84	705.97 ± 169.04
	MH	51.59 ± 20.61	15.22 ± 4.95	1861.14 ± 759.09	4485.1 ± 670.31

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8 Sworn Statement

I declare in lieu of oath that I have researched and written this Master's thesis myself (statement of authorship), no passages of text have been taken from third parties or own exam papers without having been identified as such and that all tools, personal notifications, and sources used by the applicant have been indicated in the Master's thesis.

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The Master's thesis has not yet been submitted as an examination paper for state or other academic examinations.

Jena, March 7, 2014

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Franziska Eberl