The Roles of Vegetative Volatiles in Plant Defense and Other Interactions

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1. General Introduction

One of the most fascinating characteristics of plants is their ability to produce an enormous variety of chemical compounds. Some of these are small organic molecules, which, thanks to their high vapor pressure, leave the plant and disperse in the surrounding atmosphere. Plant volatile organic compounds (VOCs) belong to several different chemical classes: terpenes, phenylpropanoids/benzenoids, fatty acid derivatives, and amino acid derivatives. To date, approximatively 1700 VOCs from more than 90 plant families have been identified (Dudareva et al., 2006).

Complex volatile mixtures make up the scent and aroma of flowers and fruits, but they can also be released from vegetative plant parts. The most common vegetative VOCs are green leaf volatiles (GLVs, C_6 fatty acid derivatives, which form the typical odor of cut leaves), and terpenes (C_{10} monoterpenes and C_{15} sesquiterpenes).

Vegetative VOCs have been shown to have very diverse functions in plants (Dudareva et al., 2006). They can protect the plant against abiotic stresses, like oxidative stress and heat (Loreto and Schnitzler, 2010). They can mediate plant-plant communication (Kessler et al., 2006) and they can function as hormone-like signals within a single plant (Heil and Silva Bueno, 2007). Moreover, they mediate allelopathy and affect plant competition (Kegge and Pierik, 2010). However, since volatile emission is abundant upon herbivory, vegetative volatiles have been widely investigated for their role in anti-herbivore defense. VOCs can act directly against herbivores by being toxic or repellent, or attract herbivore enemies, strategies known as direct and indirect defense, respectively (Unsicker et al., 2009) (Fig. 1.1). Furthermore, as defense compounds VOCs can be separated into constitutive and induced. While constitutive defense compounds are always present in the plant, production of induced defenses is triggered by initial attack of herbivores or pathogens.

1.1 Volatiles as direct plant defenses

Plant direct defenses have been defined as the "characteristics of a plant […] that negatively affect the physiology or behavior of herbivores" (Dicke and Baldwin, 2010). The most evident of these characteristics are physical barriers such as thick cuticles, lignified stems, thorns or hairs which make plant surfaces less accessible to herbivores.

Another less visible but no less effective layer of defense is made up of chemical compounds, like plant volatiles.

VOCs can act as direct defenses against insect herbivores by deterring oviposition by adult foliar feeders. For example, caterpillar-infested *Nicotiana tabacum* plants release a volatile blend dominated by C_6 compounds, which is repellent to *Heliothis virescens* moths. This blend is released during the night, when *H. virescence* females are actively searching for an oviposition site (De Moraes et al., 2001).

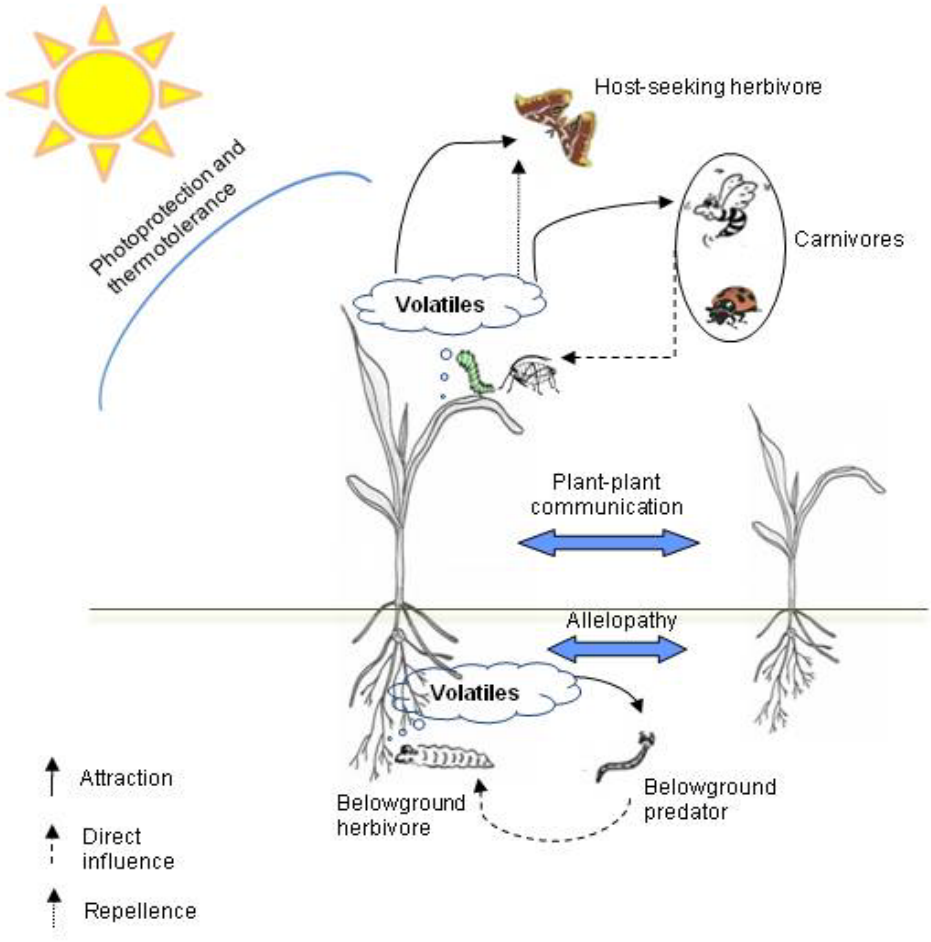

Fig. 1.1: Vegetative volatiles mediate the interaction of the plant with its environment in multiple ways. Interactions with animals include attraction of herbivore enemies both above and belowground, and attraction or repellence of host-seeking herbivores. Volatiles also elicit or prime defenses in neighboring undamaged plants, and act as allelopathic agents in the rhyzosphere. As defenses against abiotic stresses, volatiles confer tolerance to transient increases of temperature and oxidative stress. Modified from Unsicker et al., 2009, drawing of the aphid © Copyright D G Mackean.

Kessler and Baldwin (Kessler and Baldwin, 2001) demonstrated that volatile emission can significantly reduce the oviposition rate of foliovores in the field. In a pioneering study on the effect of volatiles of *Nicotiana attenuata* plants in their natural habitat, the authors found a lower number of eggs of *Manduca quinquemaculata*, a major pest of *Nicotiana* plants, on herbivore-attacked plants emitting a complex blend of volatiles or treated with synthetic linalool (a monoterpene) as compared to control, untreated plants.

Besides discouraging oviposition, vegetative VOCs can also deter caterpillars from feeding. Isoprene, a very common C_5 volatile emitted by many plant species, has been shown to deter feeding of *Manduca sexta* on transgenic isoprene-emitting *Nicotiana tabacum* plants and on artificial diet containing isoprene. Surprisingly, the deterrent effect was caused by isoprene emission rates even lower than the average emission rate of plants in natural conditions (< 6 nmol m^{-2} s^{-1}). Although the significance of feeding deterrence by isoprene in nature is not yet clear, isoprene emission could be a factor that influences herbivore food choice (Laothawornkitkul et al., 2008).

Herbivores are not the only enemies from which plants have to defend themselves. Pathogenic microorganisms such as viruses, bacteria, protozoa, and fungi can diminish plant fitness and sometimes lead to death (Agrios, 2005). There is ample *in vitro* evidence that vegetative volatiles, such as monoterpenes, have antimicrobial activity against numerous fungal and bacterial species. Plant essential oils that are mixtures of volatile compounds have been tested in bioassays and the efficacy of their components extrapolated. However, evidence that volatiles may actually have a defensive role against pathogens *in planta* is still scarce. One of the few studies addressing this question is by Mendgen and colleagues (Mendgen et al., 2006), who tested the effect of different volatiles on agriculturally important species of rust fungi. All the fungi tested were negatively affected by exposure to farnesyl acetate. When an inoculated plant was incubated with this volatile, the number of haustoria significantly decreased, and the number of colonies was reduced by 48%. However, farnesyl acetate is emitted together with nonanal and decanal, which instead promoted the development of rust fungi haustoria. Thus the question whether the production of this volatile is useful to the plant as an antifungal defense is left unanswered.

Two other studies considered the effect of GLVs on pathogenic necrotrophic fungi. The results showed a correlation between the the GLV emission of the plant and the reduction of disease development (Kishimoto et al., 2008; Shiojiri et al., 2006). Since GLVs are known signaling molecules that trigger the defensive machinery of the plant, it is possible that the reduction of the fungal performance observed in these studies may not be due to a

direct toxic action of GLVs, but rather to the elicitation of other defensive pathways (De Vos et al., 2005; Kishimoto et al., 2005). Kishimoto et al. (2008) experimentally disproved this hypothesis, and so the reduced fungal growth in this study can be attributed to the direct fungicidal activity of GLVs. However, in the study by Shiojiri et al. (2006), the possibility of GLVs as signaling molecules was not tested.

1.2 Volatiles as indirect plant defense

Since the earliest studies of Dicke, Turlings and others (Dicke et al., 1990; Turlings et al., 1990), evidence has accumulated showing that herbivore-induced volatiles can be used by arthropod predators and parasitoids to locate their host or prey. As predation or parasitation reduces plant damage, volatiles are considered mediators of a tritrophic defense strategy known as indirect defense.

The attraction of herbivore enemies to plant VOCs has been demonstrated for a restricted number of crop plants, belonging to the families of Poaceae, Fabaceae, Brassicaceae and Solanaceae (Unsicker et al., 2009). For example, maize (*Zea mays*, Poaceae), a well-studied species for tritrophic interactions, emits a characteristic volatile blend upon herbivore damage which is attractive to braconid parasitoid females (Schnee et al., 2006). Another example is given by Lima bean (*Phaseolus lunatus*, Fabaceae) which, when attacked by phytophagous spider mites, release volatiles that help predatory mites find their prey (de Boer et al., 2004). Although the attraction of carnivores to VOCs is usually measured with laboratory olfactometer experiments, a few studies on non-crop species have been performed in the field. Kessler and Baldwin (2001), in their study on *N. attenuata* in its natural habitat, showed that an increased emission of typical herbivore-induced volatiles leads indeed to the attraction of natural enemies. Interestingly, Poleman and colleagues (2009) have found a correlation between the attractiveness of *Cotesia* spp. to *Brassica* odor in olfactometer experiments conducted in the laboratory and parasitism rates in the field. This example shows how the results of carefully controlled laboratory experiments can predict a field situation.

While it is established that VOCs can attract herbivore enemies and that this attraction leads to a decrease in plant damage, it is not yet clear whether volatile emission actually benefits the plant in terms of fitness (Dicke and Baldwin, 2010). The only report of a beneficial effect of parasitism on plant fitness in a natural environment is a study by Gomez and

Zamora (1994) conducted on wild populations of a woody crucifer (*Hormatophylla spinosa*) in the mountains of the Sierra Nevada (Spain). The system included three parasitoid species and their host, a weevil seed-predator. Weevil larvae feeding on *H. spinosa* seeds remain paralyzed after parasitation by the chalcidoid wasp, thus stopping feeding. Parasitation, in this case, directly affected the number and viability of seeds, which are direct measures of plant fitness. However, in this system it is not known whether VOCs play a role in parasitoid attraction and, therefore, whether the increase in fitness observed after parasitoid attack is to be attributed to the action of volatiles.

Other studies on the correlation of plant fitness and parasitism were performed on folivores under greenhouse conditions. Van Loon and coworkers (2001) showed that *Arabidopsis thaliana* plants fed upon by parasitized *Pieris rapae* caterpillars maintained a significantly higher leaf area compared to plants treated with intact caterpillars, resulting in a 250% increase in seed number (van Loon et al., 2000). A similar experiment was conducted with maize, and again young plants fed upon by a single parasitized moth larva produced about 50% more seeds compared to non-parasitized controls (Hoballah and Turlings, 2001; van Loon et al., 2000).

Not only herbivore feeding, but also egg deposition can induce the emission of volatiles that attract carnivore arthropods. The first studies on egg-induced volatiles were conducted by Meiners and Hilker (1997, 2000), who demonstrated that the eulophid egg parasitoid *Oomyzus gallerucae* is attracted by volatiles produced by the field elm (*Ulmus minor*) after *Xanthogaleruca luteola* (Coleoptera: Chrysomelidae) oviposition. Similar results were obtained in studies on the eulophid egg parasitoid *Chrysonotomyia ruforum*. Oviposition by the sawfly *Diprion pini* in Scots pine's needles (*Pinus sylvestris*) enhances the production of (*E*)-β-farnesene, thus making the emitted volatile blend attractive to the egg parasitoid (Hilker et al. 2002, Mumm and Hilker, 2005). In both the elm and the pine systems, neither artificial wounding of the plant nor herbivore eggs alone resulted attractive to the parasitoids, which specifically responded to the odor of egg-infested plants (Fatouros et al., 2008).

Besides attracting herbivore-enemies, volatiles can induce another sophisticated indirect defense system. Several plant species rely on ants and other carnivorous arthropods for protection against herbivores, with ants being attracted by extrafloral nectar produced mainly upon herbivore damage. It has been show that VOCs released by herbivore-damaged Lima bean leaves are able to elicit the production of extrafloral nectar on neighboring tendrils (Kost and Heil, 2006). The induction of both VOCs and extrafloral

nectar caused a significant reduction of herbivore damage under field conditions (Heil, 2004).

1.3 Consitutive volatiles and their role in plant defense

Since herbivore damage induces a massive release of volatiles in plants, most research on the defensive role of volatiles focused on herbivore-induced VOCs. Nevertheless, many plant species, especially trees, are known to emit volatiles constitutively (Kesselmeier and Staudt, 1999). For example, poplars (*Populus* spp.) can invest more than 10% of their photosynthetically fixed carbon in the emission of isoprene (Brilli et al., 2009). However, not much is known about the role of constitutive volatiles both in indirect and direct defense.

Experiments on constitutive volatiles in indirect defense have shown that constitutive VOCs can be either attractive or repellent to herbivore's enemies. Attraction of insect enemies to uninfested plants has been documented for some aphid parasitoids (Hymenoptera: Braconidae) (reviewed by Hatano et al., 2008) and for two lepidopteran parasitoids (the braconid *Microplitis croceipes* and the ichneumonid *Campoletis sonorensis*) towards cotton plants (*Gossypium hirsututm* L.) (Elzen et al., 1986, 1987). Instead, isoprene-emitting plants were repellent to the parasitoid wasp *Diadegma semiclausum* (Loivamaki et al., 2008). Egg parasitoids (*Trichogramma* spp.) have been shown to respond to volatiles of undamaged plants, mostly arresting rather than attracting the wasps (Fatouros et al., and references therein).

Constitutive volatiles do not provide any information on the presence of suitable hosts or prey to insect herbivores. However, they may be used by 'searching' parasitoid and predators as cues to locate their host habitat from a distance.

Volatiles are costly compounds for the plant. As opposed to chemicals that, once produced, stay in the plant, volatiles are constantly released and therefore represent a permanent loss of energy and fixed carbon for the plant (Schoonhoven et al., 2005). Moreover, for terpene volatiles the complex enzymatic biosynthetic machinery and the extensive chemical reduction of the products may entail significant costs (Gershenzon, 1994). Constitutive volatiles as defenses may thus be cost-effective only for plants that experience very frequent attack. They could act as: 1) toxins or feeding deterrents, causing the death of the insect or reduced food consumption and consequent retarded development, or 2) repellents,

causing avoidance of the volatile-emitting plant as food source. However, not much research has been done on the anti-herbivore activity of constitutive volatiles apart from the already mentioned study on feeding deterrence by isoprene. In contrast with the antifeedant effect of isoprene found by Laothawornkitkul et al. (2008), Loivamaki and coworkers (2008) showed that *Pieris rapae* and *Plutella xylostella* caterpillars were not affected by isoprene emission from transgenic Arabidopsis plants: they fed equally on isoprene-emitting and wild-type plants, and this volatile did not influence their growth rates.

In contrast to insect herbivores, airborne fungal pathogens can threaten the plant with large loads of spores that land and germinate on aboveground organs over extended periods of time. Therefore, it is possible that constitutive vegetative volatiles could act as anti-fungal defenses.

If the development of fungi is likely to be affected by plant volatiles, then volatiles could in turn be affected by fungal infection. In fact, willow leaves infected by a rust fungus (*Melampspora epitea*), produced less isoprene but significantly more mono- and sesquiterpenes compared to non-infected leaves (Toome et al., 2010). Such alterations may also occur after infection of the plant with beneficial fungi, like arbuscular mycorrhizae. Arbuscular mycorrhizal fungi (AMF) are obligate symbionts which live in association with the roots of about 80% of the terrestrial plants. Mycorrhization is commonly considered a symbiosis because the fungus provides the plant with nitrogen, phosphates and water, and receives photosynthates from the plant (Smith and Read, 1997). Through this exchange, the plant can withstand nutrient scarcity and drought, and grow better overall. The establishment of such an intimate association requires a molecular cross-talk between plant and fungus. At the early stages of the infection, defense responses similar to the ones provoked by biotrophic pathogens are triggered in the plant (reviewed by Garcia-Garrido and Ocampo, 2002). The physiological reaction caused by mycorrhization may then modify the plant's defense signalling altering both constitutive and induced defenses, including volatile emission. Mycorrhization is known to enhance the transcription of genes involved in terpene biosynthesis in several species including *Medicago truncatula* L., *Nicotiana tabacum* L., *Zea mays* L., *Lycopersicon esculentum* Mill., and *Triticum aestivum* L. (Walter et al. 2000, 2002). However, this AMF-mediated activation of the terpene pathway has not yet been examined with respect to the formation of monoterpene volatiles.

Nutritional alterations caused by AMF can also influence plant defenses. A greater nutrient supply from the AMF may provide the plant with more resources to allocate to defense. On the other hand, if the outflow of photosynthates to the fungal symbiont is greater than the

increase in productivity due to enhanced nutrient supply, there may be a net decrease in carbon supply that could lead to a decline in defense metabolism. Constitutive volatiles may therefore act as direct or indirect plant defenses, and they are likely to be altered by the widespread association of plants with AMF.

1.4 Approaches to study the function of volatiles

The function of plant volatiles can be studied using different experimental approaches.
Synthetic volatile standards are widely employed both in laboratory and field behavioral assays with arthropod carnivores. They are sometimes applied to odorless plants, or released by dispensers in olfactometer or wind tunnel experiments (e. g. Kost and Heil, 2008; Whitman and Eller, 1992). To maintain constant emission and at realistical levels, volatiles are often imbedded in a lanolin paste, or released from a rubber septum (e.g. De Moraes et al., 2001; Kost and Heil, 2006). These techniques are useful to test one or few compounds for which synthetic standards are commercially available or can be conveniently synthesized in the laboratory. But natural volatile blends often contain compounds that cannot be purchased, or are difficult to synthesize. For these reasons, transgenic organisms modified to express the genes responsible for volatile production have proved to be a very useful tool for testing VOC function (e.g. Loivamaki et al., 2008; Schnee et al., 2006). *A. thaliana* plants, in particular, are suitable for transformation because they do not emit significant levels of VOCs in the rosette stage and can be genetically engineered following standard procedures. Moreover, the wealth of knowledge already present about the biology of this model plant facilitates the characterization of important traits that might be affected by transformation.
Another elegant way to study the role of VOCs consists in knocking out the genes coding for the volatiles of interest, thus generating non-emitting plants. This approach avoids the introduction of a new trait in a plant species and the undesirable epistatic and metabolic effects caused by transgene expression. However, gene knock-out is often difficult to achieve, especially for monocotyledons such as maize (Schnee, Ph.D. Dissertation).
In this work, we used *A. thaliana* plants overexpressing terpene synthase genes as tools for investigating the function of vegetative volatiles.

1.5 Biosynthesis of terpene volatiles in plants

Despite being so abundant, all terpenes share a common basic building block: a five-carbon-unit structure that can be either isopentenyl diphosphate (IPP), or dimethylallyl diphosphate (DMAPP) (Fig. 1.2).

There are two routes leading to the production of IPP and DMAPP in higher plants: the mevalonate pathway, which takes place in the cytosol, and the methylerythritol phosphate (MEP) pathway, which takes place in the plastids (Fig. 1.2). Sesquiterpenes usually derive from the mevalonate route, while monoterpenes and diterpenes, as well as carotenoids, usually derive from the MEP route. The compartmental separation of the two pathways is not absolute, since there can be limited exchange of IPP and DMAPP or a common downstream intermediate (Eisenreich et al., 2001; Hemmerlin et al., 2003).

Terpene synthases are enzymes that catalyze the carbon skeleton-forming reactions in the terpene biosynthetic pathway. Although these reactions are often followed by many other transformations in terpene metabolism, for volatile terpenes, terpene synthases are often the final biosynthetic step. These enzymes make up a large family of which nearly half can form multiple products (Degenhardt et al., 2009).

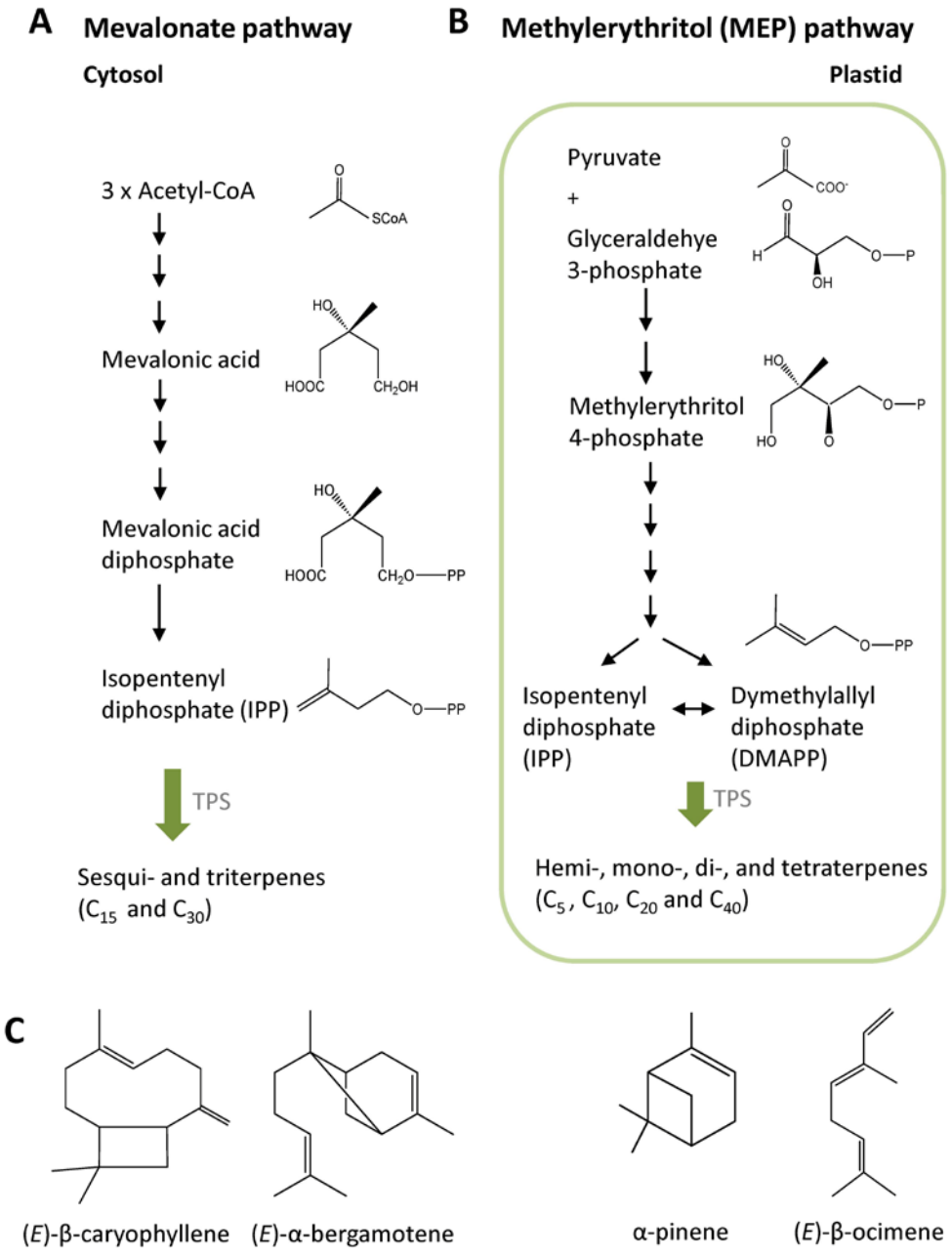

Fig. 1.2: Overview of the biosynthetic pathways leading to the production of isopentenyl diphosphate (IPP) and dimethylally diphosphate (DMAPP), the basic units of terpenes (A, B). Structure of common plant sesqui- and monoterpenes volatiles (C). TPS: terpene synthase.

1.6 Aim of the thesis

In this work we investigated the function of vegetative volatiles in plant defense against insect herbivory and fungal pathogen attack (Fig. 1.3). In particular, we aimed to understand:

1. The potential function of a complex constitutive sesquiterpene volatile blend in direct defense against a generalist lepidopteran herbivore and a fungal necrotrophic fungus (chapter I).
2. The interaction of constitutively emitted terpene volatiles with herbivore-induced ones in parasitoid attraction (chapter II).
3. The modification of plant defense metabolites, including volatiles, in a naturally-occurring association (plant - arbuscular mycorrhizal fungus) (chapter III).

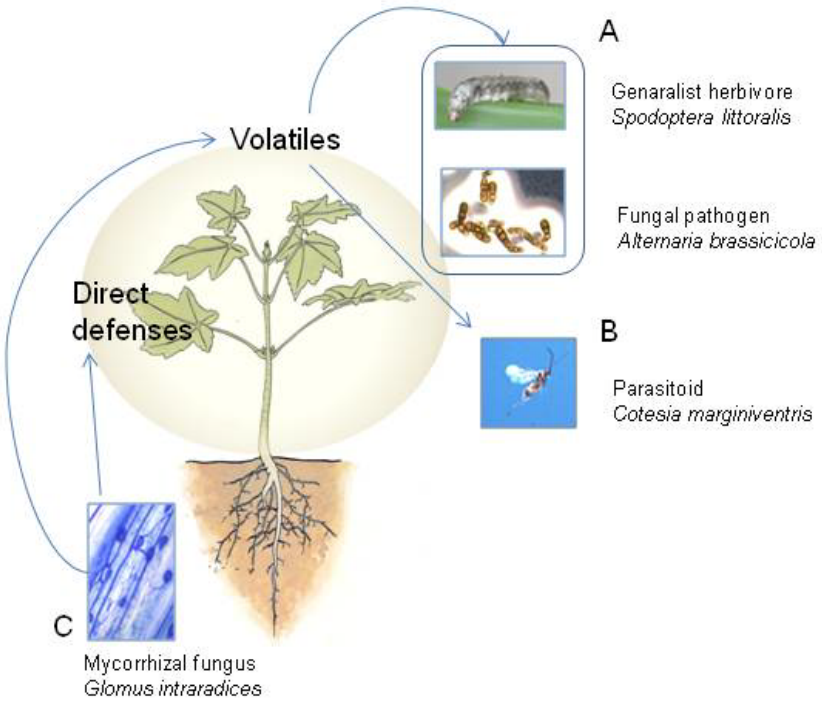

Fig. 1.3: Scheme of the interactions studied in this work. We investigated: A. How volatiles affect the development of an herbivorous insect and a fungal pathogen (chapter I); B. The role of constitutive volatiles in parasitoid attraction (chapter II); C. How the common association with an arbuscular mycorrhizal fungus can influence plant volatile emission and other potential anti-herbivore defenses (chapter III). Drawing of the plant © Copyright D G Mackean.

2. Chapter I

Volatile sesquiterpenes produced by the terpene synthase 8 (TPS8) from maize are involved in defense against fungal pathogens*

Abstract Low concentrations of mono- and sesquiterpenes (present in microgram or nanaogram levels per gram fresh weight) are found in the vegetative parts of almost all plants. Although these volatiles are known to possess antimicrobial properties, their efficacy against fungal pathogens *in planta* has not been studied. Maize seedlings of the inbred line B73 produce low concentrations of a complex terpene blend that is dominated by germacrene D. We identified the terpene synthase TPS8 which is responsible for the formation of this terpene blend. Biochemical characterization of this multiproduct enzyme revealed that 54 structurally diverse sesquiterpenes are formed from the FPP substrate. TPS8 is expressed throughout the leaves, sheath tissue and roots of the undamaged maize plant but is increased by herbivore attack and to a lower degree by infection with pathogenic fungi. We investigated the functional role of the maize TPS8 terpenes utilizing transgenic Arabidopsis that overexpressed TPS8 in an effort to separate TPS8 products from other maize terpenes and to conduct bioassays with isogenic plant lines. The feeding behavior by the generalist herbivore *Spodoptera littoralis* was not affected by the expression of TPS8 terpenes in Arabidopsis. When challenged with the nectrotrophic fungus *Alternaria brassicicola,* the transgenic Arabidopsis plants contained a lower amount of fungal biomass per gram fresh weight compared to the wild-type. The reduction in fungal growth was independent of other changes in antifungal defense compounds like glucosinolates or camalexin. No evidence was obtained that TPS8 sesquiterpenes act via JA-mediated formation of other defensive metabolites, so pathogen inhibition may be due to direct anti-fungal activity of the enzyme products. Our results suggest that sesquiterpenes present constitutively throughout the plant and emitted to the atmosphere can increase plant resistance against pathogenic fungi.

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2.1 Introduction

Many plants accumulate terpenes to fend off their enemies which include many types of herbivores as well as bacterial and fungal pathogens, (Gershenzon and Dudareva, 2007; Unsicker et al., 2009). To store these substances at high concentrations (0.1 – 5%) while avoiding autotoxicity, many plants contain specialized structures to produce and store terpenes, e.g. peppermint (*Mentha x piperita*), a Lamiaceae species which accumulates terpene-rich oils in glandular trichomes on the leaf surface, thus creating a barrier against herbivores and pathogens (Edris and Farrag, 2003; Turner and Croteau, 2004). Conifers produce and store high amounts of mono- and diterpenes in resin ducts which rupture on herbivore attack deterring attackers and associated fungal invaders (Phillips and Croteau, 1999).

However, most plants lack specialized storage structures and produce volatile mono- and sesquiterpenes in low levels (microgram or nanogram amounts per gram fresh weight) throughout their vegetative organs. These terpenes cross membranes readily and thus may diffuse freely through plant tissues and volatilize into the surrounding airspace. For example, undamaged leaves of maize (*Zea mays* L.) seedlings emit about 100 ng h^{-1} g fresh $weight^{-1}$ of sesquiterpenes (Köllner et al., 2004a). Volatile terpenes have been thought to be plant defenses also, but have usually been studied as deterrents for herbivores or attractants for herbivore enemies, rather than as direct toxins (Degenhardt, 2009).

Terpenes have been frequently reported as direct herbivore toxins (Gershenzon and Dudareva, 2007), and have also been implicated as anti-microbial factors. The antifungal activity of terpenes has been frequently demonstrated by the use of terpene-rich essential oils in *in vitro* bioassays (e.g Mueller-Riebau et al., 1995; Pitarokili et al., 2003; Tuberoso et al., 2005). Surprisingly, in some of these studies the vapor phase of essential oils could inhibit fungal growth more efficiently than the liquid phase (Cavanagh, 2007 and references therein). Terpene volatiles might directly interact with the aerial mycelium and reach the non-aerial hyphae after diffusing through the growth medium (Inouye et al., 2000, 2001). It is thus difficult to accurately establish the quantity of active compounds with which the target fungus comes into contact and to estimate whether the assay accurately simulates the *in vivo* situation. It would be much more realistic to test the anit-microbial activity of volatile terpenes by manipulating their production and emission in intact plants.

Maize (*Zea mays*) produces approximately 100 volatile mono- and sesquiterpenes that are emitted in blends of different complexity from its vegetative organs. An analysis of the sesquiterpene hydrocarbons in the inbred line B73 identified at least five groups of compounds that are differentially regulated throughout the plant (Köllner et al., 2004a). Each of the groups appears to be formed by a multiproduct terpene synthase which converts farnsesyl diphosphate, the precursor of most sesquiterpene compounds, into a characteristic blend of sesquitperpenes. For example, leaf herbivory by lepidopteran larvae induces the release of a sesquiterpene blend dominated by (*E*)-β-farnesene and (*E*)-α-bergamotene (Köllner et al., 2004a) which is produced by terpene synthase TPS10 (Schnee et al., 2006). These volatiles can attract parasitoids of lepidopteran larvae and thereby benefit the plant in an interaction termed 'indirect defense' (Hoballah et al., 2004; Schnee et al., 2006). Meanwhile, maize roots attacked by larvae of *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) emit (*E*)-β-caryophyllene under catalysis of TPS23 and this sesquiterpene attracts entomopathogenic nematodes, a natural enemy of the larvae of *D.*

virgifera virgifera (Rasmann et al., 2005). A second group of root-borne volatiles expressed constitutively is dominated by (*S*)-β-bisabolene and (*S*)-β-macrocarpene which are produced by the terpene synthases TPS6 and TPS11 (Köllner et al., 2008). In contrast, the terpene synthases TPS4 and TPS5 are developmentally regulated and only show activity in leaves of mature plants (Köllner et al., 2006).

In our continuing effort to elucidate the biosynthesis and ecological functions of terpenes, we identified maize terpene synthase 8 (TPS8), a multiproduct enzyme that is responsible for the production of a complex terpene blend present constitutively in almost all tissues of the plant and released to the atmosphere. We utilized transgenic Arabidopsis plants overexpressing TPS8 to demonstrate that these volatile terpenes increase plant resistance towards a necrotrophic fungus like *Alternaria brassicicola* Schw. but do not defend the plant against attack of generalist herbivores.

2.2 Methods and materials

2.2.1 Isolation, characterization and heterologous expression of the maize terpene synthase TPS8

Plant and insect material Seeds of the maize (*Zea mays*) inbred line B73 were provided by KWS Seeds (Einbeck, Germany). Plants were grown in commercially available potting soil in a climate-controlled chamber with a photoperiod of 16 h, 1 mmol $(m^2)^{-1}$ s^{-1} photosynthetically active radiation, a day/night temperature cycle of 22°C/18°C and 65% relative humidity. Caterpillars of *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae) (Syngenta, Basel, Switzerland) were reared on artificial bean diet (Fontana et al., 2009) at 21°C.

cDNA library construction One gram of leaf material was finely ground in a mortar in liquid nitrogen. 10 mL of Trizol reagent (Invitrogen, Carlsbad, CA) were added to the leaf powder. The mixture was treated with a Polytron (Kinematika AG, Luzern, Switzerland) for 1 min and incubated for 3 min on ice. Total RNA was isolated according to the manufacturer's instructions. The mRNA was isolated from about 80 µg of total RNA by using poly-(T)-coated ferromagnetic beads (Dynal, Oslo) and transcribed into cDNA for the construction of a Marathon RACE library according to the manufacturer's instructions (BD Bioscience, Palo Alto, CA).

Isolation of the maize terpene synthase* tps8 *cDNA A sequence with high similarity to plant terpene synthases was identified in a BLAST search of a public EST database (ZmDB, Gai et al., 2000). To isolate the full-length cDNA, the sequence was extended toward the 5' end by the Marathon RACE procedure (BD Bioscience Clontech) with the cDNA library described above. The complete sequence ORF of 1620 bp was designated *tps8* and deposited in GenBank (www.ncbi.nml.nih.gov) with the accession number AY928080.

***Heterologous expression of the terpene synthase gene* tps8** For expression with an N-terminal strep tag, the complete ORF of *tps8* was amplified with the primers tps8fwd (ATGGTACGTCTCAGCGCATGGCGCCGAAGACTGTGTGG) and tps8rev (ATGGTACGTCTCATATCAGCAGAGGGGAACATGGTTGACG) and cloned as a *Bsm*BI fragment into the bacterial expression vector pASK-IBA7 (IBA-GmbH, Göttingen, Germany). The construct was introduced into *Escherichia coli* strain TOP10 and fully sequenced to avoid errors introduced by DNA amplification. Liquid cultures of the bacteria harbouring the expression construct were grown at 37°C to an OD_{600} of 0.5. The expression of the construct was induced by adding anhydrotetracycline (IBA GmbH) to a final concentration of 200 µg L^{-1}. Cells were incubated for 20 h at 18°C, then collected by centrifugation and disrupted by 4 x 30-s treatment with a sonicator (Bandelin UW2070, Berlin, Germany) in chilled extraction buffer (50 mM MOPSO, pH 7.0, with 5 mM MgCl, 5 mM sodium ascorbate, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM dithiothtreitol and 10% (v v^{-1}) glycerol). The cell fragments were removed by centrifugation at 14,000*g* and the supernatant was desalted into assay buffer (10 mM MOPSO, pH 7.0, 1 mM dithiothreitol, 10% (v/v) glycerol) by passage through a Econopac 10DG column (Bio-Rad, Hercules, CA).

Assay for terpene synthase activity The assay contained 50 µL of the bacterial extract and 50 µL of assay buffer with 10 µM (*E*,*E*)-farnesyl diphosphate, 10mM $MgCl_2$, 0.05 mM $MnCl_2$, 0.2 mM $NaWO_4$, and 0.1 mM NaF in Teflon-sealed, screw-capped 1mL glass vials. The assay was overlaid with 0.1 mL of pentane to trap volatile products and incubated for 30 min at 30°C. The reaction was stopped by mixing and 1 µL of the pentane phase was analyzed by GC-MS.

GC and terpene identification A Hewlett-Packard model 6890 gas chromatograph was employed with the carrier gas He at 1 mL min^{-1}, splitless injection (injector temperature 220 °C, injection volume 1 µL), a Chrompack CP-SIL-5 CB-MS column ((5%-phenyl)-methylpolysiloxane, 25 m x 0.25 mm i.d. x 0.25 µ film thickness, Varian, USA) and a temperature program from 40°C (3 min hold) at 5°C min^{-1} to 240°C (3 min hold). The

coupled mass spectrometer was a Hewlett-Packard model 5973 with a quadrupole mass selective detector, transfer line temperature 230°C, source temperature 230°C, quadrupole temperature 150°C, ionization potential 70 eV and a scan range of 40-350 atomic mass units. Products were identified by comparison of retention times and mass spectra with authentic reference compounds as described by (Köllner et al., 2004a). Quantification was performed with the trace of a flame ionization detector (FID) operated at 250°C. A nonyl acetate internal standard was utilized to determine the average and standard error of three independent samples.

Generation of transgenic* Arabidopsis thaliana *plants The ORF of *tps8* was amplified from the pASK-IBA7 construct with the primers tps8fwd1 (TCAGGATCCTATGGCGCCGAAGAC) and tps8rev1 (ACTGGTACCTCTAGCAGAGGGGAAC) and inserted as a *Bam*HI–*Kpn*I fragment between the 35S promoter of the cauliflower mosaic virus (CaMV) and a nopaline synthase terminator into the binary vector pBIN420 (Spychalla et al., 1997). The obtained construct was introduced into the *Agrobacterium tumefaciens* GV3101 strain, which was used to transform Arabidopsis (ecotype Col-0) plants by the floral dip method (Clough and Bent, 1998).

Transgenic lines were selected by kanamycin resistance, and transformation was additionally confirmed by PCR analysis.

2.2.2 TPS8 transcipt level analysis in maize after herbivory and *Colletotrichum graminicola* infection

Plant, insect and fungus material The transcript levels of TPS8 in maize plants were analyzed by RNA gel blot and real time quantitative PCR (RT-qPCR). For the RNA blot, leaves, sheaths and roots were collected and immediately frozen in liquid nitrogen. The herbivory treatment consisted of feeding by three third-instar on two-week-old plants for about 14 h. Caterpillars were enclosed in a cage made of two modified halves of a 9-cm diameter Petri dish on the middle portion of the plant (Rose et al., 1996). Leaves and husks of undamaged adult plants (~12 weeks old) grown in a greenhouse were collected in the same way.

For the RT-qPCR assays, two-week-old seedlings were divided in three groups of three or four plants that underwent the following treatments: Group 1 - Herbivory (H): two third-

instar *S. littoralis* caterpillars per plant were allowed to feed overnight on the foliage. The caterpillars were enclosed in cages as described before. Group 2 - Fungus (F): leaves were infected with 150 µL of a *Colletotrichum graminicola* (Ces) Wils. spore suspension. The plants were then enclosed within a cellophane bag (205 x 380 mm, Unipack, Germany) to prevent cross-inoculation. Infection took place four days before the herbivory treatment. Group 3 - Control (C): control plants received no herbivory or no fungal infection. Plants were harvested at once by cutting leaves and sheaths and flash-freezing in liquid nitrogen. The roots of these plants were immediately washed to remove soil and flash-frozen. All the material was stored at -80°C. *C. graminicola* (strain DSMZ 63127) was grown at 26°C on oatmeal agar medium obtained by mixing 50 g of finely ground commercially available oat flakes (Fortin Mühlenwerke, Düsseldorf) with 12 g agar-agar (Roth, Karlsruhe, Germany) and 1 L deionized water. The substrate was sterilized for 8 min at 121°C before inoculation with the fungus. Spores were collected by flooding the plates with ca. 2 mL deionized water and scratching gently with an inoculating loop. Spores were washed twice with deionized water and their concentration was adjusted to 5 x 10^6 spores mL^{-1}. A 0.5 µl mL^{-1} portion of Tween-20 was added to the suspension prior to plant infection.

RNA gel blot Plant RNA was prepared with the RNeasy plant mini kit (Qiagen, Hilden, Germany) according the manufacturer's instructions. A 610 bp fragment containing the N-terminal part of *tps8* was used as a probe, generated by linear PCR with the primer tps8rev2 (TGTCGTCGGCACTGCTGTATTCC) and the complete ORF as a template. The probe was labeled with ^{32}P-adenosine triphosphate using the Strip-EZ PCR procedure (Ambion, TX, USA). Blotting on a Nytran-Plus nylon membrane (Schleicher & Schuell, Germany), hybridization and washing were carried out following standard procedures (Sambrook et al., 1989).The blots were scanned with a Storm 840 phosphoimager (Molecular Dynamics, Sunnyvale, CA). All RNA hybridization experiments were performed in two biological replicates.

RNA isolation for RT-qPCR assays Total RNA was isolated from 100 mg aliquots of leaf, sheath or root material finely ground in liquid nitrogen with a Quiagen RNeasy plant mini kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was digested on column for 15 min with Dnase I (Quiagen). Purified RNA was stored at -80°C. RNA concentration and 260:280 and 260:230 nm ratios were determined spectrophotometrically and the quality of the RNA assessed with an Agilent 2100 Bioanalyzer (Palo Alto, California, USA) using a RNA 6000 Nano LabChip®.

Reverse transcription and cDNA purification For cDNA synthesis, Superscript III reverse polymerase (Invitrogen, Carlsbad, California, USA) was used according to the manufacturer's instructions, with reverse transcription of 3 µg total RNA. RT-qPCR experiments were performed on a Stratagene Mx3000P (La Jolla, California, USA) using SYBR® green I with ROX as an internal loading standard. Each 25-µl reaction contained cDNA corresponding to 5 ng total RNA. Controls included non-RT controls (using 5 ng total RNA without reverse transcription to monitor for genomic DNA contamination) and non-template controls (water template). PCR thermocycles started with denaturation at 96°C for 6 min followed by 40 cycles of 30 s at 95°C, 1 min at 60°C, and 1 min at 72°C, followed by one last cycle of 30 s at 95°C, 1 min at 55°C, and 30 sec at 95°C. Fluoresence was read following each annealing and extension phase. All runs were followed by a melting curve analysis from 55 to 94°C. The products of each primer pair were cloned and sequenced ten times to verify primer specificity. The linear range of template concentration to threshold cycle value (C_t value) was determined by performing a series of sixfold dilutions using cDNA from three independent RNA extractions analyzed in three technical replicates. Primers of the reference gene for maize RNA polymerase II largest subunit (accession AF 519538) were: RP2fwd (GCTGGATGATGAGAATTGGAGACC), RP2rev (GCTTGAGGTTCACAGGCATAGG). Primers for *tps8* (accession AY 928080) were tps8fwd2 (ACGGGAGGGATCGGATTGTTG), and tps8rev3 (GCACTCAGGTAGAAGAGAAATATCATCAC). Primers were designed using BeaconDesigner (version 5.0; PremierBiosoft, Palo Alto, California, USA) and HPLC purified (Invitrogen). Primer efficiencies for all primer pairs were calculated using the standard curve method (Pfaffl, 2001). All amplification plots were analyzed with the MX3000P™ software to obtain C_t values.

2.2.3 Insect bioassays on *Arabidopsis thaliana*

Feeding assay Feeding assays were performed in order to investigate the performance of *S. littoralis* larvae on transgenic *A. thaliana* overexpressing TPS8 in comparison to wild-type Arabidopsis controls.

The plants were grown in the same conditions as described below for the fungus bioassay, with the difference that the plants were not transferred to glass vials. In order to control for possible position effects of the TPS8 overexpression lines, three lines derived from independent transformation events were chosen.

For each line, five four-week-old plants were randomly chosen and five neonate *S. littoralis* larvae were set on each plant. In order to prevent caterpillars from escaping, each plant was enclosed in a perforated plastic bag (30 x 20 cm, holes ca. 0.7 mm diameter, 0.5 cm distant to one another) that was tightened with a rubber band around the pot. After seven days, the weight of each caterpillar was assessed for the first time and it was recorded every second day for the following week. If the caterpillars consumed more than 70% of the leaf area of one plant during this time, all plants in the experiment were substituted with undamaged ones. At the end of the second week, all plants were substituted with undamaged ones and the caterpillars were reduced to two per plant by removing the heaviest and the lightest ones, so that the variability within groups of caterpillars feeding on each line was reduced. From this day on, the caterpillar weight was recorded daily until all insects pupated. The weight of the pupae and of the emerged adults was recorded and the percentage of adults emerged on the initial number of larvae calculated. The number of days from the beginning of the experiment to pupation and to adult emergence was also recorded. This experiment was repeated three times.

Choice experiments A choice experiment was performed to test the attraction of *S. littoralis* caterpillars to two lines of TPS8 overexpressing plants and wild type control plants. In each experiment, 7 or 10 third-instar caterpillars were individually put in the middle of an arena and given the choice between two plants positioned opposite to one another. The arena consisted of a Plexiglas square surface with six holes of 4 cm diameter cut at regular distance to one another along a 20 cm diameter circumference. Plants were positioned in the holes so that the pot remained below the Plexiglas surface, while the rosette rested above. The arena was put in a dedicated growth chamber at 21°C, 55% relative humidity and 250 μmol s^{-1} m^{-2} photosynthetic active radiation. Every caterpillar had 30 min to choose one of the plants, i.e. to come into contact with a leaf, before being removed from the arena. The arena was cleaned with acetone and water and dried with a tissue between the releases of the larvae. The plants and their position in the arena were changed in each experiment, which was repeated six times.

A second experiment was conducted to calculate the feeding deterrence of TPS8 volatiles on *S. littoralis* caterpillars. Three TPS8 overexpressing plants and three Col-0 plants were alternated in the arena. Ten caterpillars were placed in the centre of the arena and allowed to choose any plant and feed on it. After 24 h, the caterpillars were removed, the rosettes harvested and an image of each leaf was acquired by scanning. The total and consumed leaf

areas were calculated by analysis of the images with the software SigmaScan Pro 5.0. The experiment was repeated three times with three independent lines of TPS8 overexpressing plants.

2.2.4 *Alternaria brassicicola* bioassays on *A. thaliana*

Plant and fungus material *A. thaliana* plants (three TPS8 independent lines and wild-type ecotype Col-0) were grown singularly in 7 x 7 x 8 cm pots in commercially available potting soil. Plants were kept in a climate chamber with 8 h photoperiod at 21°C, 55% relative humidity and 250 µmol s^{-1} m^{-2} photosynthetic active radiation.

Four weeks after emergence, each plant was transferred to a 125 mL glass container (5 cm diameter) closed with a vented lid (Magenta Corporation, Chicago, USA) filled with ca. 3 cm of autoclaved potting soil. The lid was necessary in order to prevent cross contamination after infection of the leaves with the fungal pathogen as described below. The day after transplantation, half of the plants (six to eight) of each line were infected with 80 µL of an *Alternaria brassicicola* (Schweinitz) Wiltshire spore suspension containing 3.2 x 10^6 spores mL^{-1} in sterile water and 0.5 µl mL^{-1} Tween-20 ("infected plants"). The other half of the plants was mock-inoculated with 80 µL of sterile water and the same volume of Tween-20 ("non-infected plants"). *A. brassicicola* (strain MUCL 20297) colonies were grown at room temperature on malt-agar medium obtained by mixing 20 g agar-agar and 50 g of commercially available barley malt extract (Lindenmeyer, Heilbronn, Germany) with 500 mL deionized water. The substrate was sterilized for 20 min at 121°C before inoculation with the fungus. Spores were collected by flooding the plates with ca. 2 mL deionized water and scratching gently with an inoculating loop and were washed twice with deionized water. Four days after infection, all plants were cut at the rosette base, the aboveground part put in 2 mL Eppendorf tubes and immediately frozen in liquid nitrogen. Plants were then freeze-dried for at least 24 h, weighed and reduced to a fine powder by grinding the tissue in liquid nitrogen. The plant material was used for the extraction of chitin, chitosan, and glucosinolates.

For the time course analysis of camalexin concentration and for the RT-qPCR experiments, two TPS8 overexpressing lines and Col-0 wild-type were grown and infected or mock-inoculated as described before, with the exception that the treatments took place four days after transplantation to the glass vials. Three plants per each line were not treated at all and harvested right before the start of the experiment. These plants were used as normalizers in the RT-qPCR experiments. Three to four infected and mock-infected plants per line were

harvested at 6, 12, 24, 48 and 72 h after infection. Harvest consisted of cutting the plants at the rosette base and flash-freezing them in liquid nitrogen. The plant material was then kept at -80°C until used.

Chitin and chitosan extraction and D-glucosamine analysis Chitin and chitosan were used as indicators of the fungal biomass present in the plant (Osswald et al., 1995). A 1 mL portion of a 2% NaOH solution was added to the freeze-dried and finely ground leaf material. The samples were thoroughly shaken and heated at 98°C for 1 h to achieve deproteinization. The alkali-insoluble fraction was separated by centrifugation at 6000 *g* for 10 min and the supernatant was discarded. The pellet was washed twice with deionized water. Finally, 1 mL 6 N HCl was added and the samples were left to heat overnight at 98°C in order to monomerize chitin and chitosan to D-glucosamine (Synowiecki and Al-Khateeb, 1997). The acidic suspension was then filtered through a 0.2 µm mesh filter (Sarstedt, Nümbrecht, Germany) and diluted 1:100 in a 0.2 M borate buffer (pH 10.4) to reach a basic pH.

Samples were derivatized with 1/3 vol. of a 0.2 M boric acid solution (pH 10.4) containing 1.14% (w v^{-1}) O-phthaldialdehyde and 1.01% (v v^{-1}) 2-β-mecaptoethanol. After 2 min, 30 µL of each derivatized sample were injected in an Agilent HP1100 Series HPLC equipped with a Supelcosil LC-18-DB column (25 cm x 4.6 mm, 5 µm, Supelco, USA), kept at 28°C. D-Glucosamine was separated by using a solution containing 85% 0.02 M citric acid buffer (pH 5.5) and 15% methanol:acetonitrile (65:35) (solvent A) and a solution containing 10% of the 0.02 M citric acid buffer and 90% of the (65:35) methanol-acetonitrile solution (solvent B) as solvents. The flow was adjusted to 1 mL min^{-1}. The gradient for solvent B was the following: 0-27.7% (25 min), 27.7-100% (0.5 min, hold 4 min), 100-0% (0.5 min, hold 5 min). The eluent was monitored by fluorescence detection from 220 to 380 nm (5 nm interval). Additionally, the identity of D-glucosamine was confirmed by LC-MS. Concentrations of D-glucosamine were calculated using response curves generated with an external standard. The experiment was replicated four times with three different TPS8 lines. For each experiment and for each line, the differences between the D-glucosamine content of each infected plant and the mean D-glucosamine content of the non-infected plants were calculated and used in the statistical analyses.

2.2.5 Determination of *A. thaliana* secondary metabolites

Glucosinolate extraction and profile Glucosinolates were extracted and quantified following the procedure described in (Burow et al., 2006) and modified as follows: 1) 50

mg of ground leaf material were used for extraction, 2) 600 µL of the extract was loaded on DEAE columns, 3) desulfated glucosinolates were eluted with 0.5 mL ultrapure water.

Camalexin extraction and analysis Camalexin was extracted from 20-70 mg flash-freezed plant material, finely ground in liquid nitrogen with a 50% methanol, 0.25% formic acid solution. The volume added to each sample was adjusted to the ratio 500 µL solution/100 mg plant material. Samples were vortexed, extracted for 30 min at room temperature, and centrifuged at 15700 *g* for 15 min. The supernatant was collected and analyzed by LC-MS-MS as follows. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent, Germany). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B respectively. The elution profile was: 0-0.5 min, 5% B; 0.5-1 min, 5-100% B in A; 1-2 min 100% B and 2.1-4.5 min 5% B. The mobile phase flow rate was 0.8 mL min^{-1}. The column temperature was maintained at 25°C. An API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source was operated in the positive ionization mode. The instrument parameters were optimized by infusion experiments. The ion spray voltage was maintained at 5500 V. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 70 psi, curtain gas at 35 psi, heating gas at 70 psi and collision gas at 2 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion: *m/z* 201.09 → 59.01 with collision energy 45 V and declustering potential 51 V. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing. Linearity in ionization efficiencies was verified by analyzing dilution series of samples containing camalexin.

2.2.6 LOX2 transcript level analysis in *A. thaliana* plants

RT-qPCR assays Plants were grown and harvested as described before (see A. brassicicola bioassays). RNA extraction, reverse transcription and cDNA purification were performed as described above for maize. Primers of the reference gene for A. thaliana adenine phosphoribosyltransferase 1 (sequence number At1g27450) were: APT1 Fwd: GTTGCAGGTGTTGAAGCTAGAGGT, APT1 Rvs. TGGCACCAATAGCCAACGCAATAG (Phillips et al., 2009). Primers for LOX2 (accession AT3G45140.1) were LOX2fwd (GTTTCTGGAGGGCATAACTTGGTC), and LOX2rev (TGGTATTGGTTCTGAATCTTGATGGC).

2.2.7 Statistical analyses

1. Insect feeding assays. For each experimental replicate, the influence of the plant line was assessed separately on a) the caterpillar growth rate, b) the pupal weight, c) the adult weight, d) the time to pupation and e) the time to adult emergence by analysis of variance (ANOVA).
2. First insect feeding choice experiment. To determine the effect of terpene production on the caterpillar's choice, a generalized linear model (GLM) with a quasipoisson error distribution was used.
3. Glucosinolate and camalexin analyses. The effects of fungal infection and of plant type (TPS8 and Col-0) on the amount of aliphatic and indole glucosinolates, and on the amount of the two indole glucosinolates 4-hydroxyindol-3-ylmethylglucosinolate (4OHI3M) and 4-methoxyindol-3-ylmethylglucosinolate (4MOI3M) were tested by two-way ANOVA. The effects of fungal infection and of plant type (TPS8 overexpression and Col-0) on the level of camalexin at each time point were also tested by two-way ANOVA. Camalexin quantities were log-transformed when needed to fulfil the requirements for ANOVA.
4. D-glucosamine analysis. The effect of the plant type on the relative glucosamine content was tested by analysis of covariance (ANCOVA), where plant type (TPS8 overexpression or Col-0) was considered as fixed factor and plant weight was fitted in the model as a continuous covariate.

Interaction between factors was always tested in two-way ANOVA and ANCOVA. Whenever possible, the models were simplified by removing non-significant terms (Crawley, 2007). All the analyses were performed with the software package R (R Development Core Team (2008), R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

2.3 Results

2.3.1 TPS8 is a multiproduct terpene synthase that produces 54 sesquiterpenes

To identify genes responsible for terpene production in maize, we screened a public EST database (ZmDB, Gai et al., 2000) for sequences with similarity to terpene synthases from other plants. One of the resulting sequences, designated *tps8*, contained an open reading

frame of 1620 bp. The encoded protein, TPS8, showed highly conserved elements of plant terpene synthases including the aspartate-rich region DDxxD and the NSE/DTE motif which both are involved in metal cofactor binding. The deduced amino acid sequence of TPS8 was similar to that of other terpene synthases from maize (Fig. 2.1). In a dendrogram analysis, TPS8 showed most sequence similarity with two previously identified monoterpene synthases, TPS19 (STC1) and TPS26 (Lin et al., 2008). In contrast to both monoterpene synthases, TPS8 contained a shorter N-terminus that indicated no presence of signal peptides, suggesting that TPS8 resides in the cytosol in the presence of farnesyl diphosphate (FPP), and acts as a sesquiterpene synthase *in planta*.

Heterologous expression of *tps8* in *Escherichia coli* yielded a protein which was able to convert FPP into a complex mixture of 54 sesquiterpene hydrocarbons and alcohols (Table 1, Fig. 2.2 A). The main hydrocarbon products were identified as germacrene D (19.2% of total products), α-copaene (7%), (*E*)-β-caryophyllene (5.6%), and δ-cadinene (5.0%). The substrates geranyl diphosphate (GPP) and gernanylgeranyl diphosphate (GGPP) were not accepted by the enzyme. The estimated K_m values for the FPP substrate and the cosubstrates magnesium and manganese (Table 2.2) were comparable to those of other terpene synthases from maize (Schnee et al., 2002; Köllner et al., 2004b; Koellner et al., 2008).

To rationalize the high number of reaction products formed by TPS8, we propose a reaction mechanism that focuses on the seven major products (Fig. 2.2 B). The reaction starts with the ionization of the diphosphate moiety of FPP. The resulting carbocation can undergo a 1,10- or 1,11-cyclization leading to the formation of the carbon skeletons of the germacrane-type products or caryophyllane-, and humulane-type products, respectively. Alternatively, the isomerization of the primary carbocation and subsequent 1,10-, 1,11-, 1,6-, and 2,7-cyclizations (the numbering of carbon atoms refers to that of the FPP substrate) results in the formation of cadinane- and copaane-type compounds. The final carbocations are deprotonated to yield the respective olefinic products or they are captured by water forming sesquiterpene alcohols.

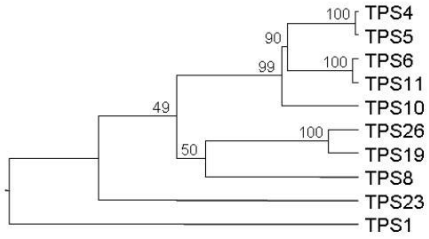

Fig. 2.1 Amino acid sequence aligment of maize terpene synthases. TPS8 is similar to other terpene syntases of maize. The dendrogram represents the sequence relationships of 10 terpene synthases from maize. Sequence alignment was performed by the Clustal W method with default settings in the program module MegAlign of the DNASTAR package. Bootstrap values resulting from 1000 trials are indicated at each node. The accession numbers for published protein sequences are: TPS1 (AAO18435), TPS4 (AAS88571), TPS5 (AAS88574), TPS6 (AAS88576), TPS10 (AAX99146), TPS11 (ACF58240), TPS19 (AAG37841), TPS23 (ABY79206), TPS26 (ABR09292)

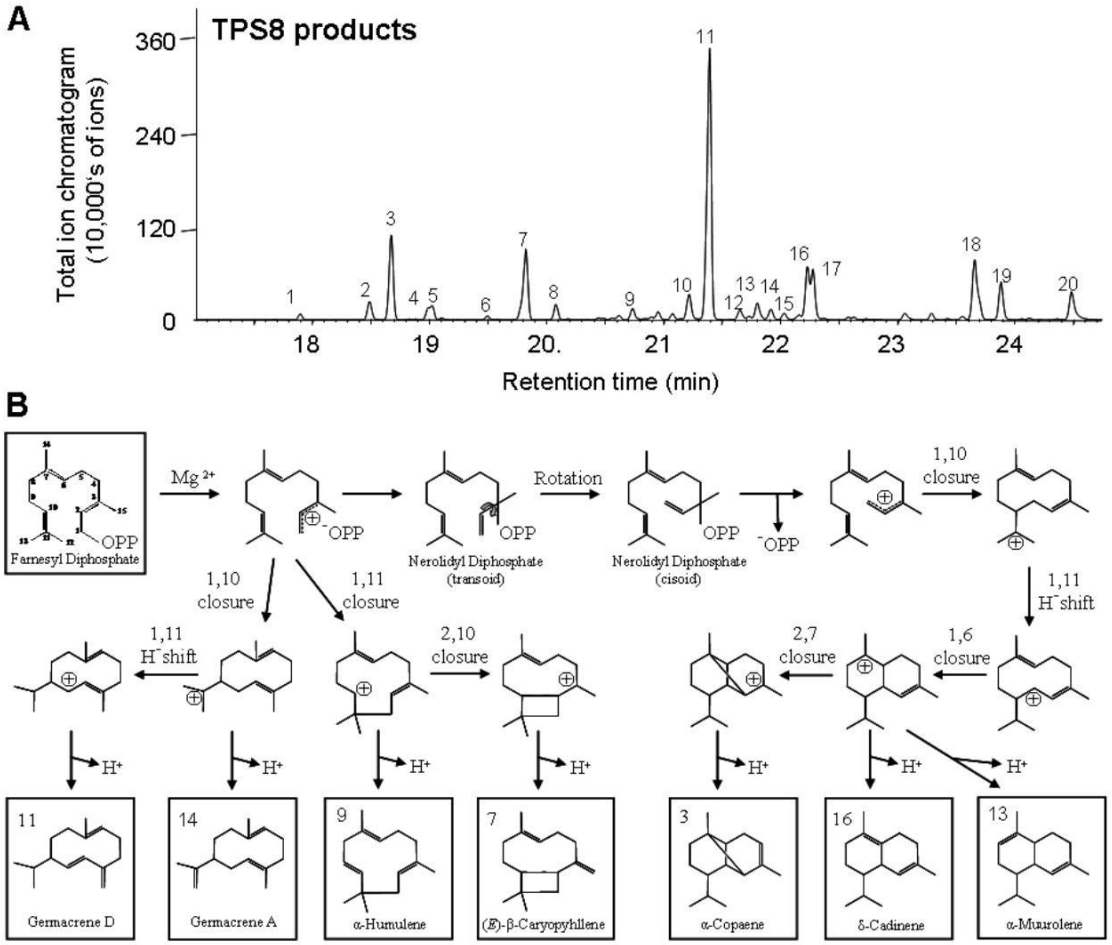

Fig. 2.2 *tps8* encodes a multiproduct terpene synthase. Products of TPS8 overexpressed in *Escherichia coli* (A) and proposed mechanism of formation of the principal compounds (B). The identity and the relative amounts of the peaks in A are shown in Table 2.1. Bacteria were incubated with farnesyl diphosphate and the volatile products were collected by SPME and analyzed by GC-MS using a DB-WAX column.

Table 2.1 Sesquiterpene products of TPS8-B73

peak	product	%
1	α-Cubebene	0.9
2	unknown	1.4
3	α-Copaene[a]	7.0
4	β-Cubebene	1.4
5	β-Elemene[a]	1.1
6	α-Gurjunene	0.5
7	(*E*)-β-Caryophyllene[a]	5.6
8	β-Copaene	1.7
9	α-Humulene	1.5
10	γ-Muurolene	1.5
11	Germacrene D[a]	19.2
12	Bicyclosesquiphellandrene	1.3
13	α-Muurolene[a]	1.5
14	Germacrene A[a]	0.9
15	γ-Cadinene[a]	1.3
16	δ-Cadinene[a]	5.0
17	oxygenated sesquiterpene 1	9.8
18	oxygenated sesquiterpene 2	10.1
19	oxygenated sesquiterpene 3	7.2
20	oxygenated sesquiterpene 4	7.0
--	34 unknown, each < 1 %	14.1

[a]Compounds were identified by matching GC retention time and MS to authentic standards. All other compounds were tentatively identified based on the mass spectrum and Kovat's retention index. The percentages refer to the total amount of terpene products formed by the enzymes and were obtained from measurements on GC-FID.

2.3.2 TPS8 is expressed throughout the maize seedling but regulated differently in roots and aboveground parts

To elucidate the spatial expression pattern of *tps8* throughout the maize plant, we analyzed transcript accumulation in the different organs and developmental stages of the maize inbred line B73. Both the RNA gel blot and the RT-qPCR analysis indicated the presence of *tps8* transcripts in the root, sheath and leaf tissue of undamaged, herbivore-treated or fungus-infected juvenile plants, but only traces were present in leaves and husks of mature plants (Fig. 2.3 A, B). In seedlings, the site of highest *tps8* transcript levels were the roots of undamaged plants. Herbivore damage by *S. littoralis* or fungal infection by *C. graminicola* affecting the leaves of the seedling reduced this level about 13-fold or 30-fold, respectively (Fig. 2.3 B). The levels of *tps8* transcripts in leaves of undamaged seedlings

were much lower than in the roots. In leaves, herbivore damage increased transcript levels approximately 5-fold while the increase after fungal infection was only marginal. In sheath tissue, *tps8* transcript levels were increased to a similar extent but *C. graminicola* infection caused no induction of *tps8* (Fig. 2.3 A, B). The products of TPS8 were detected in leaves, sheath and roots of seedlings of the inbred line B73 (Fig. 2.4). In leaves and sheath tissue, the terpene concentration increased after herbivory, thereby corresponding with *tps8* transcript levels. In roots, no reduction was observed, but instead a slight increase in terpene concentration indicating that terpene production in roots may be regulated not by the amount of expressed enzyme but by FPP substrate availability or competition with other terpene synthases like TPS6 or TPS11.

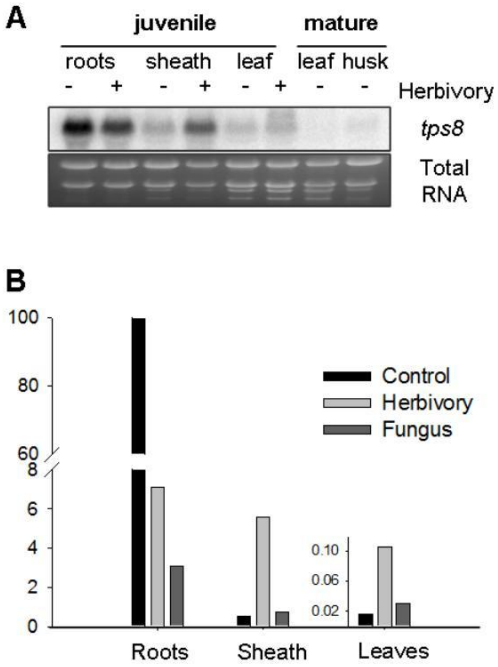

Fig. 2.3 The transcript levels of TPS8 are affected by herbivory and fungal infestation. (A). RNA gel blot analysis of TPS8 transcript levels in roots, sheaths and leaves of two-week-old B73 plants, untreated and after *S. littoralis* feeding, and in leaves and husks of an untreated mature plant. (B) Transcript levels of TPS8 in the different organs of B73 juvenile plants were also analyzed by RT-qPCR. Plants were untreated (Control), subjected to overnight *S. littoralis* feeding on the leaf (Herbivory) or infected by the fungus *C. graminicola* for four days (Fungus). Fold-changes in gene expression have been calculated and normalized to 1 to leaves of control plants. Graph depicts the fold-change percentages relative to the highest value (the concentration in roots of control plants was designated 100%)

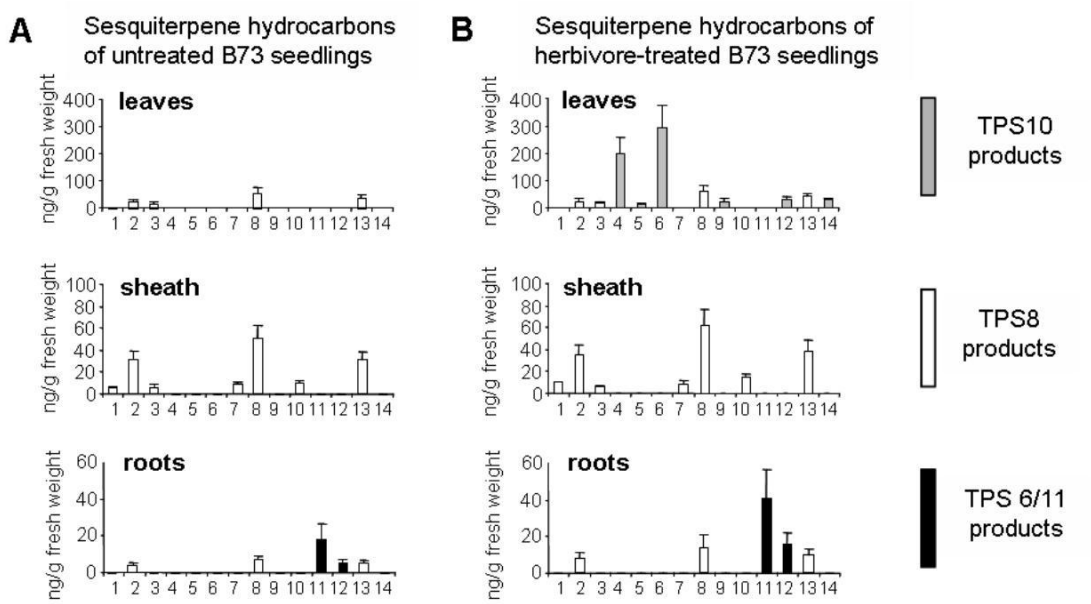

Fig. 2.4 TPS8 sesquiterpenes are present throughout the maize seedling. Sesquiterpene hydrocarbons in leaves, sheath and roots of maize seedlings of the inbred line B73, untreated (A) and after overnight feeding of *S. littoralis* caterpillars (B). Volatiles were extracted with pentane, identified by GC-MS by comparison of mass spectra and retention times with those of authentic standards, and quantified by comparison of FID responses to those of an internal standard. The identity of the compounds is shown in Table 2.1

2.3.3 The TPS8 sesquiterpene blend did not affect the development of the generalist herbivore *S. littoralis*

In order to investigate the role of TPS8 volatiles *in planta* without the interference of other maize terpenes, we transformed *A. thaliana* with *tps8* under the control of the cauliflower mosaic virus 35S RNA promoter. In their rosette stage, the transgenic plants released a sesquiterpene blend identical to that after heterologous expression of *tps8* in *E. coli* (Fig. 2.2 A, 2.5 A). The emission rate of total sesquiterpenes was between 10 and 14 ng gFW^{-1} h^{-1} at 1 mmol $(m^2)^{-1}$ s^{-1} photosynthetically active radiation which is about one third of the rate emitted by maize plants at optimal light conditions (Köllner et al., 2004a). Arabidopsis harboring the expression vector without terpene synthase did not produce any terpenes (Fig. 2.5 B).

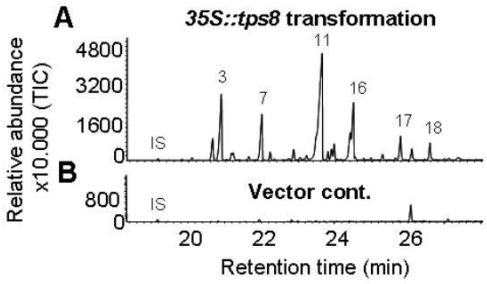

Fig. 2.5 Transgenic Arabidopsis plants overexpressing TPS8 produce the TPS8 sesquiterpene blend. Volatile profile of *A. thaliana* plants tranformed with *tps8* (A) and with an empty vector (B). The identity of the compounds is shown in Table 2.1

Table 2.2 Kinetic constants for TPS8-*B73* heterologously expressed in *E. coli*

	FPP [a]	Mg^{2+} [b]	Mn^{2+} [b]
K_m [µM]	1.6	75.3	4.6

[a]Values for FPP were measured in the presence of 10 mM Mg^{2+} and 0.05 mM Mn^{2+}

[b]Values for Mg^{2+} and Mn^{2+} were measured with 10 µM FPP

We investigated the effects of TPS8 terpenes on the development of the generalist insect herbivore *S. littoralis*. Caterpillars were reared on three lines of transgenic Arabidopsis overexpressing TPS8 and wild-type Col-0 plants. No significant differences were found in any of the experimental replicates between TPS8 and Col-0 plants for growth rate of the caterpillar, time until pupation, pupal weight, time until emergence and weight of the adult moths (Fig. 2.6, Table 2.3).

Attraction of *S. littoralis* caterpillars to TPS8-overexpressing Arabidopsis versus wild-type plants was assayed in a choice experiment. The caterpillars chose transgenic and wild-type plants almost equally (40.5% Col-0 and 45.5% TPS8), while 14% made no choice (Fig. 2.7 A). Thus the production of TPS8 volatiles did not affect larval choice (t= 0.26, P= 0.797).

A second choice experiment tested for a deterrent effect of TPS8 volatiles on feeding by *S. littoralis* larvae. Of the total leaf area consumed, 40 to 49% was of TPS8 plants versus 51

to 60% of Col-0 plants, indicating that these sesquiterpenes were only marginally deterrent to the herbivores at best (Fig. 2.7 B).

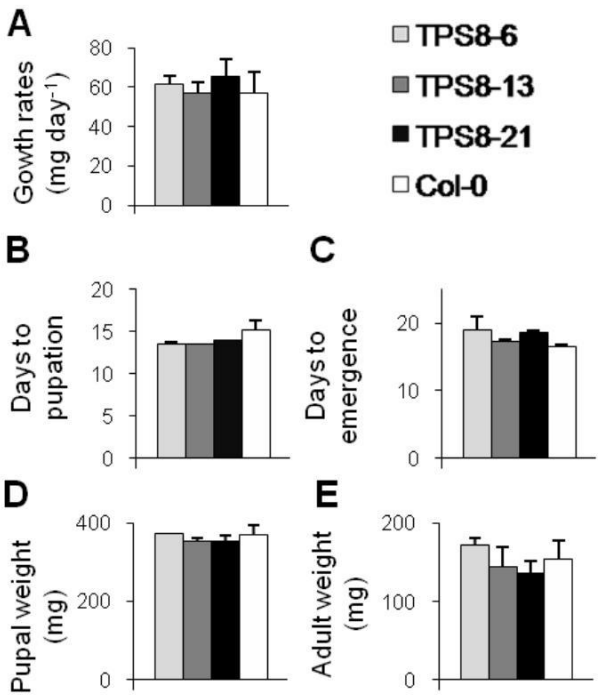

Fig. 2.6 Development of the generalist herbivore *S. littoralis* is not affected by TPS8 sesquiterpenes. Growth rates (A), days to pupation (B), days to emergence (C), pupal (D) and adult (E) weight of *S. littoralis* caterpillars fed on TPS8 (three independently transformed lines) and Col-0 Arabidopsis plants. Growth rates were calculated from the start of the experiment to the day when the first caterpillar pupated. The experiment was repeated three times with similar results. Bars represent means ± SE (N=5)

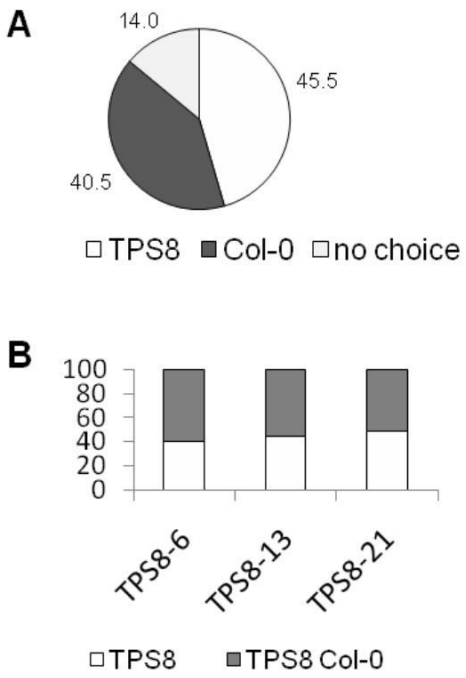

Fig. 2.7 Attraction of *S. littoralis* caterpillars to Arabidopsis is not altered by TPS8 terpene products. A. Percentages of *S. littoralis* caterpillars choosing TPS8 or Col-0 *A. thaliana* plants in an experimental arena. Seven to ten caterpillars were released per experiment, which was repeated six times (total number of caterpillar tested= 57). No significiant choice was made (t= 0.26; P= 0.797). B. Percentage of TPS8 vs. Col-0 leaf area eaten by *S. littoralis* caterpillars in choice experiments

Table 2.3 ANOVA summary table for the effects of plant type (TPS8, Col-0) on the performance of *S. littoralis* caterpillars

Factor: "Plant type" (df=1)	Exp. 1			Exp. 2			Exp. 3		
	F	*P*	df	*F*	*P*	df	*F*	*P*	df
Growth rate	0.24	0.629	18	0.02	0.878	18	1.68	0.211	18
Days to pupation	4.10	0.078	8	0.11	0.749	13	2.38	0.14	18
Pupal weight	0.22	0.646	11	0.24	0.63	14	0.09	0.766	13
Days to emergence	7.19	**0.023**	10	1.33	0.27	12	0.26	0.624	10
Adult weight	0.01	0.907	12	0.01	0.926	12	0.01	0.936	10

2.3.4 The sesquiterpenes produced by TPS8 decrease fungal growth *in planta*

To test for anti-fungal effects of the TPS8 products, transgenic and wild-type Arabidopsis were inoculated with the fungal pathogen *A. brassicicola* for four days. The transgenic plants contained significantly lower D-glucosamine levels compared to Col-0 plants. The levels were determined relative to the mean glucosamine content of non-infected plants ($F_{1,52}$=9.95, P=0.003) (Fig. 2.8). This result demonstrates that fungal biomass was lower in TPS8 than in Col-0 plants.

The cofactor "dry weight" included in the statistical analysis was significant ($F_{1,52}$=7.44, P=0.009), indicating that the concentration of D-glucosamine varied with the plant weight. However, no significant interaction of dry weight and plant type (TPS8, Col-0) was found ($F_{1,51}$=0.05, P=0.83), meaning that the effect of the plant type on the D-glucosamine content did not vary according to plant biomass. Moreover, TPS8 and Col-0 plants did not significantly differ in their dry weight ($F_{1,53}$=2.88, P=0.10).

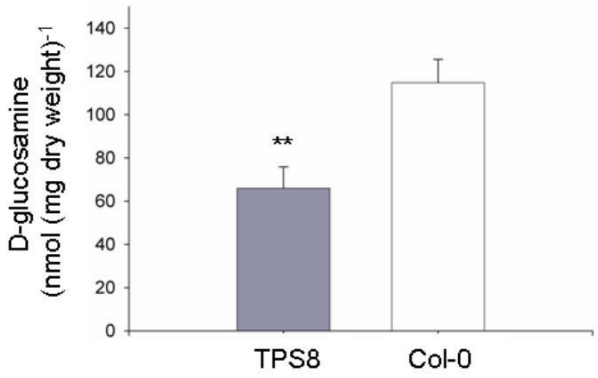

Fig. 2.8 Biomass of the fungus *Alternaria brassicicola* is reduced in Arabidopsis overexpressing TPS8. Fungal biomass was measured by chitin and chitosan extraction from leaves of infected and non-infected plants followed by acidic hydrolysis to glucosamine and HPLC analysis. Quantities are relative to the mean glucosamine content of non-infected plants. Three independent TPS8 lines were tested in four separate experiments. Since no significant effect of the lines on the relative glucosamine amount was found (ANOVA, $F_{3,50}$=9.06, P=0.26), the data were merged. Bars represent means ± SE. Asterisks represent significant differences according to ANCOVA (**P<0.005) (N=27-28)

2.3.5 Transgenic Arabidopsis overexpressing TPS8 are not altered in the production of aliphatic and indole glucosinolates or camalexin

To exclude the possibility that TPS8 products could have acted by influencing the concentration of other antifungal compounds in transgenic Arabidopis, we analyzed the most relevant secondary metabolites in transgenic and wild-type plants, both untreated and challenged with the fungus *Alternaria brassicicola*. The total amount of aliphatic and indole glucosinolates did not differ between TPS8 and Col-0 plants (aliphatic: $F_{1,27}$= 0.12, P=0.732; indole: $F_{1,27}$=0.04, P= 0.850) and was not influenced by fungal infection (aliphatic: $F_{1,27}$=0.31, P=0.582; indol: $F_{1,27}$= 3.05, P=0.092) (Fig. 2.9 A, B). The influence of plant type and infection on the two indole glucosinolates, 4-hydroxyindol-3-ylmethylglucosinolate (4OHI3M) and 4-methoxyindol-3-ylmethylglucosinolate (4MOI3M), was analyzed separately. There is in fact evidence that 4MOI3M plays a role in plant defense against fungi (Bednarek et al., 2009) and 4OHI3M is one of its precursors in the biosynthetic pathway (Pfalz et al., 2009). 4OHI3M was present in higher amounts in TPS8 plants compared to Col-0 plants, but did not vary with pathogen infection (factor plant: $F_{1,27}$=17.95, P<0.001, factor infection: $F_{1,27}$=4.01, P=0.055) (Fig. 2.9 C, above). On the contrary, 4MOI3M content did not vary between the plants, but was enhanced by fungal infection (factor plant: $F_{1,27}$=2.57, P=0.121, factor infection: $F_{1,27}$=40.09, P<0.01) (Fig. 2.9 C, below). The experiment was repeated with a second, independent TPS8 overexpressing line with similar results.

Camalexin was extracted at five time points after *A. brassiciola* infection. As expected, the levels of camalexin increased dramatically in infected plants within 24 h after fungal inoculation (Table 4, Fig. 2.10). However, no significant differences in the quantity of camalexin were found between TPS8 and Col-0 plants at any time point (Table 2.4).

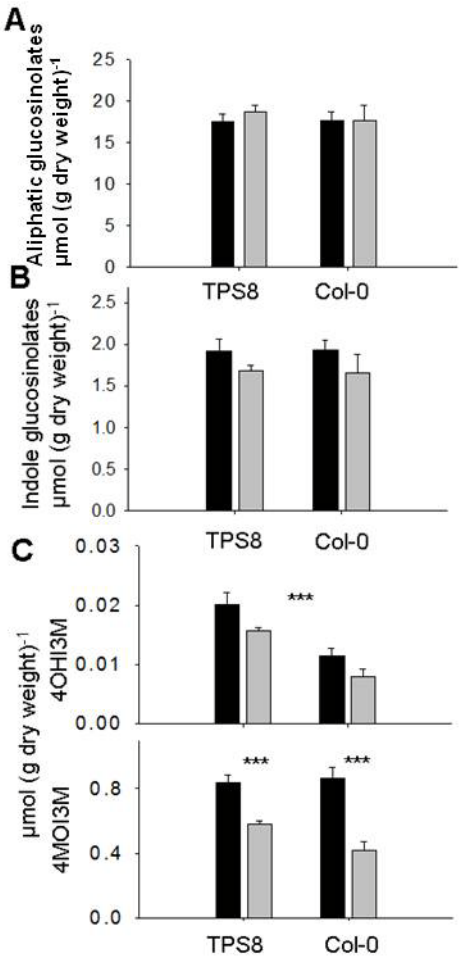

Fig. 2.9 TPS8 overexpression does not alter the glucosinolate content of Arabidopsis. Glucosinolate content of TPS8 and wild-type *A. thaliana* plants (Col-0) infected with the fungus *A. brassicicola* (black bars) and non-infected (grey bars). Bars represent means ± SE (N=7-8). Asterisks indicate a significant difference between TPS8 and Col-0 plants (C, above) and between infected and non-infected plants (C, below) according to two-way ANOVA (***P< 0.001)

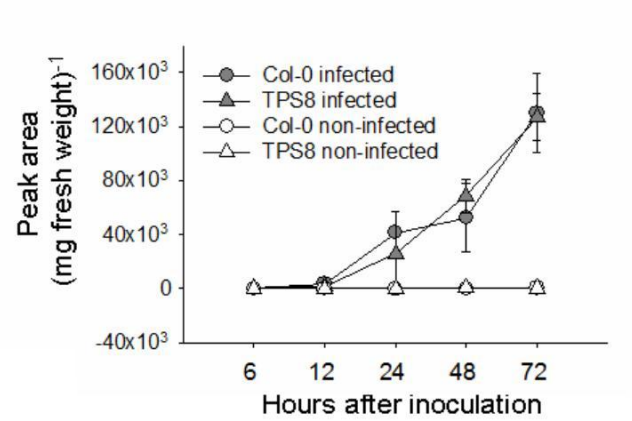

Fig. 2.10 Camalexin production of TPS8-overexpressing and Col-0 Arabidopsis during infection with *A. brassicicola.* Plants were either inoculated with a spore suspension (grey symbols) or mock-inoculated (open symbols). Plants were harvested at five different time points after inoculation. Camalexin was extracted and quantified by LC-MS-MS. Each symbol represents a mean ± SE (N=2-7)

Table 2.4 ANOVA summary table for the effects of plant type (TPS8, Col-0) and fungal treatment on the production of camalexin

Time point	Factors				df residuals
	Plant type (df = 1)		Treatment (df = 1)		
	F	*P*	*F*	*P* [a]	
6 h	0.001	0.980	0.13	0.670	13
12 h	2.09	0.178	2.29	0.161	10
24 h	0.95	0.346	387.15	**<0.001**	14
48 h	1.18	0.293	100.72	**<0.001**	17
72 h	1.57	0.228	531.92	**<0.001**	16

[a] Bold numbers indicate significant effects

2.3.6 TPS8 sesquiterpenes did not alter JA-mediated plant signaling

To test for effects of TPS8 sesquiterpenes on JA-mediated signaling after infection by necrotrophic fungi, the transcript levels of LOX2 were analyzed in TPS8-overexpressing plants and Col-0 plants which were either untreated or challenged with *A. brassicicola*. Non-infected plants had LOX2 transcripts <3% compared to transcripts of Col-0 plants 24 h after infection (=100%). The level of LOX2 transcripts in the TPS8 lines and in Col-0 plants increased between 12 and 24 h after inoculation. However, TPS8 lines had 30% and 90% fewer transcripts compared to Col-0 at 24 h (Fig. 2.11) which might be a consequence of less severe fungal attack on the TPS8-overexpressing lines.

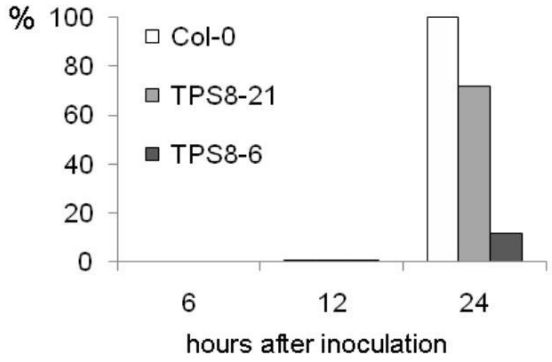

Fig. 2.11 LOX2 expression in two independent TPS8-overexpressing lines and Col-0 *Arabidopsis thaliana* plants after challenge with *A. brassicicola*. Plants were collected 6, 12, and 24 hours after pathogen inoculation. LOX2 expression in *tps8* and Col-0 mock-inoculated plants was <3% (not shown). Transcript level was measured by RT-qPCR. N= 3

2.3.7 The activity of TPS8 is highly conserved among maize and its wild relatives

To test whether *tps8* as an important defense trait against pathogens is conserved in the wild relatives of maize, we isolated apparent *tps8* orthologs from the teosinte species *Zea mays mexicana*, *Z. m. huehuetenangensis*, *Z. m. parviglumis*, and *Z. diploperennis*. The ORFs were cloned and expressed in *E. coli*. All four of the apparent TPS8 orthologs accepted the FPP substrate and produced a sesquiterpene blend identical to that of TPS8 in B73 (Fig. 2.12). However, the ratios between several compounds in the product mixtures varied between the different species. Whereas the apparent orthologs isolated from *Zea m. mexicana* and *Z. m. parviglumis* produced a compound spectrum nearly identical to TPS8 of B73 with germacrene D as the major product, the enzymes isolated from *Z. m.*

huehuetenangensis and *Z. diploperennis* produced δ-cadinene and an unidentified sesquiterpene as major compounds.

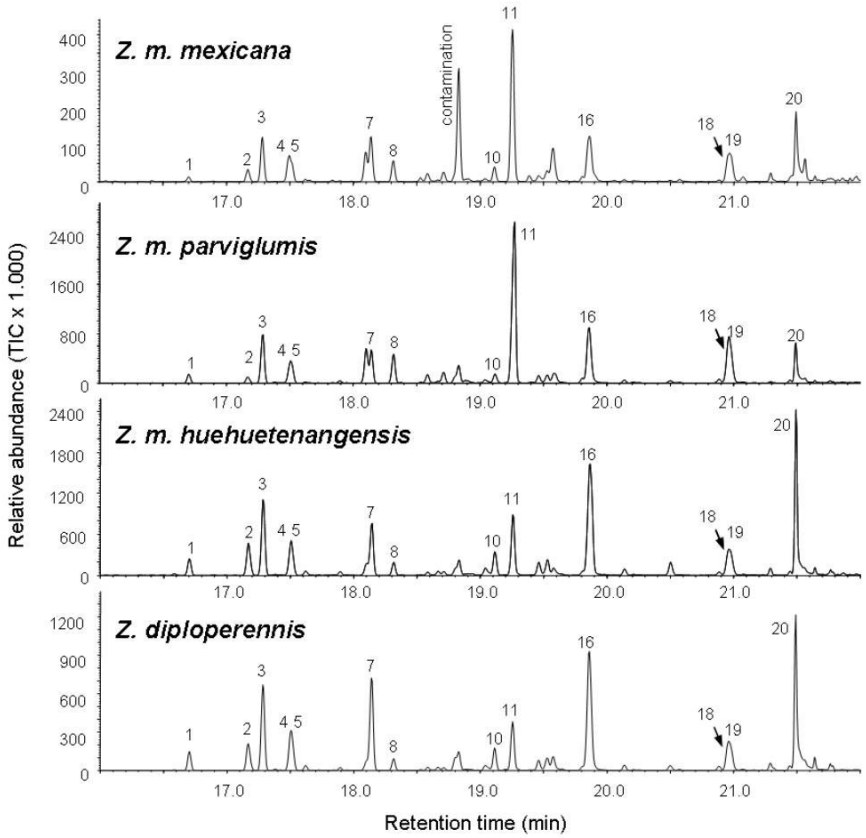

Fig. 2.12 Products of apparent TPS8 orthologs from different teosinte species. The enzymes were overexpressed in *E. coli.* Major products are labeled according to Table 1. Bacterial extracts were incubated with FPP and the volatile products were collected by SPME. The volatiles were analyzed by GC-MS

2.4 Discussion

Nearly all plants that have been analyzed for their terpene content have been found to have low, constitutive concentrations of monoterpenes or sesquiterpenes (ng-µg per gram fresh weight) that are often detected in the headspace. Although these volatiles are known to possess antimicrobial properties, their efficacy against fungal pathogens *in planta* has not been studied. Here, we show that a sesquiterpene blend emitted by maize seedlings may play a role in direct defense against pathogenic fungi.

Most tissues of maize inbred line B73 contain a complex terpene blend that is dominated by germacrene D. These terpenes are produced by the multiproduct terpene synthase TPS8 which forms 54 sesquiterpenes from the substrate FPP. The relative ratios of the terpene products do not change in repeated experiments, suggesting a complex but highly controlled reaction mechanism. A model of the proposed reaction mechanism demonstrates that the enzyme catalyzes a 1,10- or 1,11-cyclization after the initial ionization of the diphosphate moiety. Subsequently, the carbocation can undergo isomerizations and further 1,10-, 1,11-, 1,6-, and 2,7-cyclizations until it is deprotonated or captured by water. The resulting sesquiterpenes are of the germacrane-, caryophyllane-, humulane-, cadinane- or copaane-type and thereby have a high structural diversity. A detailed analysis carried out on two other multiple product maize sesquiterpene synthases, TPS4 and TPS5, suggested that this diversity arises because discrete steps of the enzymatic reaction sequence are controlled by different active site pockets and that conformational chages of the carbocation intermediates cause a shift from one pocket to the other (Köllner et al., 2006). Studies that model the TPS8 active site cavity and subject critical amino acids to site-directed mutagenesis would give insight into how structural diversity is produced by this enzyme. The apparent orthologs of TPS8 from teosinte species show a conservation of enzyme activity that is as high as that between maize TPS10 and TPS23 and their respective teosinte orthologs (Köllner et al., 2008; Köllner et al., 2009). This functional conservation suggests an important role of the TPS8 sesquitepenes during the evolution of maize and related grasses. The production of a high number of structurally diverse sesquiterpene, a very striking feature of TPS8, might be an important aspect of its function since mixtures have unique properties in plant defense (Gershenzon and Dudareva, 2007). TPS8 is expressed throughout the leaves, sheath tissue and roots of the undamaged maize plant. An increased emission of TPS8 products after herbivore damage is mirrored by increased transcript levels of *tps8* in leaves and sheaths. It is conceivable that the plant benefits from an increased production of antifungal terpenes after herbivore attack since herbivore damage provides a convenient entry point for many fungal pathogens. In roots, TPS8 production did not correlate with transcript levels, suggesting that terpene production is limited by low substrate concentrations and/or competition with other terpene synthases, such as the root-specific TPS6 and TPS11.

We investigated the functional role of the maize TPS8 terpenes utilizing transgenic Arabidopsis that overexpressed TPS8. This approach allows the TPS8 terpenes to be studied separately from other maize terpenes, especially the herbivore-induced ones. In

addition, comparison of transformed Arabidopsis with untransformed controls provides direct information about the function of the TPS8 terpenes. Transformation of Arabidopsis with terpene synthase genes has been shown to faithfully reproduce complex blends of terpenes from other species without altering the relative proportion of individual compounds. The TPS8 transformants were tested with larvae of the generalist herbivore *S. littoralis*. However, these compounds were found to be neither attractant nor repellent. Nevertheless, there are many reports of sesquiterpene hydrocarbons with antifeedant activity. For example, in wild tomato species (*Lycopersicon* spp.), zingeberene is associated with resistance to the Colorado potato beetle (*Leptinotarsa decemlineata* Say) and beet armyworm (*Spodoptera exigua* Hubner) (see Antonious and Kochhar, 2003) and references therein). Wild tomato sesquiterpenes are toxic to *L. decemlineata*, with an LD_{50} of 7 µg/larva. Since sesquiterpenes in wild tomato are stored in concentrated amounts in glandular trichomes, the LD_{50} is reached by a larva feeding on only 10-20 mm^2 of leaf surface (Carter et al., 1989). To ingest the same amount of TPS8 sesquiterpenes, a larva would have to consume about 50 g of maize leaves, or 500 g of TPS8-transformed Arabidopsis which may help explain why TPS8 sesquiterpenes did not have any anti-herbivore activity in our *in planta* experiments.

Plants have to constantly defend themselves against a vast array of pathogens. In particular, polycyclic airborne fungi can deposit vast amounts of spores on above ground parts that germinate over extended periods of time. Our results indicate that the presence of terpenes may provide young maize plants with a basal level of protection against those fungi. TPS8 Arabidopsis plants challenged with the nectrotrophic fungus *Alternaria brassicicola* contained a lower amount of fungal biomass per gram fresh weight compared to wild-type plants (Fig. 2.10). In order to determine whether this anti-fungal activity arises from possible pleiotropic effects of TPS8 terpenes on other anti-fungal defenses, we measured the content of glucosinolates and camalexin, the main secondary metabolites known to play a role in antifungal defenses in Arabidopsis. Total aliphatic and indole glucosinolate content did not significantly vary between Col-0 and TPS8 plants, and was not altered by *A. brassicicola* infection (Fig. 2.8 A, B). Although glucosinolates are best known as constitutive anti-herbivore defenses of Brassicaceae, many studies have provided evidence for their antimicrobial activity as well (e.g. Manici et al., 1997; Brader et al., 2006). In particular, the indole glucosinolate 4-methoxyindol-3-ylmethylglucosinolate (4MOI3M) has been shown to play a major role in antifungal defense of Arabidopsis. This compound is involved in the activation of callose deposition in response to pathogen attack (Bednarek

et al., 2009; Clay et al., 2009). Consistent with these results, we found that 4MOI3M was induced by fungal infection both in wild-type and in TPS8 plants (Fig. 2.8 C). Camalexin was also induced after infection with *A. brassicicola*, starting between 12 and 24 hr after inoculation, but this occurred in both TPS8-overexpressing plants and wild type.

The reduced fungal growth observed in TPS8-overexpressing Arabidopsis may be an effect of a direct antifungal action of sesquiterpenes. Since our biassays were carried out with intact plants, it is not clear whether the fungus is affected by the TPS8 terpenes within the plant or those that have diffused into the airspace around the plant. An analysis of fungal development on the leaf surface might elucidate the mechanism of terpene toxicity. There are many reports of *in vitro* antifungal activity of sesquiterpene hydrocarbons. For example, Caccioni et al. (1998) tested the essential oils from six *Citrus* species against two common post-harvest citrus fruit pathogens (*Penicillium digitatum* Sacc. and *P. italicum* Whem.) and found a significant positive correlation between sesquiterpenes content of the oil and antifungal activity. In another study, the growth inhibitory fractions isolated from extracts of different ecotypes of wild *Daucus carota* L. subsp. *carota*, were found to be rich in sesquiterpene hydrocarbons and characterized by a high concentration of β-bisabolene (17-51%) (Maxia et al., 2009). These oils inhibited the growth of 12 fungal species among yeasts, dermatophytes, and moulds, suggesting that terpenes have an unspecific mode of action against fungi.

An alternative explanation for the antifungal effects we observed is that terpenes act as volatile signaling agents in the plant to promote the activation of other antifungal defenses. This can occur via a direct induction or via priming, in analogy with the way that exposure to green leaf volatiles does not cause an immediate response, but primes the plant to mount a more rapid and extensive response to subsequent herbivory (Engelberth et al., 2004; Ton et al., 2007) or pathogen attack (Kishimoto et al., 2005). Signaling can occur within the same plant, but also between different conspecifics (Heil and Bueno, 2007). In Arabidopsis, necrotrophic fungi like *A. brassicicola* elicit the jasmonic acid (JA) defense cascade (Glazebrook, 2005). Thus, we measured the expression of *LOX2*, a gene of the JA pathway, in TPS8 overexpressing lines and Col-0. There was no difference between these plants in the activation of *LOX2* in response to fungal infection indicating that sesquiterpenes do not exert their defensive effect against *A. brassicicola* by signaling via a JA-mediated pathway. Moreover, of two TPS8-overexpressing lines tested, one even had a 90% lower *LOX2* expression after 24 hr compared to Col-0 plants, suggesting reduced JA signaling, which may be due to the lower level of infection in the transgenic plants.

In conclusion, our results point to a novel defensive role for volatile plant terpenes that are present in low concentrations. This role may be widespread since the presence of monoterpene or sesquiterpenes at sub-microgram or nanogram levels is commonplace among plants. However, further studies employing other types of pathogens with terpenes in *in planta* bioassays are needed to firmly establish a role for these metabolites in protection against microbes.

3. Chapter II

Attractiveness of natural maize sesquiterpene blends to the parasitic wasp *Cotesia marginiventris* (Cresson).*

Abstract Plant volatiles induced by herbivore attack have been demonstrated to provide a signal to herbivore enemies like parasitic wasps that use these volatiles to locate their host larvae. However, in addition to herbivore-induced volatiles, plants often release low levels of volatiles constitutively. We assessed the interaction between herbivore-induced and constitutively released maize volatiles in the attraction of the wasp *Cotesia marginiventris* that parasitizes herbivorous lepidopteran larvae feeding on maize. Experiments were carried out in a six-arm olfactometer in which the sources of volatiles were transgenic *Arabidopsis thaliana* plants overexpressing maize sesquiterpene synthases. We found that the constitutively released volatiles of maize terpene synthase 8 (TPS8) were attractive to *C. marginiventris*, just like those of the herbivore-induced TPS10 in an earlier study. But, a mixture of both TPS8 and TPS10 volatile blends was more effective in parasitoid attraction, indicating that the constitutively released TPS8 sesquiterpenes synergize the attraction of the herbivore-induced TPS10 sesquiterpenes. While *C. marginiventris* did not distinguish between these and another maize sesquiterpene blend (TPS5), the attraction of the wasp appears to increase with the complexity of the blend.

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3.1 Introduction

Plants emit a plethora of volatile organic compounds (VOCs) from their vegetative organs into the surrounding atmosphere. The most common compounds emitted are green leaf volatiles (GLVs, C_6 alcohols, aldehydes, and derivatives), and terpenes, especially monoterpenes and sesquiterpenes (Dudareva et al., 2004). VOCs are released abundantly after herbivore attack, but also undamaged plants, especially tree species, show high emission rates (Kesselmeier and Staudt, 1999). For example, poplars (*Populus* spp.) can invest more than 10% of their photosynthetically fixed carbon in the emission of isoprene (Brilli et al., 2009). The majority of studies on vegetative VOCs have focused on herbivore-induced volatiles and provided ample evidence for their role in attraction of herbivore enemies. These studies include agronomically important crops (e.g. maize, tomato, soybean, cabbage) as well as wild species, both herbaceous and woody (reviewed by Unsicker et al., 2009). Much less is known about the role of constitutively emitted volatiles in plant indirect

defense, as these have rarely been studied separate from the herbivore-induced ones. Attraction of insect enemies to uninfested plants has been documented for some aphid parasitoids (Hymenoptera: Braconidae) (reviewed by Hatano et al., 2008) and for two lepidopteran parasitoids (the braconid *Microplitis croceipes* (Cresson) and the ichneumonid *Campoletis sonorensis* (Cameron)) towards cotton plants (*Gossypium hirsututm* L.) (Elzen et al., 1986, 1987).

Herbivore-induced and constitutive VOCs are often released in complex blends (Dicke et al., 2009). Since these natural mixtures are difficult to manipulate or to reproduce synthetically, most studies tested arthropod attraction to either whole blends, or to single VOCs available in pure form. For example, the GLV (*E*)-3-hexenol is known to be attractive to the braconid wasps *Apanteles* (=*Cotesia*) *kariyai* (Watanabe) (Takabayashi et al., 1991), and the sesquiterpenes γ-bisabolene and (*E*)-β-caryophyllene have been proved to attract *C. sonorensis* females, which oviposit in noctuids that feed on cotton plants, rich in sesquiterpenes (reviewed by Rutledge, 1996). Single volatiles responsible for the attraction of arthropod herbivores have been identified also in a few aphid-parasitoid systems (reviewed by Hatano et al., 2008) and in one mite - predatory mite system (de Boer and Dicke, 2004; de Boer et al., 2004). However, there are cases in which the whole volatile blend released by a plant is necessary for the parasitoid to locate its host's habitat. A study with the egg parasitoid *Chrysonotomyia ruforum* (Krausse) (Hymenoptera: Diprionidae) demonstrated that the VOC blend emitted by Scots pine (*Pinus sylvestris* L.) attracts the parasitoid after oviposition by the herbivorous sawfly *Diprion pini* (L.) (Mumm and Hilker, 2005). This blend differs from that of uninfested plants by having greater amounts of (*E*)-β-farnesene. However, neither (*E*)-β-farnesene alone, nor the odor of uninfested plants were attractive to the parasitoids; both were required.

Maize (*Zea mays* L.) is one of the best studied species for the role of volatiles in indirect plant defense. The volatile bouquet emitted by seedlings after feeding by generalist lepidopteran caterpillars (*Spodoptera littoralis* (Boisd.), *S. exigua* (Hübn.)) has been well characterized and studied for over two decades (D'Alessandro et al., 2009; Turlings et al., 1990). Important components of this odor are GLVs (esters and aldehydes) with their abundance dependent on the extent and intensity of herbivore damage. The monoterpene alcohol linalool, the C_{11} homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and indole are often also present in high amounts. However, the biggest proportion of the blend is made up of (*E*)-β-farnesene, (*E*)-α-bergamotene and other sesquiterpene hydrocarbons (Turlings and Ton, 2006). This herbivore-induced blend was found to be attractive to

parasitic wasps (Turlings et al., 1991; Turlings et al., 1990), raising the question of whether the entire mixture or individual components are responsible for the attraction. In order to tackle this question, the volatile mixture has been fractionated (D'Alessandro and Turlings, 2005) and plants have been engineered to emit only a specific fraction of the terpene blend. Transgenic *Arabidopsis thaliana* plants emitting the volatiles of terpene synthase 10 (TPS10), which make up most of the herbivores-induced sesquiterpenes of maize leaves, were more attractive than untransformed controls to the parasitoid wasp *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) in olfactometer experiments (Schnee et al., 2006).

In addition to herbivore-induced terpenes, many lines of maize emit an additional sesquiterpene mixture, which is constitutively emitted by the roots and aboveground vegetative tissues of young plants. These terpenes are synthesized by TPS8 (Fig. 3.1a), a terpene synthase that is expressed independent of herbivory in the inbred line B73 as well as in several other cultivars and wild ancestors of maize (Köllner et al., 2008; Köllner et al., 2009; Köllner et al., 2004b). The ecological role of maize TPS8 products has been previously investigated in the context of plant-pathogen interactions and deterrence of insect herbivory, and these substances have been shown to act as defenses against fungi, but not against herbivores (Chapter I). The terpene blend produced by TPS8 is extraordinarily complex and one major component of this mixture, (*E*)-β-caryophyllene, has been implicated in the attraction of herbivore enemies both above and below ground (Köllner et al., 2008).

Here, we studied the function of constitutively emitted TPS8 terpenes in an indirect plant defense system in maize that involves the attraction of the generalist parasitic wasp *C. marginiventris* to oviposit on lepidopteran herbivores.

These constitutively emitted TPS8 volatiles may act 1) synergistically with the herbivore-induced (TPS10) terpenes to strengthen attraction of the wasps; 2) antagonistically with the herbivore-induced signal to weaken the parasitoid attraction; or 3) without effect neither enhancing nor diminishing the parasitoid response to the herbivore-induced odor. To test these hypotheses, we performed a series of olfactometer experiments where we measured the attraction of *C. marginiventris* females to the TPS10 or TPS8 terpene blends, either singly or in combination, emitted from transgenic *A. thaliana* lines. We also tested whether the parasitoid's responses to the terpene blends are preformed or learned by association with an oviposition experience. It has been shown that *C. marginiventris* females can be attracted to different odors (among which the one of herbivore-induced maize) only after

they had associated them to an oviposition experience (Hoballah and Turlings, 2005; Schnee et al., 2006). However, it is not known whether the parasitoids learn the whole blend or only some of its components, and how specifically. Finally, using a third maize terpene blend, that of TPS5 which is expressed in the husks and, at lower levels, in the leaves of mature maize plants (Köllner et al., 2004a), we investigated whether complexity of the mixture is an important variable in parasitoid attraction.

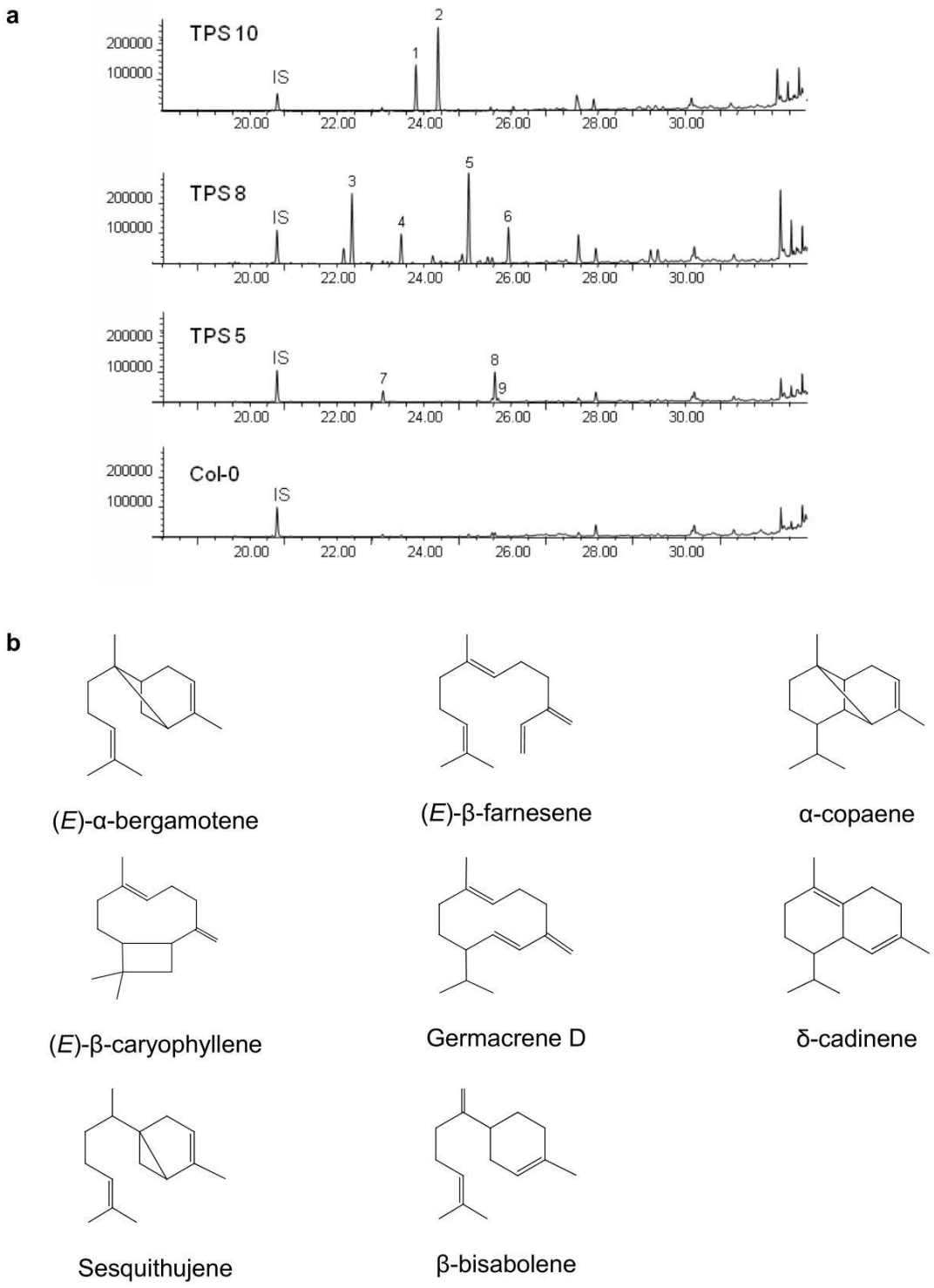

Fig. 3.1 (a) Volatile profile of *Arabidopsis thaliana* plants transformed with TPS10, TPS8, TPS5, and of Col-0 plants. The main volatile compounds are identified as: 1. (*E*)-α-bergamotene; 2. (*E*)-β-farnesene; 3. α –copaene; 4. (*E*)- β-caryophyllene; 5. Germacrene D; 6. δ-cadinene; 7. sesquitujene; 8. β-bisabolene. Their structures are shown in (b)

3.2 Methods and Materials

3.2.1 Generation of transgenic TPS5 *Arabidopsis thaliana* plants

Arabidopsis thaliana *Plants* The open reading frame (ORF) of *tps5* from *Zea mays* var. Delprim (*tps5-Del1*) was amplified from the pASK-IBA7 construct (Köllner et al., 2004b) with the primers forward (*tps5*fwd): 5′-ATGGCGTCTCCTCCAGCACATCG-3′ and reverse (*tps5*rvs): 5′-TCATTCGGGTATTGGCTCCACAAACAG-3′ and cloned into the pCR-TOPO vector (Invitrogen, Carlsbad, CA) for sequencing. After sequence analysis, the 1665 bp ORF was re-amplified from the sequencing vector and cloned using Gateway technology (Karimi et al., 2002) into the plant expression vector pB2GW7 between the p35S promoter and the T35S terminator of the cauliflower mosaic virus. The resulting construct was introduced into the *Agrobacterium tumefaciens* strain GV3101 which was then used to transform *Arabidopsis thaliana* L. (ecotype Col-0) plants using the floral dip method (Clough and Bent, 1998) supplemented with vacuum infiltration. Since the construct had a *bar* selectable marker gene that confers resistance to the herbicide Basta, seeds from T_0 plants were screened for the transgene by applying Basta on soil-germinated young seedlings. To select for transgenic lines emitting the expected TPS5 volatile sesquiterpenes, headspace volatiles were collected from detached leaves of the Basta survivors with SPME (Solid Phase Micro Extraction) and analyzed by GC-MS (see *Volatile Collection and Analysis* for details). Transformation was additionally confirmed by PCR analysis.

3.2.2 Plant and insect material

A. thaliana plants overexpressing one of the maize sesquiterpene synthases TPS10, TPS8 or TPS5 (Schnee at al, 2006; Chapter I), and wild-type (ecotype Col-0) plants were grown in 7 x 7 x 8 cm single pots on fertilized potting soil and grown in a climate chamber at 21°C, 55% relative humidity, and 150 µmol s^{-1} m^{-2} photosynthetically active radiation (PAR) under short day conditions (8 hr light day^{-1}).

Maize plants var. Delprim were grown in a climate chamber at 23±2°C, 60% relative humidity, 16 hr light day^{-1}, and 900 µmol s^{-1} m^{-2} PAR. Eggs of the Egyptian cotton armyworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) were obtained from Syngenta rearing facilities (Stein, Switzerland). After emergence, the larvae were reared on a wheatgerm-based artificial diet (also supplied by Syngenta) at room temperature. The solitary endoparasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) was

reared as described in Turlings et al. (2004). Female and male adult parasitoids were kept together in plastic cages (30 x 30 x 30 cm) (MegaView Science Education Services Co. Ltd., Taiwan), at a ratio of approximately 2 : 1 in incubators at 25 ± 1°C with 16 hr light day^{-1} and supplied with moist cotton and honey. Mated, 2- to 5-day-old females were used in the olfactometer experiments.

3.2.3 Olfactometer experiments

The behavioural response of *C. marginiventris* females to three maize sesquiterpene blends and their combinations was tested using transgenic *A. thaliana* plants as odor sources in six-arm olfactometer experiments. The six-arm olfactometer (described in Turlings et al., 2004) was set with incoming air at 1.2 l min^{-1} per vessel. A portion of the air was pulled out from each vessel at 0.6 l min^{-1} through a volatile collection trap. Each of the six vessels contained either a plant as the odor source or was left empty. Purified, humidified air carried the volatile odors from the vessels to the corresponding arm. When combinations of two or three terpene blends were tested, two or three *A. thaliana* plants were used as odor sources. The same number of plants was used in all the odor-carrying arms in order to have a comparable plant biomass in all the vessels. The transgenic plants were tested to confirm volatile emission prior to each experiment and different confirmed individuals were randomly chosen in each experimental replicate (see *Volatile collection and Analysis*).

Groups of six *C. marginiventris* females were released in the center of the olfactometer and were given 30 min to choose one of the arms. Wasps that did not enter an arm within this time were considered as "no choice". After the choice of the wasps had been recorded, the wasps were removed from the olfactometer. Each group of parasitoids tested was either naïve (with neither oviposition experience nor exposure to any of the tested odors) or experienced (for details on the number of releases per experience and experimental replicates, see Table 3.1). Oviposition experiences were given by placing a single female parasitoid in a glass enclosure containing about 20 second- or third-instar *S. littoralis* larvae and connected on one side to a glass vessel containing a given odor source, which was either a transgenic Arabidopsis plant or a maize seedling. The wasps were allowed to oviposit in presence of the odor at least three times before they were removed from the enclosure. To induce volatile production in the maize seedling used for these experiments, the plants were enclosed in a glass vial with a vented lid and one third-instar *S. littoralis* caterpillar was allowed to feed overnight.

Every experiment was replicated 3 to 8 times. At the end of each replicate, all parts of the olfactometer were washed with water, acetone and hexane and the glass parts were dried in an oven at 250°C. In each replicate, 1 to 4 groups of six wasps with the same experience were released (Table 3.1).

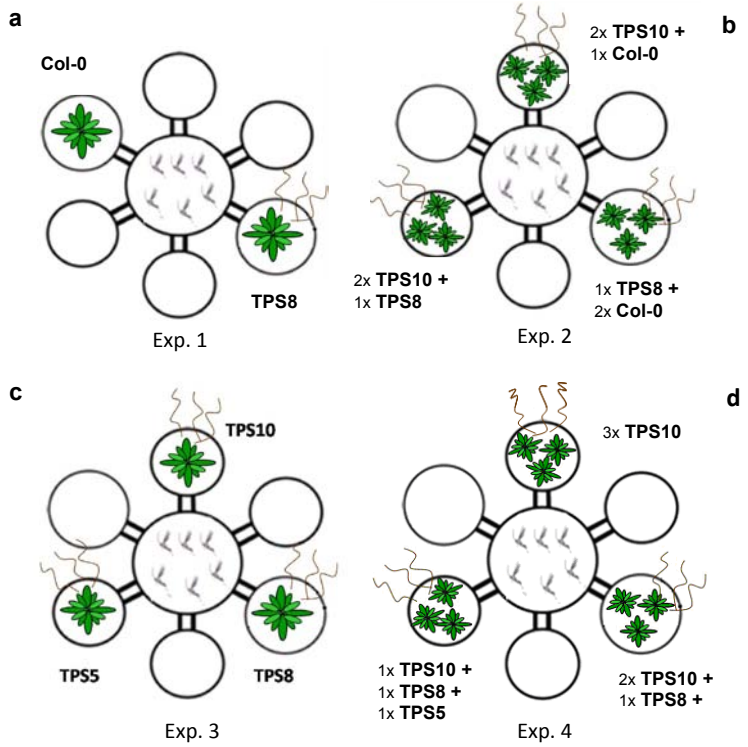

Fig. 3.2 Position of the odor sources in the olfactomenter experiments (Experiment 1 (a), Exp. 2 (b), Exp. 3 (c), Exp. 4 (d)). The schematic plants represent *A. thaliana* individuals, either transgenic or wild-type (ecotype Col-0)

3.2.4 Volatile collection and analysis

All transgenic Arabidopsis plants were screened for volatile production before being used in the olfactometer experiments. A leaf was detached from ca. three-week-old plants, put in a 1.5 ml glass vial and incubated for about 20 min at 40°C with an SPME fiber (100 µm Polydimethylsiloxane coating, Supelco, USA). The collected volatiles were then analyzed by GC-MS (GC: Hewlett-Packard 6890; carrier gas: He, 1 ml min^{-1}, splitless injection (injection T: 220°C), column: DB-5MS (30 m x 0.25 mm x 0.25 µm film, J & W Scientific, Folsom, USA), temperature program: 80°C to 180°C at 10°C min^{-1}, 180°C to 220°C. MS: Hewlett-Packard 5973, quadrupole mass selective detector; transfer line T: 270°C; ionization potential: 70 eV; scan range: m/z 40-350).

During the second olfactometer experiment, volatiles were collected from the vessels in order to assess the effect of the quantity of volatiles on the wasps' response Odors were trapped with 25 mg Super-Q adsorbent (Alltech Associates, Inc., Deerfield, Illinois, USA),

and eluted with 150 µl dichloromethane; 200 ng nonylacetate were added as an internal standard. Volatiles were analyzed by GC-MS GC: Agilent 6890 Series GC system G1530A, carrier gas: He, 1 ml min-1, splitless injection (injection T: 230 °C),column: HP-1 (Alltech Associates, polydimethylsiloxane, 30 m x 0.25 mm x 0.25 µm film), temperature program: 40 °C for 3 min, 40 °C to 100 °C at 8 °C min-1, 100 °C to 200 °C at 5 °C min-1, 5 min at 250 °C. MS: Agilent 5973 Network Mass Selective Detector, electron impact mode, transfer line T: 230 °C, ionization potential: 70 eV, scan range: amu 33-280. The relative quantity (%) of the sesquiterpenes was calculated based on their peak area relative to the peak area of the internal standard.

3.2.5 Statistical analysis

In order to analyze the influence of the odors tested and of the experiences on the parasitoids' choices, a generalized linear model (GLM) with a log-link function and a quasipoisson error distribution was used (Turlings et al., 2004). "No choice" wasps were not included in the analyses. The model was fitted by maximum quasi-likelihood estimation and its adequacy was assessed through likelihood ratio statistics and examination of residuals. Odor source and experience of the wasps were fitted to the model as fixed factors. We tested the interactions between the factors as well as the effect of the single factors on the wasps' choice. The effect of the odor was tested separately on naïve and experienced wasps in Exp. 1 and 2.

The response of the wasps to the relative amounts of volatiles offered in Exp. 2 was assessed with the GLM described before.

All the analyses were performed using the software package R (R Development Core Team (2008), R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org).

3.3 Results

3.3.1 Constitutively produced maize sesquiterpenes are attractive to experienced *C. marginiventris* parasitoids (Exp.1)

In order to test the attractiveness of the constitutively-produced maize sesquiterpenes to naïve and experienced parasitoids, we compared the attraction of *A. thaliana* plants

overexpressing TPS8 with wild-type Col-0 plants in olfactometer experiments (Fig. 3.2a). Experienced *C. marginiventris* females that had had a previous oviposition experience in the presence of TPS8 volatiles were significantly more attracted by *A. thaliana* plants producing TPS8 terpenes than by wild-type plants (t=2.98, P=0.004), while naïve parasitoids did not show a preference for either plant (t=0.15, P=0.88) (Fig. 3.3a, b). Both naïve and experienced wasps preferred arms containing a plant to empty arms (naïve: $F_{2,69}$=14.40, P<0.001, experienced: $F_{2,69}$=45.63, P<0.001). Overall, 49% of the parasitoids responded to the plant odors (i.e., they entered an arm), while 45% made no choice (Fig. 3.3c).

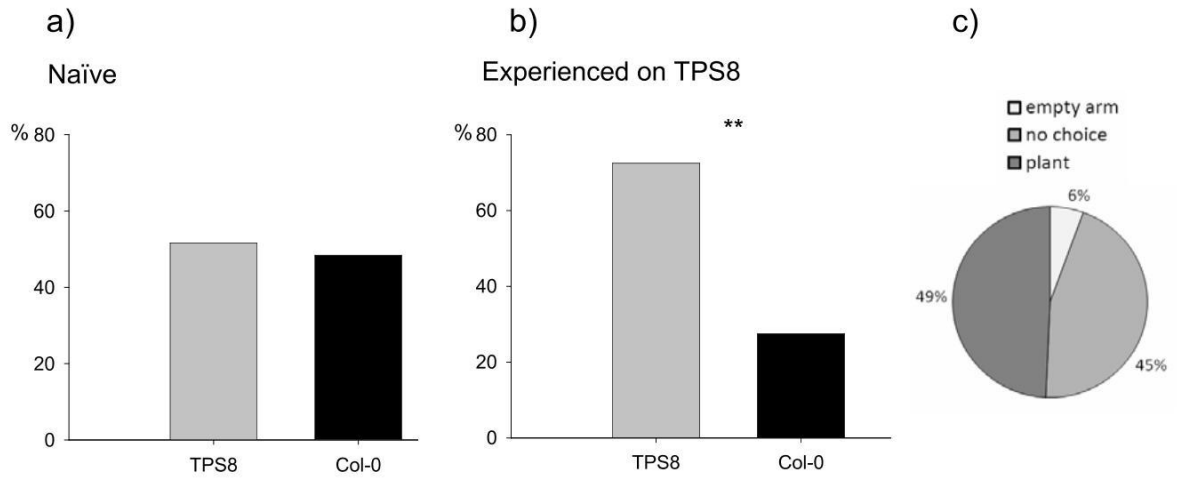

Fig. 3.3 Percentage of naïve (a) and experienced (b) *C. marginiventris* females that were attracted to a terpene-emitting (TPS8) or a wild-type (Col-0) *A. thaliana* plant in a six-arm olfactometer. Asterisks represent a significant difference between total number of experienced wasps that chose the terpene-producing plant and the wild-type plant (P=0.004). Responsiveness of the wasps is shown in c)

3.3.2 Experienced *C. marginiventris* females prefer the full sesquiterpene blend of an herbivore-induced maize plant including constitutive volatiles (Exp.2)

Since experienced *C. marginiventris* were attracted to the constitutively released TPS8 volatiles of a maize plant, we wanted to test whether this attraction is similar to that of herbivore-induced TPS10 volatiles (Schnee et al., 2006) and whether there is any interaction between the two terpene blends. The volatile blend of herbivore-damaged maize seedlings contains about twice as many herbivore-induced volatiles as constitutive TPS8 compounds. To mimic these proportions, two TPS10-emitting plants were combined with

one TPS8-expressing plant (Fig. 3.2b, Exp.2). This blend was compared to the volatiles of two TPS10 plants combined with one Col-0 plant and the volatiles of one TPS8 plant with two Col-0 plants. The parasitic wasps were given different oviposition experiences (Table 3.1). The combination of TPS10 and TPS8 sesquiterpenes was preferred by experienced wasps over the TPS8 volatiles alone (t=-2.65, P=0.009), but not over the TPS10 volatiles (t=-1.51, P=0.13) (Fig. 3.4b). Experienced *C. marginiventris* preferentially oriented towards arms carrying plant odors than towards empty arms ($F_{1,190}$=38.93, P<0.001). Naïve wasps did not significantly prefer any of the odors offered ($F_{2,21}$=0.85, P=0.44) (Fig. 3.4a), but they chose arms carrying plant volatiles more frequently than empty arms ($F_{2,21}$=0.85, P=0.03). The different oviposition experiences had no influence on the parasitoids' choice ($F_{1,46}$=5.16, P=0.87). The wasps' responsiveness was low: 40% of the parasitoids chose a plant odor, while 51% made no choice (Fig. 3.4c). The relative amount of the volatiles collected from the plant-containing vessels did not influence the distribution of the wasps in the olfactometer ($F_{1,22}$=6*10^{-4}, P=0.98).

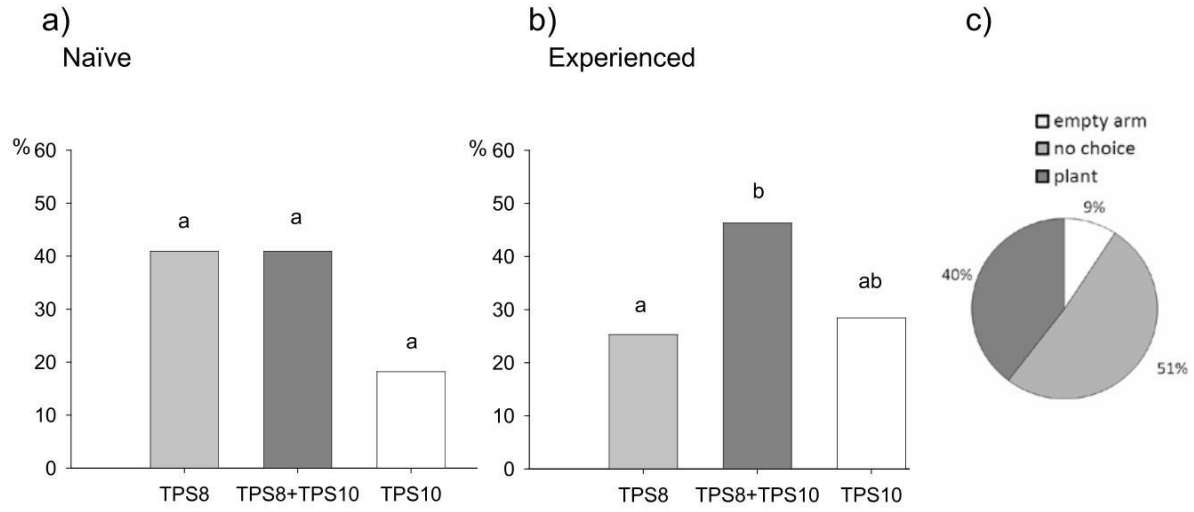

Fig. 3.4 Percentage of naïve (a) and experienced (b) *C. marginiventris* females that were attracted to three maize sesquiterpene blends produced by transgenic *A. thaliana* plants in six-arm olfactometer experiments. TPS8: background odor of an intact young maize plant; TPS8+TPS10: sesquiterpene volatiles of an herbivore-induced maize plant; TPS10: herbivore-induced sesquiterpenes of maize. Different letters represent significant differences (P=0.009). Responsiveness of the wasps is shown in c)

Table 3.1 Summary of details of the olfactometer experiments, including types of oviposition experience used for *Cotesia marginiventris* prior to experiment, number of releases per experience, number of experimental replicates and total number of wasps used. Six wasps were used in each release. Naïve wasps had no prior oviposition experience

	Experiences					Releases per experience	Replicates of the experiment	Total number of wasps
	Naïve	TPS8	TPS10	TPS8+TPS10	HI* maize			
Exp. 1	x	x				4	3	144
Exp. 2	x	x	x	x	x	1	8	240
Exp. 3			x			3 - 4	4	84
Exp. 4			x			5 - 6	7	216

3.3.3 Experienced *C. marginiventris* females do not discriminate between different maize sesquiterpene blends (Exp.3)

The previous experiments suggested that the association of volatiles with oviposition may not be dependent on specific sesquiterpenes. To test this hypothesis, we offered parasitoids experienced on TPS10 *A. thaliana* plants a choice between *A. thaliana* plants overexpressing TPS8, TPS10 or TPS5 (Fig. 3.2c, Exp. 3). The products of terpene synthase TPS5 are sesquiterpenes found only in the leaves and husks of mature plants which parasitoid wasps are not likely to experience in the field. The parasitoids significantly preferred arms containing a plant to empty arms ($F_{1,82}$=9.23, P=0.003), but none of the sesquiterpene blends offered was preferred over the others (TPS10 vs. TPS8: t=0.23, P=0.82; TPS5 vs. TPS8: t=0.98, P=0.33; TPS10 vs. TPS5: t=-0.76 P=0.45) (Fig. 3.5a). Again, the responsiveness was low: 30% of the parasitoids chose a plant odor, while 61% made no choice (Fig. 3.5b). These results indicated that *C. marginiventris* do not associate specific sesquiterpenes with oviposition and chose either of the three blends for host finding.

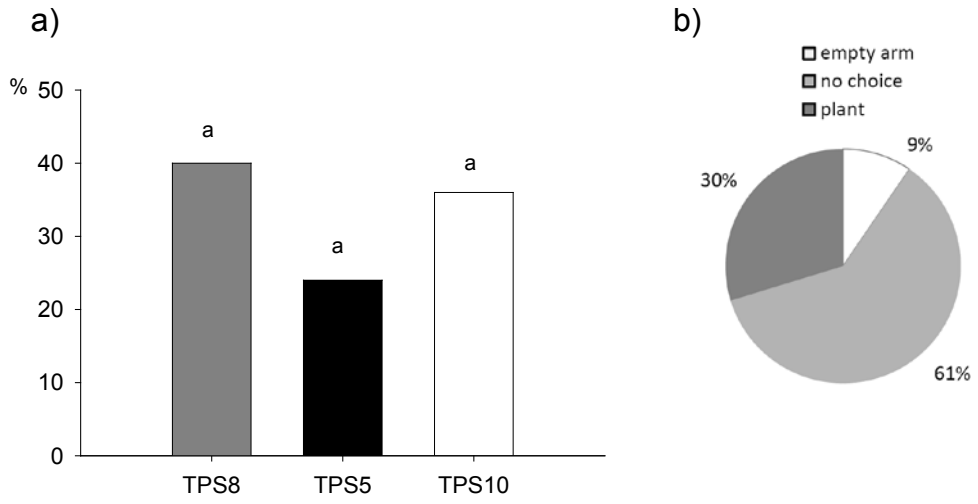

Fig. 3.5 Percentage of *C. marginiventris* females that were attracted to sesquiterpene blends from maize produced by three lines of transgenic *A. thaliana* plants in six-arm olfactometer experiments (a). The terpene composition of the blends is detailed in Fig. 3.3.1. Responsiveness of the wasps is shown in b)

3.3.4 Experienced *C. marginiventris* females tended to orient towards more complex sesquiterpene blends (Exp.4) Since specific sesquiterpenes are not recognized by *C. marginiventris* (Exp.3), and the blend of TPS8 and TPS10 sesquiterpenes combined are more attractive than each separately (Exp. 2), greater complexity of the blend may increase its attraction to the wasps. In order to test whether more complex blends are more attractive than simpler blends, groups of parasitoids experienced on TPS10 *A. thaliana* plants were given the choice between the products of one (TPS10), two (TPS8 and TPS10), and three (TPS8 and TPS10 and TPS5) terpene synthases (Fig. 3.2d, Exp.4). The first group consisted of two TPS10 and one wild-type plants, the second group of two TPS10 and one TPS8 plants, the third group of one plant of each TPS8, TPS10, and TPS5. Parasitoids significantly preferred arms containing a plant to empty arms ($F_{1,95}$=6.72, P=0.011) (Fig. 3.6b). Although there was no statistically significant preference for any of the blends offered, the wasps tended to orient towards the most complex blend (TPS8+TPS10+TPS5) (t=1.82, P=0.07) (Fig. 3.6a). Overall, 25% of the parasitoids responded to the plant odors, while 66% made no choice.

a) b)

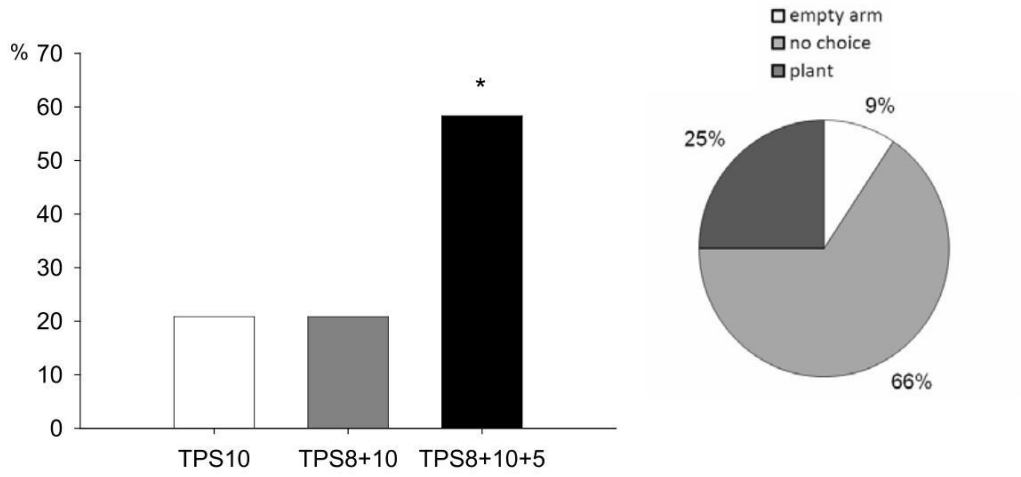

Fig. 3.6 Percentage of *C. marginiventris* females that were attracted to sesquiterpene blends of growing complexity in six-arm olfactometer experiments (a). The odor sources were *A. thaliana* plants transformed with one maize sesquiterpene synthase gene (*tps8* or *tps10* or *tps5*). The asterisk represents a trend in the wasps' preference towards the more complex blend (P=0.07). Responsiveness of the wasps is shown in b)

3.5 Discussion

Herbivore-induced plant volatiles have been the focus of many studies on indirect defense (Dicke, 2009; Heil, 2008; Takabayashi et al., 1991; Turlings et al., 1990). These studies do not take into account that hymenopteran parasitoids using herbivore-induced plant volatiles to search for their lepidopteran host larvae also come into contact with many VOCs emitted by undamaged plant parts. A successful host finding strategy of the parasitoid might therefore involve both constitutive and herbivore-induced plant volatiles.

Utilizing *A. thaliana* plants genetically transformed with maize terpene synthases to emit partial blends of maize volatiles, we showed that constitutively released volatiles can be attractive to experienced *C. marginiventris* females (Exp.1, Fig. 3.3). A similar response to odors from undamaged plants that support their hosts has been documented for several parasitoids, including the aphid parasitoid *Aphidius funebris* Mackauer (Hymenoptera: Braconidae) which responded to uninfested *Centaurea nigra* L. (Asteraceae) (Pareja et al.,

2007), and the lepidopteran parasitoids *Microplitis croceipes* and *Campoletis sonorensis* which responded to cotton plant volatiles (Elzen et al., 1986; Elzen et al., 1987).

In the experiments described here, experienced *C. marginiventris* females parasitoids were given a choice between terpene blends that reproduced: 1. the constitutive odor of an undamaged maize plant, 2. the odor produced by herbivore induction, or 3. the complete odor of an herbivore-infested plant. They showed a preference for the complete sesquiterpene composition of an herbivore-infested plant including both constitutive and induced odors (Exp. 2, Fig. 3.4b). This shows that constitutively produced VOCs act synergistically with herbivore-induced VOCs and can therefore benefit the indirect defense of the plant. The constitutively emitted TPS8 terpenes may represent a 'background odor' that indicates the general habitat of the host larvae. This background odor reinforces the particular 'signal odor' released by maize only upon herbivore feeding and enhances parasitoid attraction. Such a synergistic action of volatiles cues has also been described for *Leptopilina boulardi* (Barbotin, Carton & Kelner-Pillault) (Hymenoptera: Eucoilidae), a larval parasitoid of *Drosophila* species, which is attracted towards the odors of pear and banana fruits (Couty et al., 1999). When this odor was provided together with damp filter papers impregnated with the smell of *Drosophila* flies, the parasitoids significantly preferred this combination to the smell of the flies alone. The importance of the background odor for a parasitoid has also been highlighted by Mumm and Hilker (2005) in their study with the egg parasitoid *Chrysonotomyia ruforum*, which did not respond to the signal of oviposition-induced pine twigs ((*E*)-β-farnesene) unless it was offered in combination to the odor of an uninfested twig. Another interesting interaction between odor blends has been reported by Fukushima and coworkers (2002), who found a synergistic effect in the attraction of conditioned *Cotesia kariyai* (Hymenoptera: Braconidae) parasitoids between a blend of four volatiles typically emitted after herbivory (geranyl acetate, (*E*)-β-caryophyllene, (*E*)-β-farnesene, and indole), and a non-specific blend made up of three GLVs, β-myrcene and linalool. A limitation of this and other studies on volatiles is the authenticity of such partial plant volatile blends artificially constructed by mixing purified compounds as these often lack critical minor compounds. The use of transgenic *A. thaliana* plants transformed with TPS genes resulted in formation of both major and minor components, allowing a much more accurate dissection of the complex maize terpene blend and its ecological function.

The preference of experienced *C. marginiventris* females for the combination of two terpene blends observed in Exp. 2 could also be explained by the fact that more complex

mixtures of terpenes are more attractive to wasps. We tested the effect of mixture complexity on the wasps by giving them the choice between three terpene blends with an increasing number of components (Exp. 4). Here the parasitoids tended to orient towards the most complex blend, suggesting that the number of compounds of a terpene mixture may play a role in the attraction of *C. marginiventris* females. Similar results have been obtained in field experiments where the number of insect herbivores attracted by a volatile blend positively correlated with the number of chemicals present in the blend (Szendrei and Rodriguez-Saona, 2010). Complex volatile blends are known to play important roles in plant-insect interactions besides attraction of herbivore enemies. In a recent study, Riffell and coworkers investigated the perception and behavioral response of the moth *Manduca sexta* (L.) (Lepidoptera: Sphingidae) to the floral volatiles of the Sacred Datura (*Datura wrightii* (Regel), Solanaceae) (Riffell et al., 2009). Of the over 60 components of the floral scent, many of them terpenes, nine elicited a neural response in the moth, and, among these, four were monoterpenes (linalool, nerol, β-myrcene and geraniol), and two sesquiterpene hydrocarbons ((*E*)-caryophyllene and α-farnesene). Strikingly, the nine compounds were attractive to the moths only when offered as a mixture, but not when tested singularly.

The parasitoids used in our experiments, *C. marginiventris,* did not prefer the TPS10 sesquiterpene mixture that they were trained on over the sesquiterpene mixtures formed by TPS8 and TPS5 although there are no major sesquiterpenes common to the three odors. This suggests that *C. marginiventris* may be attracted by the presence of sesquiterpenes in general but does not distinguish between individual sesquiterpenes. A study on *M. croceipes* investigated the ability of this egg parasitoid to distinguish between aliphatic alcohols differing in the carbon chain-length and the position of the functional groups. Their results suggested that a difference of at least two C-units is necessary for the wasps in order to discriminate between two aliphatic alcohols. It is possible that *C. marginiventris*, like *M. croceipes*, is not able to distinguish among different C-15 hydrocarbon compounds like sesquiterpenes and therefore does not distinguish between the different blends offered, once they have learned one.

The responsiveness of the parasitoids to the odors offered was low in our experiments, ranging from 25-49% (Fig. 3.3b, 3.4c, 3.5b, 3.6b). A responsiveness of *C. marginiventris* females of 42% was recorded by Turlings et al., (2004) in experiments with a six-arm olfactometer exploring the odor of female wasps. Even lower responsiveness was found by Girling et al., (2006) in Y-tube olfactometer experiments with an aphid parasitoid (*Diaretiella rapae* (M'Intosh) (Hymenoptera: Aphiididae)) and *A. thaliana* plants. In this

study, the percentages of parasitoids responding to the odors offered were in some cases as low as 22.5%. From this result, the authors concluded that the corresponding odors were not especially attractive to *D. rapae*. Studies on naïve *C. marginiventris* females utilizing the complete herbivore-induced volatiles of a maize plant demonstrated a responsiveness of up to 80% (D'Alessandro and Turlings, 2005). These much higher rates of attraction to the complete blend might be due to one or more volatile compound that were not present in our blends and have not been identified yet (D'Alessandro et al., 2009; D'Alessandro and Turlings, 2005). To fully understand the importance of both constitutive and herbivore-induced volatile terpenes in parasitoid host finding, experiments should be carried out with a greater range of parasitoid species with different degrees of host specialization.

4. Chapter III

The effects of arbuscolar mycorrhizal fungi on direct and indirect defense metabolites of *Plantago lanceolata* L.*

Abstract Arbuscular mycorrhizal fungi can strongly influence the metabolism of their host plant, but their effect on plant defense mechanisms has not yet been thoroughly investigated. We studied how the principal direct defenses (iridoid glycosides) and indirect defenses (volatile organic compounds) of *Plantago lanceolata* L. are affected by insect herbivory and mechanical wounding. Volatile compounds were collected and quantified from mycorrhizal and non-mycorrhizal *P. lanceolata* plants that underwent three different treatments: 1) insect herbivory, 2) mechanical wounding, or 3) no damage. The iridoids aucubin and catalpol were extracted and quantified from the same plants. Emission of terpenoid volatiles was significantly higher after insect herbivory than after the other treatments. However, herbivore-damaged mycorrhizal plants emitted lower amounts of sesquiterpenes, but not monoterpenes, than herbivore-damaged non-mycorrhizal plants. In contrast, mycorrhizal infection increased the emission of the green leaf volatile (*Z*)-3-hexenyl acetate in untreated control plants, making it comparable to emission from mechanically wounded or herbivore-damaged plants whether or not they had mycorrhizal associates. Neither mycorrhization nor treatment had any influence on the levels of iridoid glycosides. Thus, mycorrhizal infection did not have any effect on the levels of direct defense compounds measured in *P. lanceolata*. However, the large decline in herbivore-induced sesquiterpene emission may have important implications for the indirect defense potential of this species.

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4.1 Introduction

About 80% of all terrestrial plants form associations with arbuscular mycorrhizal fungi (AMF) (Wang and Qiu, 2006). These are fungal symbionts that are well known to improve plant nutritional status by enhancing the uptake of essential nutrients such as phosphorous and nitrogen and by improving the water supply through an increase in root surface area (Smith and Read, 1997). In return, fungi receive carbon in the form of photosynthates from the plant.

For both the establishment and the maintenance of the symbiotic association between plants and AMF, it is essential that both partners recognize each other. These recognition processes are initiated via molecular cross-talk mediated by changes in the gene expression and production of signal compounds in both partners (Harrison, 2005). On the plant side, altered gene expression in the presence of AMF can influence other aspects of metabolism and even result in the induction of chemical defenses (Gange et al., 2007). A number of

studies in the recent past have reported the effects of AMF on plant defensive compounds, such as volatile terpenoids (Akiyama and Hayashi, 2002; Rapparini et al., 2008), essential oils (Copetta et al., 2006; Khaosaad et al., 2006) and glucosinolates (Vierheilig et al., 2000). Furthermore, effects of AMF on salicylic acid (SA) and jasmonate dependent signaling pathways have been reported, suggesting that AMF modulate plant defenses (review by Pozo and Azcòn-Aguilar, 2007 and references therein). AMF invasion triggers a general plant response to pathogen attack (Dumas-Gaudot et al., 2000), causing a transient accumulation of SA and activation of the SA-dependent signaling pathway at the early stages of the association. These responses then seem to be repressed once the compatibility of the symbionts is recognized (reviewed by Garcìa-Garrido and Ocampo, 2002).

Two major forms of plant anti-herbivore defenses can be distinguished: direct defenses, which are toxic to the herbivore or deter feeding, and indirect defenses, which protect the plant by attracting natural enemies of the herbivore, either parasitoids or predators. Direct and indirect defenses can be constitutively expressed or induced by mechanical damage or herbivore feeding. The literature on mycorrhizal influence on direct and indirect defenses and the consequences for insect herbivores is scarce (Gange et al., 2007; Hartley and Gange, 2009), although some studies that show the effects of AMF on direct defenses have been published (e.g., Marak et al., 2002; Fuchs and Bowers, 2004). A major group of indirect plant defenses are volatile organic compounds (VOCs) that consist principally of green leaf volatiles (GLVs) and mono- and sesquiterpenes (Pichersky and Gershenzon, 2002; Degenhardt et al., 2003). Herbivore-induced VOCs play an important role in attracting natural enemies of insect herbivores (e.g., Dicke et al., 1990; Turlings et al., 1990; Kessler and Baldwin, 2001). Furthermore, herbivore-induced VOCs act as both intra- and inter-plant signals, and can result in priming and induction of plant defenses (e.g. Kost and Heil, 2006; Frost et al., 2007; Heil and Silva Bueno, 2007).

Naturally occurring VOC emissions have been compared in mycorrhizal vs. non-mycorrhizal plants. For example, mycorrhization of *Artemisia annua* L. with two AMF species did not affect the amount of total terpenes emitted, but there were slight changes in the relative quantities of single compounds (Rapparini et al., 2008). In addition, an unspecialized fungal root endophyte (*Acremonium strictum*) reduced terpene emission of tomato plants with consequences for insect oviposition preference (Jallow et al., 2008). However, no study to date has investigated how herbivore damage alters the production of defenses in AMF vs. non- AMF plants, even though herbivory is known to have marked effects on VOC emission profiles and the levels of other defense compounds. *Plantago*

lanceolata L. is a perennial forb with a cosmopolitan distribution and commonly forms associations with a large number of AMF species (Johnson et al., 2004; Oehl et al., 2004). The main group of secondary metabolites in *P. lanceolata* is the iridoid glycosides, with two dominant compounds, namely aucubin and catalpol. These compounds function as feeding and oviposition stimulants for specialized insects, and as deterrents or toxins for generalist herbivores (e.g., Bowers and Puttick, 1988, Biere et al., 2004). Antimicrobial functions of these monoterpene derivatives also have been documented (Marak et al., 2002). The association of *P. lanceolata* with AMF can modify plant defense properties. In a study by Gange and West, the levels of the two iridoid glycosides (IGs), aucubin and catalpol, increased when the plants were associated with AMF (Gange and West, 1994). However, the effects of AMF on other groups of defensive compounds in *P. lanceolata*, such as green leaf volatiles or volatile terpenoids, have not yet been documented.

In this study, we investigated the effects of the arbuscular mycorrhizal fungus *Glomus intraradices* (N.C. Schenck & G.S. Sm.) on *P. lanceolata* by focusing on two groups of compounds, IGs and VOCs, that typically act as direct and indirect plant defenses, respectively. In an experiment with a full factorial design, we compared VOC emissions and the IG contents of AMF-inoculated and non-inoculated *P. lanceolata* individuals after mechanical wounding and caterpillar herbivory with those of non-treated control plants.

Mycorrhizal fungi could influence a plant's allocation to defense in different ways: 1) Altering nutritional status of the host plant: Greater nutrient availability could lead to an increase in primary productivity that provides more resources for the plant to use in the biosynthesis of defensive metabolites, such as IGs or VOCs. On the other hand, harboring AMF is no guarantee of increased productivity, since in return for nutrients, plants provide symbiotic fungi with photosynthates. If the outflow of photosynthates to the fungal symbionts is greater than the increase in productivity due to enhanced nutrient supply, there may be a net decrease in carbon supply that could lead to a decline in defense metabolism. This decline might affect the production of direct vs. indirect defenses differently depending on the relative value of these defensive strategies under different nutritional conditions. 2) Altering signalling pathways: Independent of plant nutritional status, the presence of microorganisms, including AMF, could alter defense signalling. Microbial infection generally is known to activate many types of defense responses, although mycorrhizal fungi usually elicit only attenuated responses (Garcìa-Garrido and Ocampo, 2002).

4.2 Methods and Materials

4.2.1 Plant, fungus and insect material

Seeds of *P. lanceolata* (Rieger & Hofmann, Germany) were sown in trays filled with commercially available sowing soil (Stender Vermehrungssubstrat A210, Stender, Germany) that was previously autoclaved for 20 min at 121°C, in order to kill potential AMF propagules. *P. lanceolata* germinated and grew in a greenhouse (day:night temperatures 20–22°C: 18–20°C, 30–55% humidity, 16 h light, photosynthetically-active radiation ca. 180 µmol $m^{-2}s^{-1}$).

To prepare a growing medium, soil from a meadow in proximity to the greenhouse (in Jena, Germany) was mixed with sand in a 1:1 (w w^{-1}) proportion, and autoclaved for 20 min at 121°C. Thereafter, 178 pots (14.4 cm diam, 1.3 l) were each filled with 300 ml of the soil-sand mixture and watered with 10 ml soil suspension (500 g fresh soil suspended in 5 l tap water, filtered through a 25 µm Whatman filter to exclude AMF propagules) in order to allow the establishment of a new microbial community in the sterile soil mixture (Schroeder and Janos, 2004). Afterwards, all pots were covered with gauze to prevent the soil from desiccation.

Glomus intraradices inoculum was purchased from Amykor (Germany) (strain AMYKOR1), and half of it was autoclaved for 30 min at 121°C. Half of the 178 pots were then inoculated with 5 g of AMF vital inoculum each ("mycorrhizal plants"), the other half were mock inoculated with the same quantity of AMF autoclaved inoculum ("non-mycorrhizal" plants). The inoculum was mixed with the upper layer (3–4 cm) of the soil-sand mixture, and the *P. lanceolata* seedlings were transplanted individually into the pots.

Due to a thrips infestation, all plants were treated once with Conserve (Dow AgroSciences LLC, USA) (Conserve 0.075 %, 0.5 l spray for all the 178 plants) 5 wk after transplantation.

Caterpillars of the generalist feeder *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae) (Syngenta, Basel, Switzerland), were reared on an artificial bean diet at 21°C. The diet was prepared by mixing 1 l tap water with 1 kg beans, 18 g ascorbic acid, 10 g 4-ethylbenzoic acid, 18 g α-tocopherol (7.1% in germ oil), 8 ml 3.7% formaldehyde, and 2.4 l of a 5% agar solution) at 21°C. Third-instar caterpillars were starved for 24 h before they were used in the experiment.

4.2.2 Experimental setup and plant treatments

Plants were divided between mycorrhizal and non-mycorrhizal treatments, and 15 plants of a single treatment were placed in one tray (60×40 cm). The trays were positioned on a greenhouse bench in two rows, one with mycorrhizal plants and the other without, in order to avoid cross infection of AMF. Tray position on the greenhouse bench was shifted weekly to control for any differences in light or temperature conditions.

The experiment started 52 days after seedling transplantation and lasted for 18 d. Treatment of the plants started in the evening, and was performed as follows: six to seven mycorrhizal and the same number of non-mycorrhizal plants were randomly chosen and moved separately to two different trays. Plants in each tray then were divided into three groups that underwent the following treatments: Group 1 – Mechanical wounding (MW): young and old leaves were evenly damaged by punching ten 4 mm holes per plant with a ticket puncher. The same treatment was repeated on the following day, before the volatile collection started. Group 2 – Herbivory (H): six third-instar *S. littoralis* caterpillars per plant were allowed to feed overnight on the foliage. Group 3 – Control (C): control plants received no damage. The foliage of both the treated and the control plants was enclosed within a cellophane bag (205x380 mm, Unipack, Germany) to prevent caterpillars from escaping. In total, 32 mycorrhizal and 32 non-mycorrhizal plants underwent the MW treatment, 29 mycorrhizal and 29 non-mycorrhizal plants underwent the H treatment, and 28 mycorrhizal and 28 non-mycorrhizal underwent the C treatment.

The following morning volatile organic compounds were collected from all plants. Cellophane bags and caterpillars were removed before the volatile collection started.

4.2.3 Plant volatiles

Volatile organic compounds emitted by *P. lanceolata* after herbivory, mechanical wounding, or control treatment were collected in a dynamic headspace collection system located in a growth chamber set at 20°C, 55% relative humidity and 85±5 μmol m^{-2} s^{-1} photosynthetically-active radiation.

Approximately 16 h after the start of the treatments (H, MW, and C), each potted plant was placed individually in a 3 l glass desiccator (Schott, Germany). Each desiccator was tightly closed with a glass lid equipped with a valve that allowed air, which was previously purified through a charcoal filter, to enter the enclosure. The incoming air flux was adjusted to 2±0.3 $lmin^{-1}$. After being in contact with the plant, the air exited the glass cylinder through a collection trap (4 mm diam glass tube containing 30 mg Super Q (ARS,

Gainesville, FL, USA), positioned in an opening in the lid.

All VOC collections were performed within a six-hour timeframe from around 10 am to 4 pm to minimize variation in volatile emissions due to plant diurnal rhythm. After 4 ½ h VOC collection, the Super Q traps were eluted with 150µl dichloromethane that contained 1500 ng nonylacetate as an internal standard.

VOCs were identified with a Hewlett-Packard model 6890 gas chromatograph employing the carrier gas He at 1 ml min^{-1}, splitless injection (injection temperature: 220°C, injection volume: 1 µl), a DB-5MS column (30 m×0.25 mm×0.25µm film, J & W Scientific, Folsom, USA), and a temperature program from 40°C (2 min hold) to 300°C (2 min hold) with a first gradient of 7°C min^{-1} to 155°C and a second gradient of 60°C min^{-1} to 300°C. Coupled to the gas chromatograph was a mass spectrometer (Hewlett-Packard model 5973) with a quadrupole mass selective detector; transfer line temperature, 270°C; ionization potential, 70 eV; and a scan range of m/z 40–350. For quantification, a GC was coupled to a FID detector operating at 250°C, using the same conditions described above.

VOCs were first identified on the GC-MS by reference spectra in the Wiley and National Institute of Standards and Technology libraries and in the literature (Joulain and König, 1998) and by comparison of retention times and mass spectra to those of standards in our collection and others kindly supplied by Wilfried A. König, Hamburg (essential oils of *Oreodaphne porosa* and *Aloysia sellowii*). Quantification of the identified compounds was carried out by comparing the peak areas in the FID traces with that of the internal standard, applying a response factor of 1 for the internal standard, 1.11 for (*Z*)-3-hexenyl acetate, 0.75 for (*E*)-β-ocimene and (*E*)-4,8 dimethyl-1,3,7-nonatriene (DMNT), and 0.74 for all the sesquiterpenes (calculated according to the effective carbon number concept (Scanion and Willis 1985)). In addition to the 6 major compounds discussed in the "*Results*" section, other compounds were identified in a subgroup of the 29 plants subjected to herbivory treatment (*N*=number of individuals from which the particular VOC was identified): limonene (*N*=11), α-copaene (*N*=12), β-elemene (*N*=5), α-humulene (*N*=5), α-muurolene (*N*=2), δ-cadinene (*N*=2). As the sum of these terpenoids never exceeded 7% of the total volatiles, they were not included in further analyses.

4.2.4 Plant performance

After VOC collection, the aboveground parts of all plants were cut at ground level, and the number of leaves and fresh weight were recorded for each plant. In order to estimate the amount of leaf area lost due to caterpillar feeding in the herbivore treatment, the leaves

from these plants were aligned on a white board together with a reference area of 2.25 cm² and photographed with a digital camera. Digital images were analyzed with Adobe Photoshop (Adobe Systems Incorporated, USA). By referring to the amount of pixels in the reference area, actual remaining leaf areas were determined. Leaf area loss due to caterpillar feeding then was reconstructed by using the remaining leaf area as a template. After photographing, leaves of all plants were frozen in liquid nitrogen and freeze dried. Then, the dry weight of each individual was measured.

4.2.5 Iridoid glycosides

Iridoid glycosides were extracted from 25 mg of freeze dried, finely ground leaf material with 1.8 ml methanol. After 6 h extraction, leaf material was centrifuged at 16000 g, and the supernatant was transferred into clean tubes and evaporated to dryness under nitrogen. The pellet was redissolved in 500 µl of a 20% methanol solution and centrifuged at 16000 g (modified from Marak et al., 2002). Separation of iridoid glycosides was achievedon a Hewlett Packard HP 1100 Series HPLC system with autosampler and diode-array detector. The procedure employed a C-18 reversed phase column (Supelcosil LC18, 250×4.6 mm i.d., 5µm particle size, Supelco) operated at 1 ml min^{-1} and 25°C. Injection volume was 20 µl. Elution was accomplished with a gradient (solvent A: 0.05% trifluoroacetic acid, solvent B: MeCN) of 0–15 % B (15 min), followed by a cleaning cycle (15–100% B in 0.5 min, 2.5 min hold,100 to 0% B in 0.1 min, 5 min hold). Eluting compounds were monitored at 200 nm, and peaks were identified by match of retention time and UV spectrum with those of commercial standards (catalpol, Wako Chemicals; aucubin, Carl Roth, Germany). Additionally, the identity of catalpol and aucubin was confirmed by LC-MS. Concentrations of iridoid glycosides were calculated by using response curves generated with external standards of catalpol and aucubin.

4.2.6 Mycorrhization rates

For the determination of mycorrhization rate, a subset of 30 mycorrhizal and 30 non-mycorrhizal plants was sampled so that an equal number of individuals from each treatment (H, MW, and C) and from each day of volatile collection were included. Immediately after removal of the leaves, roots were rinsed carefully to wash off the soil, fixed in formaldehyde–acetic-acid [FAA: 6.0% formaldehyde, 2.3% glacial acetic acid, 45.8% ethanol and 45.9% H2O (v v^{-1})], and stored at 4°C. Fixed roots were washed with distilled water, cut in segments of approximately 1.5 cm, and heated at 90°C for 10 min in 10% KOH.

Afterwards, roots were rinsed in tap water, acidified to 3.7% HCl for 10 min, and stained for 11 min in a ready-to-use lactophenol blue solution (Fluka, Switzerland) (Phillips and Hayman, 1970). The stain in excess was removed in 50% lactic acid. Total mycorrhizal colonization rates (percentage of the examined root segments with mycorrhizal structures) were determined microscopically using the line intersect method (Phillips and Hayman, 1970), modified after Schmitz et al., (1991). Stained root segments from one single plant were densely packed on a microscope slide. A minimum of 300 visual fields per slide were observed at 200×magnification.

4.2.7 Plant and soil nutrient analysis

Freeze dried, finely ground plant material (300 mg) and the sand-soil mixture in which the plants grew (500 mg) were analyzed for total carbon and nitrogen content with an elemental analyzer (Vario MAX CNS, Elementar, Hanau, Germany).

4.2.8 Statistical analysis

In order to analyze the influence of mycorrhization and treatments (H, MW, and C) on the amount of 1) the individual volatile compounds, 2) the total amount of terpenes, and 3) the amount of GLVs emitted and corrected for the dry weight of the plants at the same time, analysis of covariance (ANCOVA) was used. Type of treatment and presence of mycorrhiza were considered as fixed factors, and dry weight was fitted in the model as a continuous covariate. Data were either cube root (total terpenes and GLV) or log transformed (single volatiles), and quantities equal to zero were excluded from the analysis of single volatiles to match the prerequisites for ANCOVA. Whenever possible, models were simplified by removing non-significant terms (Crawley, 2007). Significance of differences between factor levels were tested by Tukey multiple comparisons of means.

As for volatiles, the influence of mycorrhization and treatments was analyzed on catalpol and aucubin levels. Since IGs are constitutively produced compounds known to accumulate during plant development (Barton, 2007), time was fitted to the model as a covariate. Time was indicated as the number of days (1 to 18) from the start of the experiment, i.e., from the day of the treatment of the first set of plants. Catalpol quantities were square-root transformed before analysis to fulfil the requirements for ANCOVA.

Significance of differences in aboveground biomass (dry weight), leaf number, herbivory rates, and carbon and nitrogen content between mycorrhizal and non-mycorrhizal plants was tested by Student's t-test.

All statistical analyses were performed with software package R (R Development Core Team (2008), R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.).

4.3 Results

4.3.1 Plant volatiles

Plantago lanceolata plants emitted a volatile bouquet dominated by the green leaf volatile (GLV), (*Z*)-3-hexenyl acetate, as well as terpenoids of different classes. In order to assess the effect of mycorrhization and different treatments on the volatile emission of *P. lanceolata*, we quantified the six major volatile compounds, which together make up between 70–90% of the total mixture in herbivory treated and mechanically wounded plants. These include the green leaf volatile (*Z*)-3-hexenyl acetate, the monoterpene (*E*)-β-ocimene, the C_{11} homoterpene (*E*)-4,8- dimethyl-1,3,7-nonatriene (DMNT), and the sesquiterpenes (*E*)-β-caryophyllene, (*E*)-α-bergamotene, and (*E*)-β-farnesene. The volatile organic compound (VOC) emission profile of *P. lanceolata* was significantly different among plants in the different treatments (Fig. 4.1a, Table 4.1). Most strikingly, herbivore-damaged plants emitted much higher levels of terpenoids than mechanically-damaged plants or undamaged controls (Fig. 4.1a, Table 4.1).

There were significant differences between mycorrhizal and non-mycorrhizal plants. Mycorrhizal plants emitted about 55% less total terpenoids compared to nonmycorrhizal plants in the herbivory treatment (mycorrhiza: $F_{1,171}$=5.11, P=0.039; interaction mycorrhiza:treatment: $F_{2,171}$=8.14, P<0.001; Fig. 2a). Among the classes of terpenoids, the emission of sesquiterpenes was 63% lower in mycorrhizal compared to non-mycorrhizal plants after herbivory (Table 1), but the emission of monoterpene, (*E*)-β-ocimene, was not affected by this treatment ($F_{1,83}$=0.04, P=0.845, Table 4.2).

For (*Z*)-3-hexenyl acetate, the only major GLV detected, mycorrhizal infection increased emission of undamaged plants more than 3-fold compared to undamaged nonmycorrhizal plants. Herbivory and mechanical wounding of mycorrhizal plants caused no further increase in the emission of this GLV, while these treatments did increase emission from non-mycorrhizal plants by over 5-fold (Fig. 4.1b).

The ANCOVA showed no significant interactions between the quantity of any of the

terpenes and plant dry weight. However, a significant interaction of dry weight and mycorrhization ($F_{4,170}$=4.36, P=0.038, Table 4.2) was found for (*Z*)-3-hexenyl acetate. This means that the effect of mycorrhization on GLV emission varied according to plant biomass. In small plants, mycorrhization increased (*Z*)-3-hexenyl acetate emission, while it had the opposite effect on larger plants (Fig. 4.2).

Table 4.1 Emission rate of the six major volatiles and content of the iridoid glycosides in the leaves of herbivory-treated, mechanically wounded and untreated control *Plantago lanceolata* plants.

Compound	Mycorrhizal			Non-mycorrhizal		
	Herbivory	Mechanical wounding	Control	Herbivory	Mechanical wounding	Control
Volatiles ng $(gDW)^{-1}hr^{-1}$; mean±SE						
(*Z*)-3-hexenyl acetate	376.61±97.56	552.15±65.96	364.37±99.6	581.96±104.68	376.61±71.3	97.19±13.59
(*E*)-β-ocimene	75.37±15.19	6.94±2.21	1.01±0.71	103.04±17.41	4.06±1.10	0.94±0.60
DMNT	22.05±4.64	0.19±0.19	0.00	68.65±11.71	0.70±0.52	0.00
(*E*)-β-caryophyllene	45.90±10.06	2.23±0.92	0.00	107.69±25.83	0.23±0.19	0.65[a]
(E)-α-bergamotene	25.84±5.03	1.13±0.81	0.00	70.16±12.64	0.45[a]	0.45[a]
(*E*)-β-farnesene	31.84±8.05	0.88±0.61	0.00	101.75±21.67	0.00	0.59[a]
Iridoid glycosides µg $(mgDW)^{-1}$; mean±SE						
Catalpol	2.79±0.32	2.87±0.35	3.26±0.34	3.03±0.32	2.95±0.33	2.94±0.47
Aucubin	7.13±0.58	6.61±0.54	6.89±0.49	7.35±0.51	6.54±0.60	5.37±0.54

[a] These volatiles were emitted by a single plant, therefore the SE has not been calculated

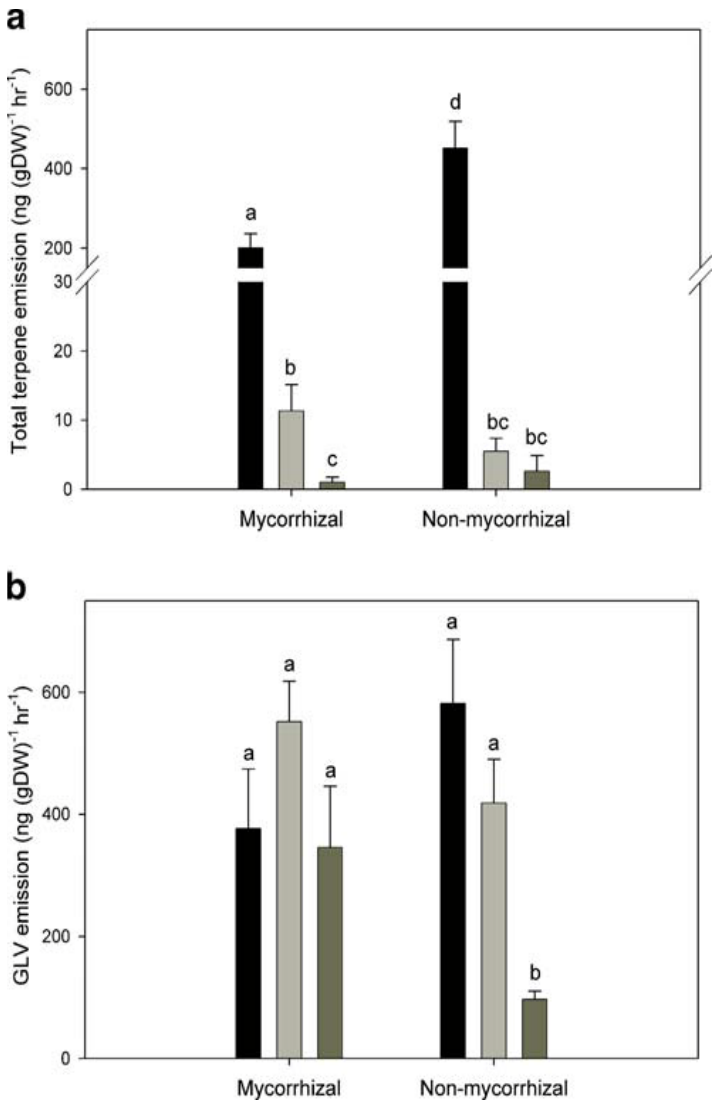

Fig. 4.1 Total emission of terpenes (**a**) and green leaf volatiles (GLV) (**b**) from *Plantago lanceolata* after herbivory (black bars) and mechanical wounding (light gray bars) in comparison to untreated control plants (dark grey bars). Bars represent means ± SE. Different letters indicate significant differences between the means according to ANCOVA followed by Tukey test (adjusted $P<0.05$)

Table 4.2 ANCOVA summary table for the effects of mycorrhization, treatment and dry weight on the VOC emission of *P. lanceolata*

VOC	Factors				Covariate		df residuals
	Mycorrhiza (df=1)		Treatment (df=2)		Dry weight (df= 1)		
	F	*P*[a]	*F*	*P*	*F*	*P*	
(*Z*)-3-hexenyl acetate	2.45	0.119[b]	15.47	<0.001[b]	1.34	0.242[b]	170
(*E*)-β-ocimene	0.04	0.845	46.85	<0.001	6.78	0.011	82
DMNT	7.68	0.008	8.55	0.005	1.20	0.279	48
(*E*)-β-caryophyllene	11.20	0.001	12.98	<0.001	3.00	0.089	50
(*E*)-α-bergamotene	18.14	<0.001	1.74	0.185	1.84	0.180	54
(*E*)-β-farnesene	7.57	0.008	1.066	0.353	1.19	0.281	46

[a] Bold numbers indicate significant effects
[b] For (*Z*)-3-hexenyl acetate two interactions were significant: mycorrhiza x treatment (F=8.41, P<0.001) and mycorrhiza x dry weight (F=4.36, P=0.038). There was no significant interaction for any of the other volatile organic compounds

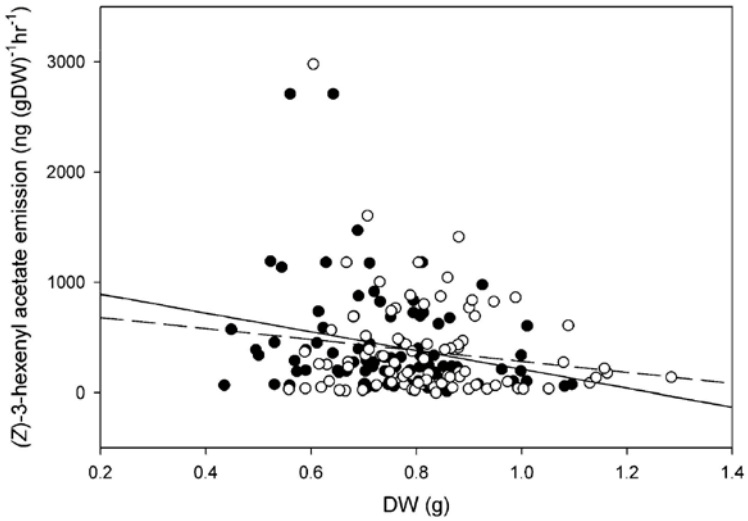

Fig. 4.2 Linear regression of (*Z*)-3-hexenyl acetate emission and plant dry weight for mycorrhizal (solid line) and non-mycorrhizal (dashed line) plants. Black dots represent all mycorrhizal plants (herbivory-treated, mechanically wounded, and untreated), while open dots represent all non-mycorrhizal plants (herbivory-treated, mechanically wounded, and untreated)

4.3.2 Plant Performance

The total aboveground biomass of mycorrhizal plants was on average 8.5% lower than non-mycorrhizal plants (fresh weight: $t_{1,175}$=−3.19, P=0.002, dry weight: $t_{1,176}$=3.87, P<0.001) (Fig 4.3). This result might be attributed to a drain of fixed carbon toward the fungus in mycorrhizal plants. In fact, mycorrhizal plants contained less carbon in their leaves compared to nonmycorrhizal plants (average C%: mycorrhizal plants= 41.4%, non-mycorrhizal plants=42.3%; Student's t-test, $t_{1,24}$=−3.98, P<0.001), but nitrogen content did not vary between leaves of mycorrhizal and non-mycorrhizal plants (N%=0.81% in both groups). The C/N ratio of the sand-soil mixture where the plants were grown was 23.15±0.35 (mean±SE, N=4). Non-mycorrhizal plants had on average a higher number of leaves compared to the mycorrhizal plants (mean non-mycorrhizal: 12.10, mean mycorrhizal: 11.40, $t_{1,162}$=−1.87, P=0.063).

Mycorrhization did not influence the feeding behaviour of *S. littoralis* caterpillars. The amount of leaf tissue consumed overnight was the same in mycorrhizal and non-mycorrhizal plants. ($t_{1,53}$=−0.83, P=0.411).

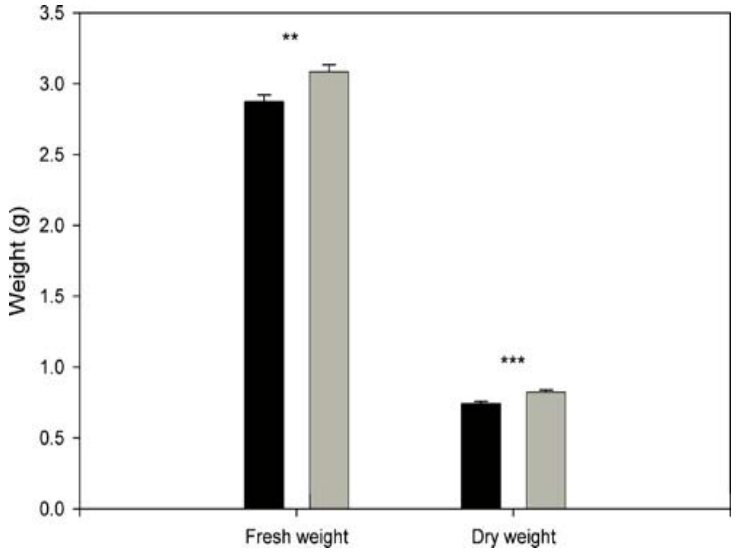

Fig. 4.3 Aboveground biomass of *P. lanceolata*. Fresh weight was recorded once for each plant, on the day the volatile collection was performed. Black bars = mycorrhizal plants, grey bars = non-mycorrhizal plants. Bars represent means ± SE. Asterisks represent significant differences according to Student's t-test (**P<0.01, ***P<0.001)

4.3.3 Iridoid Glycosides

The two main iridoid glycosides (IGs) catalpol and aucubin did not show any significant change in quantity, either between the treatments or between mycorrhizal and non-mycorrhizal plants (catalpol: treatment: $F_{2,173}$=0.065, P=0.937, mycorrhiza: $F_{1,173}$ =0.021, P= 0.884; aucubin: treatment: $F_{2,173}$=2.09, P=0.127, mycorrhiza: $F_{1,173}$=0.98, P=0.322) (Table 1). The cofactor 'time' was significant for both catalpol ($F_{1,173}$=11.91, P<0.001) and aucubin ($F_{1,173}$=8.14, P=0.005), indicating that the concentration of IGs in the leaves slightly increased over time.

4.3.4 Mycorrhization Rates

At the moment of harvest, all inoculated plants were infected by AMF. On average, we found 68% of the root system colonized by the fungus. No mycorrhizal structures were detected in roots of non-mycorrhizal plants.

4.4 Discussion

Mycorrhizal symbiosis is known to affect many plant parameters, including chemical defense (Gange et al., 2007; Hartley and Gange, 2009). To understand fully the effects of this symbiosis on chemical defense, it is necessary to investigate compounds involved in both direct and indirect defense. Indirect defense is often manifested by the herbivore-induced release of volatile organic compounds (VOCs) that attract herbivore enemies. The results from our study show that *P. lanceolata* produces a large increase in volatile terpenoids after herbivory that may serve in indirect defense. However, the association of *P. lanceolata* with the arbuscular mycorrhizal fungus (AMF)

G. intraradices significantly modifies the release of herbivore-induced VOCs from the plant by reducing the emission of the three major sesquiterpenes and the C_{11} homoterpene, DMNT. As DMNT and sesquiterpenes are prominent components of volatile blends known to act in indirect defense (Turlings et al., 1990), AMF may decrease the ability of herbivore-damaged plants to attract herbivore enemies. Consistent with these results, Jallow and coworkers (2008) recently reported that the inoculation of tomato plant roots with the endophytic fungus *Acremonium strictum* lead to a reduction in total terpenoid emission. In *Artemisia annua*, Rapparini et al., (2008) reported that infection with different *Glomus* species constitutively reduced the amounts of sesquiterpenes produced by undamaged

plants compared to non-infected control plants. In contrast, infection had no effect on the amount of monoterpenes emitted by *A. annua*. These findings are consistent with our results from caterpillar-infested *P. lanceolata* plants where the major emitted monoterpene ((*E*)-β-ocimene) did not differ in its relative release rate between mycorrhizal and non-mycorrhizal plants.

Although there is as yet no evidence in the literature that AMF can alter plant indirect defenses, there are reports of the effects of mycorrhization on the major direct defense compounds of *P. lanceolata*, the constitutively produced iridoid glycosides (IGs), aucubin and catalpol. In our study, the levels of IGs were unaffected by mycorrhization. In contrast, a positive effect of mycorrhization on the IG content of *P. lanceolata* was reported by Gange and West (1994), who recorded higher levels of IGs in mycorrhizal compared to non-mycorrhizal plants. The authors attributed this result to the relatively higher fixed carbon levels in the shoots of mycorrhizal plants compared to non-mycorrhizal plants, which allowed more substrate to be allocated to IG production. In contrast, no effect of mycorrhization by *G. intraradices* was found on the catalpol content of *P. lanceolata* leaves by Wurst et al. (2004). Moreover, these authors observed a decrease in leaf carbon content after mycorrhization. We also observed a decrease in leaf carbon levels in the present study, and saw no increase in IG content. This finding supports the hypothesis that IGs are produced in higher amounts after mycorrhization only when symbiosis leads to greater amounts of fixed carbon in the leaves.

Not only did we find a reduction of total leaf carbon content in mycorrhizal plants, but also the aboveground biomass of mycorrhizal plants was lower than that of non-mycorrhizal plants. Although positive effects of AMF on their symbionts' productivity seem to be commonplace (Johnson et al., 1997), neutral or negative effects of mycorrhization on plant growth parameters and biomass also have been documented for *P. lanceolata* (Ayer et al., 1992; Klironomos, 2003; Reynolds et al., 2005) and a number of other plant species (see Johnson et al., 1997 and references therein). That AMF can function as carbon sinks in plants has already been documented (Wurst et al., 2004; Reynolds et al., 2005; Ayres et al., 2006). If the growth of the fungus limits the carbon available to the plant for its basic metabolic requirements, the association could turn from symbiosis into parasitism, a phenomenon that has been attributed by some authors to nutrient-rich substrates, low temperatures, or limiting light conditions (reviewed by Smith and Smith, 1996; Purin and Rillig, 2008), or to particularly high mycorrhization rates (Gange et al., 1999 and references therein).

In addition, AMF can be detrimental in association with particular hosts (Klironomos, 2003), and the predisposition of a given fungal strain to parasitism rather than symbiosis seems to be at least partially under genetic control (Johnson et al., 1997). When confronted with a non-beneficial AMF association, plants might reallocate their resources among growth, defense, reproduction, and other functions. In this study of young *P. lanceolata*, there was a decline in growth, and a reduction in indirect defenses (volatile terpenes), but no change in the level of direct defenses (iridoid glycosides). These findings support our hypothesis that a net decrease in carbon availability caused by the fungus can cause a reduction in allocation to defense (in this case indirect defense). Maintaining high levels of direct chemical defenses in early developmental stages is a strategy adopted by a number of plant species, for example *Arabidopsis thaliana* (L.) Heynh. (Brown et al., 2003) and *Nicotiana sylvestris* Spegazzini and Comes (Ohnmeiss and Baldwin 2000). The reduction in indirect defenses observed can also be interpreted as a consequence of the general repression of defenses due to AMF colonization. In the first stages of the association, AMF seem to be able to modulate the defense signaling cascades in the plant, and lower the production of defense compounds (reviewed by Garcìa-Garrido and Ocampo, 2002).

The mechanisms that underlie the induction of volatile terpene emission in *P. lanceolata* after biotic stresses such as herbivore feeding have not yet been elucidated. Terpene biosynthesis is carried out by condensation of C_5 isoprenoid units, which, in plants, can derive from two pathways: the methylerythritol phosphate (MEP) pathway, considered the principal route to monoterpene production, and the mevalonate (MVA) pathway, thought to be responsible for the production of sesqui-and triterpenes. The MEP pathway also leads to the production of carotenoids and eventually apocarotenoids, some of which are responsible for the yellow-colored roots seen in a number of species after mycorrhization (Strack et al., 2003). Interest in the function of this yellow pigment has stimulated study of the MEP pathway during mycorrhizal formation. Mycorrhization enhances the transcription of genes encoding 1-deoxy-Dxylulose 5-phosphate synthase (DXS), the enzyme that catalyzes the initial step of the MEP pathway in several species including *Medicago truncatula* L., *Nicotiana tabacum* L., *Zea mays* L., *Lycopersicon esculentum* Mill., and *Triticum aestivum* L. (Walter et al., 2000, 2002). An accumulation of transcripts for 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), the enzyme immediately downstream from DXS in the MEP pathway, also has been reported in wheat roots after *G. intraradices* infection (Walter et al., 2000). This AMF-mediated activation of the MEP pathway has not yet been examined with respect to the formation of monoterpenes, assumed to be MEP pathway-

derived. After mycorrhizal infection of P. lanceolata, we observed no change in volatile monoterpene emission or the accumulation of the IGs, which derive from the same biosynthetic route as monoterpenes. Instead, a decrease in sesquiterpenes and DMNT, assumed to be derived from the MVA pathway, was detected. There are no reports in the literature on how the mevalonate pathway is altered by AMF infection. The production of IGs is known to be strongly dependent on plant age in *P. lanceolata* (Fuchs and Bowers, 2004; Barton, 2007). In the study by Gange and West (1994), who found higher levels of IGs in mycorrhizal compared to non-mycorrhizal plants, IGs were extracted from four-month-old plants, which had been associated with AMF for 14 weeks. In contrast, the plants in the study by Wurst et al., (2004), where the IG content did not change with mycorrhization, and in our study were harvested between 9 and 12 weeks of age with a mycorrhizal period of 7 or 10 weeks, respectively. According to Fuchs and Bowers (2004), the concentration of IGs in *P. lanceolata* starts increasing 9 weeks after germination, while Barton (2007) found a strong increase in constitutive levels of IGs already after 6.5 weeks, and furthermore detected a positive correlation between growth rate and daily IG production. Direct comparisons of our results with the literature are difficult because of differences in growing conditions and the genetic properties of the plant material. Nevertheless, it seems plausible that the lack of increase in IGs on mycorrhization observed in our plants is due to harvest at a relatively early developmental stage.

The production of the GLV (*Z*)-3-hexenyl acetate in undamaged mycorrhizal-infected plants was comparable to that in herbivore or mechanically damaged individuals whether or not they were infected with mycorrhizae (Fig 2). Like many volatile terpenes, GLVs have been indicated in attracting insect parasitoids (Hoballah and Turlings, 2005; Shiojiri et al., 2006), and can, therefore, be considered indirect defense signals. By increasing the emission of levels of GLVs in undamaged plants, mycorrhizal infection turns these typically induced volatiles into constitutive ones. This constitutively high GLV emission could be a possible explanation for the enhanced attractiveness of mycorrhizal vs. non-mycorrhizal plants to herbivore parasitoids observed by Guerrieri et al., (2004).

Considering the broad distribution and the importance of AMF associations in terrestrial ecosystems in general (Treseder and Cross, 2006; van der Heijden et al., 2008) and more specifically in agricultural systems (Sawers et al., 2008), there is a need for further studies to analyze the impacts of mycorrhizal associations on plant defense in a broad range of species and the regulatory mechanisms responsible for these changes. Based on the present results, such investigations should also include an assessment of the mycorrhizal effects on

the attraction of herbivore enemies. Since this attraction can vary when a combination of fungal species are associated with one plant (Gange et al., 2003), the effect of simultaneous association of different AMF on plant chemical defenses should also be investigated.

5. General Discussion

Vegetative volatiles mediate plant interactions with the environment. They help plants defend themselves against herbivores both above- (Unsicker et al., 2009) and below-ground (Ali et al., 2010; Rasmann et al., 2005). They can act as signals within or between plants, eliciting defensive responses (Heil and Bueno, 2007; Kessler et al., 2006). Since volatiles are so frequently released by plants after herbivore damage, much effort has been made to elucidate the role of herbivore-induced volatiles. However, undamaged plants of many species emit volatile organic compounds (VOCs) too. In order to fully understand the function of VOCs, it is necessary to investigate the function of both induced and constitutive volatile emission. In this work, we studied the role of constitutive volatiles in plant direct (chapter I) and indirect defense (chapter II) in relation to herbivore-induced volatiles. Moreover, we investigated how volatile emission is affected by association with arbuscular mycorrhizal fungi (chapter III).

5.1 Constitutive volatiles act as defenses against fungal pathogens but not against insect herbivores

Pathogen attack is one of the major biotic stresses that a plant has to withstand. In particular, polycyclic fungal pathogens release spores that can threaten plants' health throughout the growing season. The continuous emission of terpenes from maize seedlings could function as a protection against fungal infection.

We produced transgenic *A. thaliana* plants overexpressing the terpene synthase gene responsible for the production of constitutive volatiles in maize seedlings, and infected those and wild type plants with the nectrotrophic fungus *Alternaria brassicicola*. Quantification of the fungal biomass in the leaves four days after infection revealed that the fungus grew less in terpene-producing than in wild type plants. Hence our results show that the constitutive terpenes emitted by maize seedlings can indeed act as anti-fungal defenses *in planta* (chapter I). There are many reports of antifungal activity of sesquiterpene hydrocarbons in the literature, but mosst of these are based on *in vitro* studies. For example, Caccioni et al. (1998) tested the essential oils from six *Citrus* species against two common

post-harvest citrus fruit pathogens (*Penicillium digitatum* Sacc. and *P. italicum* Whem.) and found a significant positive correlation between the content of sesquiterpenes in the oil and their antifungal activity. In another study, Maxia and coworkers (2009) tested the growth inhibitory activity of extracts of different ecotypes of wild *Daucus carota* L. subsp. *carota*, rich in sesquiterpene hydrocarbons and characterized by a high concentration of β-bisabolene (17-51%). These oils inhibited the growth of 12 fungal species among an assortment of yeasts, dermatophytes, and moulds, suggesting that terpenes have a non-specific mode of action against fungi. There are, however, no reports in the literature on the antimicrobial efficacy of leaf terpene volatile emission *in vivo*. Mendgen and coworkers (2006) reported the antifungal activity of a natural sesquiterpenoid, farnesyl acetate. This volatile, emitted by broad bean (*Vicia faba*) leaves upon fungal infestation, caused a decrease in the number of haustoria and colony diameter of different rust fungi growing on broad bean leaves. However, farnesyl acetate is released together with other volatiles, which instead promoted fungal growth. The authors suggested that application of farnesyl acetate for rust control in crops would be promising, but left undetermined the significance of this volatile for natural plant defense. Other studies on the bioactivity of terpene volatiles focus on their potential use in the prevention of post-harvest fungal infections or for other applications in the food industry or in pharmacology (Cavanagh, 2007).

In addition to vegetative volatiles, terpene volatiles from flowers may also have antimicrobial functions. In flowers, the nectary surface is wetted by a nutrient-rich solution, which makes it an ideal site of growth for bacteria and fungi. Moreover, the openings from which the nectar exudes are a preferred site of infection for pathogens (Agrios, 2005). It is therefore possible that plants have developed defense mechanisms to prevent microbial entrance at the site of floral nectaries, and that these mechanisms include terpene volatile emission (Tholl et al., 2005). The emission of the sesquiterpene (*E*)-β-caryophyllene from *A. thaliana* stigmas, and linalool and linalool oxide from the pistils of two *Clarkia* species could function as antimicrobial defenses (Tholl et al., 2005, and references therein), but these proposals have not been tested.

The sesquiterpene volatiles of maize we tested are cyclic lipophilic compounds (chapter I). Their antimicrobial properties are considered a result of their tendency to accumulate in lipid membranes, impairing their structure and altering enzyme activity. The monoterpene α-pinene, for example, showed toxic effects on the yeast *Saccharomyces cervisiae*. Toxicity was due to loss of cellular integrity due to membrane disruption and inhibition of respiratory activity in mitochondria (see Sikkema et al., 1995, and references therein).

Maize sesquiterpene volatiles might therefore interact with cellular or organellar membranes of fungal pathogens causing a loss of functionality.

Another possible explanation for the reduced fungal growth we observed is that terpenes act as signaling agents in the plant and prime more rapid or sustained activation of antifungal defenses. Green leaf volatiles (GLVs), for example, can prime an unattacked plant, and induce a more rapid and efficient response to subsequent herbivory (Engelberth et al., 2004; Ton et al., 2007) or pathogen attack (Kishimoto et al., 2005). In Arabidopsis, necrotrophic fungi like *A. brassicicola* elicit the jasmonic acid (JA) defense cascade (Glazebrook, 2005). Thus, we measured the level of induction of lipoxygenase2 (*LOX2*), a crucial gene in the JA pathway, in transgenic and wild type Arabidopsis (chapter I). Transgenic plants did not respond to infection with a faster activation of the JA pathway than wild type plants did, suggesting that sesquiterpenes do not prime the plant to pathogen attack at least via the JA signaling cascade.

In order to rule out possible alterations of other defense traits due to pleiotropic effects in the transgenic Arabidopsis lines used, we measured the content of the main secondary metabolites known to play a role in antifungal defenses in *A. thaliana*: glucosinolates and camalexin (chapter I). We found that total aliphatic and indole glucosinolate content did not vary significantly between wild type and transgenic plants, and was not altered by *A. brassicicola* infection. Although glucosinolates are best known as constitutive anti-herbivore defenses of Brassicaceae, many studies have provided evidence for their antimicrobial activity (e.g. Brader et al., 2006; Manici et al., 1997). In particular, the indole glucosinolate 4-methoxyindol-3-ylmethylglucosinolate (4MOI3M) has been recently shown to play a major role in antifungal defense in Arabidopsis, being involved in the activation of callose deposition in response to pathogen attack in the plant (Bednarek et al., 2009; Clay et al., 2009). Consistent with these results, we have found that 4MOI3M was induced by fungal infection both in wild type and in transgenic plants. Camalexin was, as expected, highly induced by fungal infection. However, no differences in the time of induction and in camalexin increase rates were observed between transgenic and wild type plants.

Since plant susceptibility to fungal infection also depends on nutritional status, we measured the content of elemental carbon and nitrogen in transgenic and wild type plants (data not shown). While carbon content was similar in all the lines, a slight though significant increase in nitrogen content was found in one transgenic line (average N: 6.73% transgenic line, 6.15% wild type, n=6). The effects of nitrogen on *Alternaria* development are controversial. Rather old and anonymous observations of field-grown crops support the

hypothesis that fertilization promotes the development of more vigorous and hence more resistant plants. More recent reports indicate that the influence of nitrogen on *Alternaria* species is dependent on environmental conditions, or that susceptibility of leaves to infection does not correlate with nitrogen levels (see Rothem, 1994, and references therein). Since in our fungal quantification experiments three independent transgenic lines were used and all produced similar results, the effect of differences in plant nutritional status on fungus development are considered to be negligible.

We also tested the activity of the constitutive maize sesquiterpene blend on the generalist herbivore *Spodoptera littoralis* (chapter I) by assaying the possible toxic effect of volatile terpenes on the performance of this lepidopteran. VOCs had no effect on the weight gain of caterpillars until pupation, the pupal weight, the adult weight, and the time needed for pupation and emergence. This finding is in apparent contradiction with the results of Turlings and Ton (Turlings and Ton, 2006), where *S. littoralis* caterpillars fed on plants exposed to herbivore-induced VOCs grew less compared to the ones which fed on control plants. However, the decreased larval weight the authors observed was not due to a direct effect of the volatiles on the caterpillars, but rather to other herbivore-induced changes in the plants on which the caterpillars fed. In fact, a gene expression analysis of defense-related genes of plants exposed to herbivore-induced VOCs showed a faster and stronger induction of proteinase inhibitor genes after subsequent herbivore feeding, which likely resulted in poor caterpillar development.

Not only were terpene volatiles without effect on the performance of *S. littoralis* caterpillars, but they also had no influence on their food preference. No repellent effects of VOCs were observed. When caterpillars were given the choice to feed on transgenic terpene-producing *A. thaliana* plants or on wild type terpene-free plants, they chose equally between the two. Moreover, the leaf area consumed was similar between the plant types. In contrast to our results, other sesquiterpenes are reported to have insect antifeedant activity. The sesquiterpene hydrocarbon zingiberene, for example, has been associated with deterrence to the Colorado potato beetle (*Leptinotarsa decemlineata*) and beet armyworm (*Spodoptera exigua*) in wild tomato species (*Lycopersicon* spp.) (Antonious and Kochhar, 2003). These different results may be explained by the concentrations at which the larvae ingest the terpenes. In tomato, terpenes are stored in concentrated amounts in the glandular trichomes (Carter et al., 1989), while the transgenic Arabidopsis we used continuously emitted the compounds as volatiles. We calculated that a larva would have to consume

about 500 g of Arabidopsis leaves to ingest the amount of sesquiterpenes present in less than a cm^2 of tomato leaf.

5.2 Constitutive volatiles in indirect defense

Herbivore-induced volatiles from maize and from other plant species have been shown on numerous occasions to attract herbivore enemies. These volatiles, however, are released against a constitutive background odor, which could interact with the herbivore-induced volatiles in insect signaling. We performed olfactometer experiments with the parasitoid wasp *Cotesia marginiventris* to test how constitutive and herbivore-induced volatiles interact in parasitoid attraction (chapter II). Constitutive terpenes may: 1) act synergistically with the herbivore-induced terpenes, which should result in a stronger attraction of the wasps; 2) mask the herbivore-induced signal, thus weakening the parasitoid attraction; or 3) neither enhance nor diminish the parasitoid response to the herbivore-induced odor. Our results show that the constitutive volatile blend can act synergistically with the herbivore-induced one, enhancing parasitoid attraction. Therefore, constitutively-emitted volatiles can play a role in the indirect defense of the plant.

C. marginiventris females are known to be attracted to odors they have associated to an oviposition experience (a phenomenon known as *associative learning*). In our laboratory experiments, we have shown that these wasps can learn and therefore be attracted to the terpene smell of an intact maize plant. Parasitoids may exploit constitutive volatiles to locate a potential host habitat (including damaged and undamaged plants) when they are remote from the food plant of their host, and then search for more specific olfactory cues when in proximity of a plant.

Regardless of which terpene blend was present when *C. marginiventris* females had their first oviposition experience, most were attracted to the combination of both constitutive and herbivore-induced terpenes. We hypothesize that the herbivore-induced blend functions as a stronger signal for the parasitoids when it is released on its natural background odor (the constitutive terpene blend). The importance of background odor for parasitoid attraction has been highlighted by Mumm and Hilker (2005) in their study with an egg parasitoid of the pine sawfly *Diprion pini*. The sesquiterpene (*E*)-β-caryophyllene released by pine twigs after sawfly oviposition signalled the presence of a host to parasitoid females but only when offered together with the backround odor of uninfested twigs.

Although herbivore-induced volatiles vary according to plant and herbivore species (Arimura et al., 2009), blends of unrelated plant species can share many common compounds. For example, after *S. littoralis* attack, maize leaves emit a blend dominated by green leaf volatiles, and by the sesquiterpenes (*E*)-α-bergamotene and (*E*)-β-farnesene (Turlings and Tumlinson, 1991). The same volatiles were found in the herbivore-induced blend of *Plantago lanceolata* plants (see chapter III). Even the (*E*)-β-caryophyllene released by pine twigs after *D. pini* oviposition is a very common volatile, present in the herbivore-induced blends of many species, like *Medicago truncatola* (Leitner et al., 2005), *Phaseolus lunatus* (Mithofer et al., 2005), and *Populus* spp. (Frost et al., 2007). Various constitutive plant volatiles are also widely distributed among taxonomically unrelated species. However, there is evidence that insect herbivores can nevertheless use the host-specific blend of these ubiquitous VOCs to recognize their host plant (Webster et al., 2010). Such a blend could have a similar function for parasitoids of herbivore enemies. A blend that combines the background odor with the herbivore-induced signal can inform parasitoids both on the presence of potential hosts, and on the plant species they are approaching.

It is widely recognized that plants usually live in complex natural communities that, besides herbivores, herbivore enemies, and pathogens, also include associated fungi, like arbuscolar mycorrhizal fungi (AMF). Mycorrhizal association is very widespread in natural ecosystems. It is estimated that more than 80% of all terrestrial plants form associations with AMF (Wang and Qiu, 2006). Nevertheless, the effects of mycorrhization on plant defenses are poorly studied. We identified and quantified the major volatile compounds emitted by intact, herbivore-damaged and mechanically-damaged juvenile *P. lanceolata* plants and investigated how emission changed in association with the AMF *Glomus intraradices* (chapter III). Our results revealed a surprising plasticity in volatile release by this perennial grassland herb. Mycorrhization caused a significant change in emission rates of different compounds in both undamaged and herbivore-damaged plants. In particular, undamaged mycorrhizal plants emitted significantly higher levels of the GLV (*Z*)-3-hexenyl acetate compared to undamaged, uninfected controls, whereas herbivore-damaged mycorrhizal plants released much lower amounts of sesquiterpenes compared to herbivore-damaged, but uninfected controls (chapter III). Rapparini et al. (2008) reported that mycorrhization of *Artemisia annua* reduced the amounts of sesquiterpenes produced by undamaged plants compared to non-infected control plants, while Leitner and coworkers (2010) reported only minor changes in the quantitative and qualitative volatile profile of

mycorrhizal *Medicago truncatula* after insect herbivory. However, the differences observed were sufficient to be able to distinguish mycorrhizal and non-mycorrhizal plants by the volatiles emitted (Leitner et al., 2010).

The reduction in the emission of sesquiterpenes (potential direct and indirect defenses) that we observed upon mycorrhization can be interpreted as a consequence of the general repression of defenses due to AMF colonization. In the first stages of the association, AMF seem to be able to modulate the defense signaling cascades in the plant, and lower the production of defense compounds (reviewed by Garcia-Garrido and Ocampo, 2002). Another possible explanation is that mycorrhizal plants reallocate their resources among growth, defense, reproduction and other functions. In our study, mycorrhization was not beneficial for the plant, since we observed a decline in growth and in total carbon content in mycorrhizal plants. Indirect defenses may thus decrease as a consequence of the limited carbon availability caused by mycorrhization.

A major effect of AMF on VOC emission of *P. lanceolata* was on undamaged mycorrhizal plants, which released levels of (*Z*)-3-hexenyl acetate comparable to the ones induced by caterpillar herbivory or mechanical damage. Since GLVs derive from leaf damage, they are commonly considered herbivore-induced volatiles. However, our results show that mycorrhization turns these typically induced compounds into constitutive ones. GLVs have been shown to serve in indirect defense both in laboratory and field experiments (Halitschke et al., 2008; Shiojiri et al., 2006). In particular, Halitschke and coworkers demonstrated the efficacy of GLVs in herbivore-enemy attraction in the field. *Nicotiana attenuata* plants expressing antisense hydroperoxide lyase, and therefore impaired in GLV production, were less attractive to predatory bugs compared to wild type plants. Additionally, the authors tested in the same context plants silenced in the production of the sesquiterpene (*Z*)-α-bergamotene. The lack of (*Z*)-α-bergamotene emission resulted in lower predator attraction, suggesting that GLVs and terpenes share the same defensive functions.

5.3 Other roles of vegetative volatiles

In this work we focused on the role of vegetative volatiles in plant defense against biotic stress. Nevertheless, the high volatile emission observed in undamaged mycorrhizal plants

(chapter III), as well the constitutive sesquiterpene volatiles we investigated in chapter I and II, may serve other functions in the plant.

Volatile terpenes appear to be effective antioxidants *in vitro*, and there is evidence that isoprene and monoterpenes reduce damage caused by ozone and by reactive oxygen species. In particular, an inverse correlation between ozone sensitivity of poplar and its capacity to produce isoprene has been found. Also transgenic isoprene-emitting *Nicotiana tabacum* plants were more tolerant to ozone than wild-type plants (Loreto and Schnitzler, 2010). Less evidence of protection against oxidative stress has been produced for monoterpene-emitting plants than for isoprene-producing ones. However, it has been shown that chemical inhibition of monoterpene production in holm oak (*Quercus ilex*) resulted in increased ozone damage (Loreto and Fares, 2007). Protection is attributed to the direct reaction of the active oxygen species with terpenes through a conjugated double bond system. Among the constitutively-emitted maize terpenes, germacrene D has a conjugated double bond (chapter I). This chemical feature is shared by other common herbivore-induced terpenes, as (*E*)-β-ocimene, (*E*)-β-farnesene and (*E*)-4,8dimethyl-1,3,7-nonatriene (DMNT), which are released by maize (Hoballah et al., 2002), and *Plantago lanceolata* plants after herbivory (chapter III, Table 3.1). It is therefore possible that these volatiles also exert an anti-oxidative function in the herbivore-damaged tissues.

The common C_5 volatile isoprene has been shown to protect the plant from transient heat damage by making photosynthesis more tolerant to short high-temperature episodes, but the mechanisms by which this happens are as yet undetermined. It is possible that isoprene transiently resides in the thylakoid membrane preventing membrane leakage caused by heat. Another possibility is that isoprene stabilizes the large membrane-bound protein complexes (like photosystem II) in the thylakoids by enhancing hydrophobic interactions between the protein and the lipid layers (reviewed by Sharkey and Yeh, 2001). Monoterpenes could provide the same kind of protection (Sharkey and Yeh, 2001), but it is not yet known whether sesquiterpenes can have this activity.

Another important function of volatiles is in between-plant and within-plant communication. Volatiles released from the foliage of mechanically wounded, herbivore-damaged or pathogen-infected plants have been shown to influence direct and indirect defenses in neighbouring plants or in other parts of the same plant. These volatiles elicit the production of the hormones, jasmonic acid (JA) and salicylic acid, and increase the transcript levels of defense-related genes (reviewed by Heil and Karban, 2010). This process may happen as a direct induction or sometimes volatiles “prime” the regulatory

machinery so that there is a faster or greater production of defenses on subsequent damage. GLVs have been shown to activate defense genes and induce pathogen resistance in *A. thaliana* (Kishimoto et al., 2005). They can also activate JA production in maize (Engelberth et al., 2004), thus enhancing the potential production of anti-herbivore defenses. Induction or priming of plant defenses by mycorrhizal-induced GLVs may be a mechanism underlying the improved resistance to herbivores recorded by many studies (see Hartley and Gange, 2009). The increase in resources attributable to AMF is in some cases not enough to explain the enhanced resistance of mycorrhizal plants against their enemies (Kempel et al., 2010). Priming of plant defenses has been suggested to be a major mechanism conferring resistance to mycorrhizal plants (Pozo and Azcón-Aguilar, 2007). Thus it would be interesting to study whether plant volatiles are involved in defense priming of mycorrhizal plants.

Given the large variety of documented roles for plant volatiles in biotic and abiotic interactions and plant communication, the subject of VOCs is only likely to become more important for plant biologists and ecologists in coming years. Since there are still so many compounds reported from volatile collections whose functions have never been examined, it would not be surprising if other important aspects of plant growth, development and adaptation to their environment were also mediated by volatiles.

6. Summary

In addition to water vapor, oxygen, and carbon dioxide, plants release a variety of volatile organic compounds from their vegetative organs. These volatiles can mediate the plant's interaction with the environment. One of their primary roles is in plant defense against biotic attackers. Two major forms of plant defenses can be distinguished: direct defenses, which are toxic to the enemy or deter attack, and indirect defenses, which protect the plant by attracting natural enemies of the attacker. Direct and indirect defenses can be constitutively expressed, or induced by mechanical damage, herbivore feeding, or pathogen infection.

Previously, we had characterized a complex sesquiterpene volatile blend constitutively produced by maize seedlings, and isolated the enzyme responsible for its production. The aim of this thesis was to study the ecological role of this vegetative volatile blend in direct plant defense against a generalist chewing insect and a necrotrophic fungus, and in indirect plant defense. Moreover, we investigated the changes in direct and indirect plant defense metabolites in a widespread naturally-occurring association with arbuscular mycorrhizal fungi.

6.1 Vegetative volatiles play a role in direct defense against fungal pathogens, but not against herbivorous insects

The first question of this thesis was whether the constitutive vegetative volatile sesquiterpene blend of maize had a role in direct plant defense, either against pathogenic fungi or insect herbivores. In order to isolate the maize blend from its natural background for experimental purposes, we transformed *Arabidopsis thaliana* plants with the terpene synthase enzyme responsible for producing the blend, resulting in plants that emitted levels of volatiles comparable to that emitted by maize. Transgenic and wild-type, non-emitting plants were then infected with the pathogenic fungus *Alternaria brassicicola*, and the fungal biomass present in the leaves was assessed after four days. The terpene-producing *A. thaliana* plants contained a lower fungal biomass compared to wild-type plants, indicating that terpene volatiles exert a defensive activity against fungal invaders. Since the overexpression of terpene volatiles could lead to the activation of other defenses in *A.*

thaliana plants, we measured the levels of camalexin and glucosinolates, the main antifungal metabolites known in *A. thaliana*. No differences between wild-type and transformed plants were found. In addition, as necrotrophic pathogens are known to elicit jasmonic acid-mediated defenses, we measured the transcript levels of a key gene in the jasmonic acid pathway. Again, no differences between transgenic and wild-type plants were observed. These findings support the hypothesis that the reduction of fungal biomass observed in the transgenic plants is due to a direct action of the volatiles on the fungus rather than to the activation of other defenses by the volatiles.

The same volatile blend was tested for anti-herbivore activity against *Spodoptera littoralis* caterpillars. Growth, developmental time, pupal and adult weight were not affected by caterpillar feeding on volatile-producing plants. Moreover, caterpillars were not repelled by the volatiles, and, when they had a choice, fed equally on volatile-emitting, and on wild-type plants. These results indicate that the constitutive sesquiterpene volatiles of maize are probably not involved in direct anti-herbivore defense.

6.2 Constitutive vegetative volatiles reinforce the herbivore-induced signal for parasitoids

We then investigated how the constitutive volatiles of maize influence the indirect defense of the plant, by measuring the attraction of the hymenopteran parasitoid *Cotesia marginiventris*.

Olfactometer experiments revealed that the constitutive vegetative volatiles of maize can be used by these wasps as cues for host location. In particular, they strengthen the herbivore-induced volatile signal emitted by herbivore-infested maize plants, which has been previously demonstrated to be attractive to *C. marginiventris* females. These results suggest that the constitutively emitted volatiles may form a background odor against which the herbivore-induced signal becomes stronger.

The results of other olfactometer experiments indicated that parasitoids prefer more complex blends over simpler ones. This could explain why these insects preferentially oriented towards the blend made of the constitutive and the herbivore-induced sesquiterpene volatiles, rather than to simpler blends.

6.3 The common association with arbuscular mycorrhizal fungi modifies the levels of direct and indirect defense metabolites in the plant

In the last chapter of this thesis we report how the naturally occurring association with arbuscular mycorrhizal fungi (AMF) impacts the direct and indirect defenses of *Plantago lanceolata* L. Since about 80% of terrestrial plants live in association with AMF, studying how AMF affect plant defenses is important to understand how plants defend themselves under natural conditions.

We collected volatiles (potential direct and indirect defense metabolites) and extracted the iridoid glycosides aucubin and catalpol (direct anti-herbivore defenses) from mycorrhizal and non-mycorrhizal plants after insect herbivory or mechanical wounding.

Emission of terpenoid volatiles was significantly higher after insect herbivory than after the other treatments. However, herbivore-damaged mycorrhizal plants emitted lower amounts of sesquiterpenes, but not monoterpenes, than herbivore-damaged non-mycorrhizal plants. In contrast, mycorrhizal infection increased the emission of the green leaf volatile (*Z*)-3-hexenyl acetate but only in undamaged control plants, making it comparable to emission from mechanically wounded or herbivore-damaged plants. Neither mycorrhization nor treatment (herbivory or mechanical wounding) had any influence on the levels of iridoid glycosides. Thus, mycorrhizal infection did not have any effect on the levels of direct, non-volatile defense compounds measured in *P. lanceolata*. However, the large decline in herbivore-induced sesquiterpene emission after herbivore feeding in mycorrhizal plants, and the higher levels of (*Z*)-3-hexenyl acetate in intact mycorrhizal plants may have important implications for the indirect defense potential of this species and for any direct defense mediated by these volatiles.

7. Zusammenfassung

Pflanzen geben über ihre vegetativen Organe Sauerstoff, Kohlendioxid und Wasserdampf, aber auch eine Reihe flüchtiger organischer Verbindungen ab. Mit diesen Substanzen tritt die Pflanze mit ihrer Umwelt in Verbindung. Eine der hauptsächlichen Funktionen dieser Verbindungen ist die Abwehr der Pflanze gegen biotische Angreifer. Dabei kann man zwei Formen der Verteidigung unterscheiden: zum Ersten die direkte Verteidigung, bei der chemische Verbindungen toxisch für den Angreifer sind oder ihn abwehren; zum Zweiten die indirekten Verteidigung, bei der chemische Verbindungen der Pflanze die Feinde der Angreifer anlocken. Direkte und indirekte Abwehr können sowohl konstitutiv exprimiert als auch durch mechanische Verwundung, Herbivorenfraß oder pathogene Infektionen induziert werden.
In der Vergangenheit wurde von uns bereits ein komplexes Gemisch von konstitutiv exprimierten volatilen Sesquiterpenen aus Mais Sämlingen charakterisiert und die für deren Produktion verantwortliche Enzyme isoliert. Aufgabe dieser Arbeit war es, die ökologische Rolle dieser pflanzlichen Duftstoffe bei der direkten und indirekten Abwehr von kauenden Insekten und nekrotropher Pilzen zu untersuchen. Darüber hinaus wurden Veränderungen der indirekten und direkten Abwehr von Pflanzen untersucht, wenn diese in Verbindung mit der in der Natur weit verbreiteten arbuskulären Mycorrhiza treten.

6.1. Vegetative Duftstoffe spielen eine Rolle bei der direkten Verteidigung gegen pathogene Pilze, wirken jedoch nicht direkt gegen herbivore Insekten

Zuerst wurde in dieser Arbeit der Frage nachgegangen, ob konstitutiv von Mais abgegebene volatile Sesquiterpene eine Rolle bei der direkten Verteidigung der Pflanze gegen pathogene Pilze oder herbivore Insekten spielen. Diese konstitutiv abgegebenen Sesquiterpene werden von einer Sesquiterpensynthase gebildet. Um diese Sesquiterpenmischung von anderen natürlichen Duftstoffen zu trennen, wurde *Arabidopsis thaliana* mit dem Gen dieser Sesquiterpensynthase transformiert. So entstanden Pflanzen, die mit Mais vergleichbare Mengen an Duftstoffen emittierten. Transgene Pflanzen und nicht emittierende Wildtyp-Pflanzen wurden daraufhin mit dem pathogenen Pilz *Alternaria brassicicola* infiziert. Die Pilzbiomasse wurde nach Ablauf von vier Tagen bestimmt. Die terpenproduzierenden *A. thaliana* Pflanzen enthielten geringere Mengen Pilzbiomasse

verglichen zum Wildtyp. Dies läßt auf eine abwehrende Wirkung der Terpene auf pilzartige Erregen schließen. Da eine Überexpression an volatilen Terpenen zu einer Aktivierung weiterer Verteidigungsmechanismen in *A. thaliana* führen kann, wurden die Gehalte an Camalexin und Glucosinolaten, den bedeutendsten antifungalen Metaboliten, die in *A. thaliana* bekannt sind, gemessen. Es wurden jedoch keine Unterschiede zwischen transformierten Pflanzen und Wildtyp-Pflanzen gefunden. Es ist außerdem bekannt, daß nekrotrophe Pathogene die Jasmonsäure-vermittelte Verteidigung auslößen. Aus diesem Grunde maßen wir die Transkriptlevel von Schlüsselenzymen der Jasmonsäure-Biosynthese. Wir konnten wiederum keine Unterschiede zwischen transformierten und Wildtyp-Pflanzen feststellen. Diese Ergebnisse unterstützen die Hypothese, daß die Reduktion der Pilzbiomasse in den transgenen Pflanzen durch die direkte Wirkung der Duftstoffe hervorgerufen wird, und nicht auf die Aktivierung anderer Verteidigungsmechanismen zurückzuführen ist.

Dieselben Duftstoffe wurden auf ihre Wirkung gegen Raupen von *Spodoptera littoralis* getestet. Wachstum, Entwicklungszeit, Puppen- und Adultgewicht waren jedoch nicht verändert, wenn Raupen and Duftstoff-produzierenden Pflanzen gefressen hatten. Darüber hinaus wurden Raupen von den Duftstoffen nicht abgestoßen. Wenn sie die Wahl hatten, fraßen sie gleich häufig und gleich viel an Duftstoff-produzierenden Pflanzen wie an Wildtyp-Pflanzen. Diese Ergebnisse legen nahe, daß die konstitutiv abgegebenen Duftstoffe von Mais keine Rolle bei der direkten Verteidigung gegen Herbivoren spielen.

6.2. Konstitutiv abgegebene Duftstoffe verstärken das herbivor-induzierte Signal für Parasitoide

Wir untersuchten, wie die konstitutiv abgegebenen Duftstoffe von Mais die indirekte Verteidigung der Pflanze beeinflussen. Dafür maßen wir, mit Hilfe eines Olfaktometers, die Anlockung des Parasitoiden *Cotesia marginiventris* (Hymenoptera). Diese Experimente machten deutlich, daß der Parasitoid die konstitutiv abgegebenen Duftstoffe von Mais für die Lokalisierung seines Wirtes nutzen kann. Dabei verstärken die konstitutiv abgegebenen Duftstoffe die Wirkung von Herbivore-induzierten Duftstoffen, die nur von befressenen Pflanzen abgegeben werden und deren Attraktivität auf Cortesia marginiventris Weibchen schon in früheren Untersuchungen gezeigt wurde. Diese Ergebnisse weisen darauf hin, daß

konstitutiv abgegebenen Duftstoffe ein Hintergrundsignal bilden, welches das Herbivore-induzierte Signal verstärkt.
Die Resultate anderer Olfaktometer-Experimente legen nahe, daß Parasitoide komplexere Duftstoffgemische im Vergleich zu weniger komplexen bevorzugen. Dies könnte erklären, warum die Mischung aus konstitutiven und Herbivore-induzierten Duftstoffen attraktiver ist als die Herbivore-induzierten Duftstoffe allein.

6.3. Die Vergesellschaftung mit arbuskulärer Mykorrhiza verändert die Mengen an Substanzen für die direkte und indirekte Verteidigung in der Pflanze

Im letzen Kapitel der Arbeit berichten wir, wie die natürlicherweise vorkommende Vergesellschaftung mit arbuskulärer Mykorrhiza (AMF) die direkte und indirekte Verteidigung von *Plantago lanceolata* L. beeinflußt. Da fast 80% der terrestrischen Pflanzen mit AMF assoziiert sind, ist es wichtig zu wissen, wie AMF die Verteidigung der Pflanzen beeinflußt. Dies ermöglicht uns zu verstehen, wie Pflanzen sich unter natürlichen Verhältnissen verteidigen.
Wir sammelten Duftstoffe (potentielle direkte und indirekte Verteidigungsmetabolite) und extrahierten die Iridoidglykoside Aucubin und Catalpol (zur direkten Verteidigung gegen Herbivoren) aus mykorrhizierten und Mykorrhiza freien Pflanzen nach Insektenfraß bzw. mechanischer Verwundung.
Die Abgabe von Terpenen war signifikant höher nach Insektenfraß im Gegensatz zu allen anderen Behandlungen. Jedoch gaben Herbivore-induzierte mykorrhizierte Pflanzen geringere Mengen an Sesquiterpenen ab als Mykorrhiza freie Pflanzen. Der Gehalt an Monoterpenen unterschied sich nicht zwischen Pflanzen mit und ohne Mykorrhiza. Im Gegensatz dazu erhöhte sich die Abgabe des grünen Blattduftstoffes (*Z*)-3-hexenyl acetate in mykorrhizierten Pflanzen. Dies geschah jedoch nur bei den unverwundeten Kontrollpflanzen, wodurch diese eine ähnliche Emission zeigten wie mechanisch verwundete bzw. befressene Pflanzen. Weder die Mykorrhizierung noch die Behandlung (mechanische Verwundung und Herbivorie) hatten einen Einfluß auf den Gehalt an Iridoidglykoside. Demzufolge hatte die Mykorrhizierung keinen Einfluß auf die Mengen an direkten, nicht flüchtigen Verteidigungsstoffen, die in *P. lanceolata* gemessen wurden. Der starke Rückgang der Sesquiterpenemission von mykorrhizierten Pflanzen nach Insektenfraß und die höheren Gehalte an (*Z*)-3-hexenyl acetate in unverwundeten mykorrhizierten

Pflanzen mag wichtige Auswirkungen auf die indirekte und direkte Verteidigung von *P. lanceolata* haben.

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10. Selbständigkeitserklärung

Entsprechend der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena, erkläre ich, dass mir die geltende Promotionsordnung der Fakultät bekannt ist. Die vorliegende Arbeit habe ich selbstständig und nur unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen, Quellen und Literatur angefertigt. Personen, die an der experimentellen Durchführung, Auswertung des Datenmaterials oder bei der Verfassung der Kapitel beteiligt waren, sind angegeben. Ich habe weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte unmittelbar oder mittelbar geldwerte Leistungen von mir für Arbeiten erhalten, die in Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen. Ich habe die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Ferner habe ich nicht versucht, diese Arbeit oder eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation einzureichen.

Anna Fontana

11. Curriculum Vitae

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RESEARCH EXPERIENCE

Jan 2011 – present	Post-doc at the Max Planck Institute for Chemical Ecology in Jena, Germany, Department of Biochemistry
Jan 2005 – Dec 2010	Ph.D. student at Max Planck Institute for Chemical Ecology in Jena, Germany, Department of Biochemistry. Advisor: Prof. Dr. Jonathan Gershenzon Primary research focus: The defensive role of vegetative volatiles against herbivores and pathogens.
Ago 2008 and Nov – Dec 2006	Research stays at the University of Neuchâtel, Switzerland, Lab of fundamental and applied Chemical Ecology. Head: Prof. Dr. Ted C. Turlings. Research focus: Attractiveness of complex maize volatile blends to the parasitoid *Cotesia marginiventris*.
Mar 2003 – Jul 2004	Master thesis at University of Milan, Italy, Department of Plant Pathology Research focus: Study of the variability of *Cercospora beticola* Sacc. populations.

EDUCATION

Jan 2005 – present	Graduate student of the International Max Planck Research School (IMPRS), Jena, Germany

Oct 1998 – Jul 2004	Degree in Agricultural Sciences and Technologies, University of Milan, Italy
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ORAL PRESENTATIONS

S. Unsicker, **A.Fontana**, S. Hempel, M. Reichelt, J. Gershenzon (2008). The effects of an arbuscular mycorrhizal fungus on plant chemical defenses of *Plantago lanceolata* L. 93rd ESA Annual Meeting, The Ecological Society of America, Milwaukee, USA

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A. Fontana (2007). The role of maize sesquiterpene volatiles in defense against herbivores and pathogenes. IMPRS Evaluation Symposium, MPI for Chemical Ecology, Jena, DE

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POSTER PRESENTATIONS

A. Fontana, M. Held, T. Turlings, J., Gershenzon, J. Degenhardt (2008). The role of maize volatiles in defense against herbivores and pathogens. presented at 93rd ESA Annual Meeting, ESA, Milwaukee, US

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A. Fontana, J. Degenhardt, M. Held, T. Turlings, J. Gershenzon (2009).The role of maize sesquiterpene volatiles in direct plant defense. ICE Symposium, MPI for Chemical Ecology, Jena, DE

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A. Fontana, M. Held, T. Turlings, C. Assefa Fantaye, J. Gershenzon, J. Degenhardt (2008). The role of maize sesquiterpene volatiles in defense against herbivores and pathogens. SAB Meeting 2008, MPI for Chemical Ecology, Jena, DE

A. Fontana (2007). The role of herbivore-induced and constitutive maize sesquiterpenes in parasitoid attraction. 6th Biannual IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

A. Fontana (2006). Terpenes in plant-fungus interaction. 4th Biannual IMPRS Symposium, MPI for Chemical Ecology, Jena, DE

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SCIENTIFIC PUBLICATIONS

A. Fontana, M. Reichelt, S. Hempel, J. Gershenzon, S. B. Unsicker (2009): The effects of arbuscular mycorrhizal fungi on direct and indirect defense metabolites of *Plantago lanceolata* L. J Chem Ecol 35 (7): 833-843

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A. Fontana, T. G. Köllner, C. Schnee, M. Reichelt, D. Rosenberger, K. Luck, J. Gershenzon, and J. Degenhardt: Volatile sesquiterpenes produced by the terpene synthase 8 (TPS8) from maize are involved in defense against fungal pathogens, *to be submitted*

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