# Induced indirect defense in soybean and maize: Effects of ultraviolet radiation, nitrogen availability and heavy metal stress

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# 1. Introduction

Vascular plants are exposed to a diversity of biotic and abiotic stress factors. In contrast to animals, plants are not able to avoid adverse conditions by moving to another habitat. Furthermore, plants are confronted with these challenges not only consecutively but also simultaneously with divergent effects depending on the single stresses.
Thus, plants have evolved a wide variety of strategies to confront single as well as combined stresses (reviewed in Walling, 2000).
Herbivory by insects is a constant threat for most plants (Dicke, 2009). The defense mechanisms used by plants to impede or reduce herbivory can be described according to their spatiotemporal characteristics (constitutive *vs.* induced defense) and their mode of action (direct *vs.* indirect defense) (Howe and Jander, 2008).
Constitutive defenses may ward off insects by preventing or constraining an attack. Mechanical barriers like wax layers or trichomes and secondary metabolites acting as toxins or deterrents are found to provide effective protection (Wu and Baldwin, 2009). In contrast, inducible defenses may use the same means but will be up-regulated only after a plant has actually been attacked by herbivores. While defenses, such as toxins or digestibility reducers, can directly affect an herbivore's growth and feeding behavior, plants may also employ indirect defenses by attracting the herbivore's natural enemies.

## 1.1. Induced indirect defense

In this study, the induced indirect defense (IID) against herbivore attack involving plant volatile organic compounds (VOCs) was the main focus. In 1980 Price (Price et al., 1980) ask for more attention for the third trophic level (enemies of the herbivore) in insect-plant-interaction studies. In the following decades the number of studies on tritrophic interactions with the focus on indirect plant defense increased significantly. Plants were found to provide shelter, food and/or host finding cues for their insect partners in order to cope with the herbivore threat (Dicke, 2009).
Dicke and co-workers (Dicke and Sabelis, 1988) published the first evidence of a plant using induced VOCs for attracting predators as a means of self-defense. Until 1999 this phenomenon has been described for plants from at least 12 different families, responding to a great variety of herbivorous insects. The herbivore's enemies attracted by plants mainly belonged to the insect groups of predatory mites and parasitic wasps (Dicke, 1999b).

The first olfactorily noticeable response to wounding and herbivory is the emission of so-called green leaf volatiles (GLV) by the affected plant (Turlings et al., 1998). These $C_6$-based substances are products of the oxidative degradation of linolenic acid and linoleic acid (Fall et al., 1999; Hatanaka, 1993). They are supposed to have antibiotic properties (Croft et al., 1993), may reduce the fecundity of some herbivore species or attract some herbivore species (reviewed in Walling, 2000).

During feeding or oviposition herbivorous insects secrete so-called elicitors of different origin and structure into the wounded plant tissue (Pare et al., 2005). One of the best characterized is the fatty acid amide *N*-(17-hydroxylinolenoyl)-L-glutamine or volicitin (Alborn et al., 1997). The compound consists of a plant derived (linolenic acid) and a herbivore derived moiety (L-glutamine and the hydroxy group) which are conjugated by the herbivore (Paré et al., 1998), maybe with the aid of gut bacteria (Spiteller et al., 2000). In the case of chewing herbivores, these elicitors activate a jasmonic acid (JA) dependent signaling cascade within the affected tissue (Alborn et al., 1997; Arimura et al., 2005; Turlings et al., 1993). Among the first steps in the signaling cascade is the calcium–activated reversible phosphorylation of wound inducible phospholipases leading to the release of linolenic acid from the chloroplast membrane (Leon et al., 2001). Linolenic acid is the precursor of JA, which is produced via 12-oxo-phytodienoic acid (OPDA) through the octadecanoid pathway (Schaller, 2001; reviewed in Wu and Baldwin, 2009).

Increased JA levels in turn lead to a wide variety of plant responses (reviewed in Hamberg and Gardner, 1992) including the emission of *de novo* synthesized VOCs (Paré and Tumlinson, 1997; Schmelz et al., 2003a) through reversible protein phosphorylation and activation of transcription factors (Glazebrook, 2001). Induced VOCs are not only emitted locally by the wounded leaf but also systemically throughout the plant (Rose et al., 1996; Turlings and Tumlinson, 1992). The emitted compounds belong to different chemical classes and are synthesized through different pathways. Indole and methyl salicylate are derived from the shikimic acid pathway, terpenoids via isopentenyl pyrophosphate (IPP) from the mevalonate pathway (D'Alessandro et al., 2006).

Enemies of the attacking herbivore, such as parasitoids or predators, can use the VOCs as host finding cues (e.g. Dicke and Sabelis, 1988; Turlings et al., 1990) to improve their foraging efficiency. Though cues emitted directly from the herbivore may be the most reliable ones regarding potential host species and location, they are often emitted in low amounts due to the herbivore's small size and the selective pressure to avoid such treacherous signals (Vet and Dicke, 1992). Thus, herbivore-derived signals are often difficult to detect in a complex environment. Plants on the other hand have a large surface area and the ability to emit large amounts of volatile infochemicals. Already in 1991, Turlings and co-workers (Turlings et al.,

1991) found the plant to be the main source of parasitoid-attracting volatile substances. The induced volatile blends differ between plant species due to different biochemical pathways producing the VOC (e.g. Brassiccaceae *vs*. Poaceae) or different genetical background of the plants in the case of different cultivars (Dicke, 1999a).

Different blends are also induced by herbivores with different feeding types mainly due to different types of damage (reviewed in van Poecke and Dicke, 2004). Parasitoids are even able to discriminate between induced VOCs of closely related plants and herbivore species with the same way of feeding or oviposition even in the field because different herbivores excrete different elicitors and can thus induce different VOC profiles in the same plant species (e.g. De Moraes et al., 1998; Meiners et al., 2000; for overview see Dicke, 1999a; van Poecke and Dicke, 2004). Moreover, parasitoids are able to associatively learn specific herbivore induced VOCs indicating the location of their host and accordingly modulate their foraging behavior (e.g. Tamò et al., 2006; Vet and Groenewold, 1990). All species of parasitoids studied yet show an innate use of infochemicals. Specialists more frequently use specific cues and learning occurs only rarely in this group. Generalists otherwise use general cues for host finding and are able to learn (specific) cues in most cases (Steidle and van Loon, 2003). All the described mechanisms enable the parasitoids to distinguish between herbivore infested and unifested plants as well as host and non-host herbivores to improve their foraging efficiency.

The next steps until successful parasitizing of the host can be divided into host location, host recognition, and host acceptance (Vinson, 1976; Vinson, 1998). In parts they can also be affected by plant characteristics, e.g. host location by footprints of the host (Rostás and Blassmann, 2009; Rostas et al., 2008).

Predation or parasitisms of herbivores have been demonstrated to improve the plants fitness either by directly killing the herbivore (predator) or by reducing the herbivore's feeding activity (parasitoid). Hoballah and Turlings (2001) could show that parasitism of *Spodoptera littoralis* larvae by *Cotesia spp*. resulted in smaller herbivore larvae with a shortened period of growth. Maize plants attacked by the parasitized herbivore larvae had significant larger leaves, plant height and seed production than plant attacked by unparasitized larvae. A similar result concerning seed production of *Arabidopsis thaliana* attacked either by parasitized or unparasitized *Pieris rapae* larvae was published 2000 by van Loon et al.

From the herbivore's point of view the volatiles are kairomones as they have negative fitness effects on the herbivore but from the parasitoid's or predator's point of view the volatiles are synomones (Dicke and Sabelis, 1988).

Some studies found evidence that inducible defenses can save metabolic costs (Karban and Baldwin, 1997; Kessler and Baldwin, 2002) because they are only mobilized at the time of

attack. Moreover, inducibility can reduce the attractivity of plants to specialist herbivores that are attracted to defense compounds. Another advantage of inducibility is the creation of variability leading to an unpredictable environment for herbivores. The evolution of inducible defenses may be favored by some other benefits (discussed in Agrawal and Karban 1999).

On the other hand, inducibility can be of disadvantage for plant survival, especially in the case of defenses that act indirectly. At first, they need to be activated, then in a second step the appropriate predator or parasitoid needs to be attracted. The first step can take hours (Turlings et al., 1998) or even days (Kant et al., 2004) depending on the plant species and other defense mechanisms employed by the plant. The efficiency and speed of the second step depends on the population density of the respective predator or parasitoid in the plants' vicinity.

### 1.2. Abiotic factors in general

Abiotic factors may alter the quality and magnitude of plant defenses. However, whether concentrations of defensive secondary compounds are increased or decreased largely depends on the abiotic factor in question.

Gouinguené and Turlings (2002) had investigated the effects of several abiotic factors on the induced emission of one maize cultivar. All tested factors affected the emission rates of artificially induced VOCs. Changes in temperature, light intensity, fertilization rate and soil humidity, also had a qualitative effect on the blend. The most dramatic effects however were due to differences in light intensity as the induced emission of VOCs only occurred during the photophase. This light-dependency was corroborated by Rostás and Eggert (2008) in herbivore-damaged soybean and by Arimura et al. (2008) in mechanically damaged lima bean. From experiments using $^{13}C$ labelled $CO_2$, Arimura et al. (2008) concluded a photosynthetically dependent *de novo* synthesis of some of the induced compounds.

### 1.3. Ultraviolet radiation

While several studies have confirmed that the intensity of photosynthetically active radiation (PAR) is of utmost importance, it is virtually unknown how the energy-rich, ultraviolet (UV) part of the solar radiation would affect VOC-mediated plant-insect interactions. UV radiation has a wide range of effects on the herbivore defense of plants as well as on the herbivores themselves. UV radiation may have a deleterious effect on proteins, DNA and other macromolecules (Bassman, 2004). Thus, plants exposed to this radiation accumulate flavonoids and phenolic acids as UV absorbing compounds to protect themselves (Bidart-Bouzat and Imeh-

Nathaniel, 2008). These compounds not only protect the plant tissue against the harmful radiation but can also have additional ecological effects. Caasi-Lit (2005) confirmed that the UV-induced flavonoids can have direct toxics effects on herbivorous insects. Kühnle and Müller (2009) found feeding-deterrent effects, whereas Reifenrath and Müller (2008) found flavonoids to act as feeding stimulants for herbivorous insects.

Apart from the already mentioned outcomes, UV light can have a wide variety of further effects on insect herbivore feeding behavior and growth. In most cases increased UV radiation lead to decreased feeding, growth or oviposition rates mediated by an altered composition in plant secondary chemicals (reviewed in Bidart-Bouzat and Imeh-Nathaniel, 2008).

Furthermore, UV radiation and herbivory can lead to overlapping gene expression mediated by JA and/or reactive oxygen species (ROS) (Bassman, 2004; Stratmann, 2003) in plants. In *Nicotiana attenuata,* for example, enhanced UV–B leads to enhanced expression of proteinase–inhibitor (PI) genes (Izaguirre et al., 2003).

Because in some cases the effects of UV light and wounding alone on induced defenses is weak or not detectable (Stratmann et al., 2000), UV radiation may rather act as a priming agent *sensu* Bruce et al. (2007): after pre-exposure to a biotic or abiotic stress (in this case UV radiation), plants respond faster and/or stronger to a subsequent stress, such as wounding or herbivory. For plant volatiles, UV effects are poorly investigated and the scarce studies are inconsistent (Bidart-Bouzat and Imeh-Nathaniel, 2008).

### 1.4. Nitrogen

Nitrogen (N) is an important macronutrient for plants and subsequent trophic levels as it is essential for the synthesis of proteins, nucleic acids, photosynthetic pigments and nonvolatile as well as volatile secondary compounds (Amtmann and Armengaud, 2009; Maathuis, 2009). Thus N can be a factor strongly affecting plant performance, defense as well as plant–animal–interactions. Plants can respond to altered nitrogen availability with an extensive reprogramming of their primary and secondary metabolism. In *Arabidosis* short term N deficiency leads to a downregulation of a wide range of genes associated with photosynthesis, chlorophyll and amino acid synthesis pathways and even many genes assigned to secondary metabolism whereas genes encoding proteins involved in amino acid breakdown are upregulated (Scheible et al., 2004). In contrast, long term deficiency reduced relative growth rate but increased levels of some amino acids at unaltered protein content in *Arabidopsis* (Tschoep et al., 2009).

The reprogramming of the metabolism may be driven by an accumulation of sugars and starch in the leaves of N deficient plants leading to the above mentioned reduction of photosynthesis by

allosteric regulation of biochemical pathways and differential gene expression. Sugar signals may also alter root-to-shoot-ration and root architecture in N deficient plants. In these plants, root growth is accelerated and lateral root branching is increased to intensify acquisition of the deficient nutrient (Hermans et al., 2006).

Despite these physiological and morphological reactions reduced N availability may lead to a reduced N content in affected plants (Hermans et al., 2006). This in turn can affect the plant's defense ability.

Looking at VOC mediated induced defenses in relation to N availability, (Schmelz et al., 2003b) found elevated and prolonged increases of volicitin induced JA levels combined with higher ethylene sensitivity when maize plants were lacking N. This correlated with an increase in induced sesquiterpene-emission. The result of increased induced JA concentrations in low-N plants resulting in enhanced VOC emission was corroborated by Chen et al. (2008b) for cotton. In their study, parasitism of the herbivorous larvae of *Spodoptera exigua* by *Cotesia marginiventris* did not differ between the treatments of the VOC emitting plants, indicating that the wasps did not discriminate between the different odor blends. In another study using a different cultivar of cotton and *Microplitis croceipes* as a parasitoid, Olson et al. (2009) found the highest rate of induced VOC was emitted by plants that had received a medium amount of N compared to plants that had received the double amount or no N at all. Here the wasps were most attracted to plants exposed to the medium level of N, i.e. emitting the highest amounts of VOC.

In contrast, Lou and Baldwin (2004) demonstrated an unaltered emission of induced VOC in spite of a decreased JA burst for tobacco under low N conditions. In this study only N intensive direct defense and C containing nonvolatile defenses were reduced by N deficiency. The authors hypothesize an N independent signal acting in addition to JA and low resource requirement for production of indirect volatile defense signals as explanation for their results.

As described, the outcome of N deficiency concerning VOC and VOC mediated induced defense is very variable and depending on the system studied.

### 1.5. Heavy metals

To gain a deeper understanding of how abiotic factors influence VOC mediated IID, the effects of two heavy metals (HM) were analyzed. "Heavy metal" is not a clearly defined scientific term (Duffus, 2002). In this study we adhere to the definition that considers all metals with a density of 5 $g*cm^{-3}$ or more as "heavy metals".

These metals can originate from natural or anthropogenic sources. Igneous rocks are a natural source, while anthropogenic sources are, for example, industrial combustion and sewage or mining.

From the plant's point of view HM can be divided into two groups. One group contains metals that are essential for the plants' physiology; the other group contains metals that can be toxic even in low concentrations (Orcutt and Nilsen, 2000).

Iron, copper and zinc, for instance, are essential for plant growth (Wintz et al., 2002). Iron has important functions in photosynthesis as electron transporter and in chlorophyll biosynthesis. Copper (Cu) is also involved in photosynthesis as electron transporter and is integrated in many enzymes as co-factor. Zinc as well can be a co-factor for many enzymes and plays a structural role in some proteins such as the ones involved in DNA–binding (Kucera et al., 2008). Despite their necessity for plants, even essential HMs can be toxic if they occur in excessive amounts. However, most plants are able to regulate their uptake and distribution within the plant because metal ions have to be transported actively through the cellular membrane. Other mechanisms to cope with high concentrations of HM are immobilization (e.g. by complexation or deposition in the cell wall) of the ions or active ion efflux (Kucera et al., 2008; Meharg, 2005).

For non-essential HMs like cadmium, lead, mercury or arsenic no functions in the plants' physiology are known, yet. These ions may enter the plant as analogues of essential ions or through passive diffusion (Meharg, 2005) and are toxic in low concentrations (Kucera et al., 2008; Orcutt and Nilsen, 2000).

Toxic metals can interfere with membrane stability and permeability, compete with essential ions, groups or metabolites, or react with phosphate groups from ADP and ATP (Orcutt and Nilsen, 2000). These interactions in turn lead to a wide range of physiological effects on the plants like decreased chlorophyll content and therefore an inhibited photosynthesis or reduced root and shoot growth (Kucera et al., 2008). Another effect of excess HMs is the enhanced production of reactive oxygen species (ROS) in the plant tissue (Mithöfer et al., 2004; Pál et al., 2006).

ROS are important messengers in the signalling cascade transferring information about herbivore attack in plants (Leitner et al., 2005; Maffei et al., 2006; Mithöfer et al., 2004). At low levels, ROS may function directly as second messengers in different pathways (Maffei et al., 2006) whereas higher concentrations may also lead to oxidation of membrane lipids, eventually increasing JA levels in the affected tissue (Mithöfer et al., 2004). As described above, JA is a major signalling compound in the pathway of VOC induction and production.

Hence an overlapping effect of herbivory and HM stress, as described above for UV radiation, finally inducing or altering indirect defense in plants may occur (Mithöfer et al., 2004).

Abiotic stresses may affect plant morphology and physiology as well as IID in different ways. Modified composition or quantity of an induced blend can alter the parasitoids' responses towards this blend due to an altered information content of the “new” blend (Rostás and Turlings, 2008; Turlings and Wäckers, 2004). However, a modified blend does not invariably affect the behavior of the parasitoids (Rostás et al., 2006) whereas an altered behavior could not always be traced back to a detectable change in the induced VOC blend (D'Alessandro et al., 2009; Rostás and Turlings, 2008). The spectrum of VOC that can be identified and measured highly depends on the analytical method used and it is likely that relevant behavior-modifying compounds remain undetected. Moreover, the quantities of key compounds may be to low to be detected by standard GC-MS procedures while the sensitivity of insect olfactory receptors for specific compounds is known to be very high (Dicke, 1999a; Rains et al., 2004). A combination of VOC analysis and behavior assays is therefore necessary to draw correct conclusions.

Most studies on the effects of abiotic stresses that have been conducted, so far, considered only the reactions of the plant itself or the plant and its herbivore, thus neglecting the parasitoid as an important player in multi-trophic plant-insect-interactions.

The aim of this study was to investigate the effects of the three abiotic factors ultraviolet radiation, nitrogen deficiency and heavy metal stress on the plant's performance, the feeding behavior and performance of an herbivore and a parasitoid with the main focus on the VOC mediated induced indirect defense of the plant.

### 1.6. Study system

To investigate the effects of UV radiation, nutrient deficiency and heavy metal stress on tritrophic interactions I used soybean (*Glycine max* (L) Merr. cv. London) (Fabaceae) and maize (*Zea maize* (L). cv. Lambada) (Poaceae) plants. Both are among the most important crops on a global scale but also in North and South America

A major pest of both plants are the polyphagous caterpillars of the moth *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae), (Barlow and Kuhar, 2005; Kranz et al., 1977), which was used as herbivore in this study. This species occurs throughout the American continent from Argentina to the northern border of the USA and eastern parts of Canada. Because *S. frugiperda* has no diapause mechanisms, the species overwinters at the coasts of the Gulf of Mexico and adults migrate north during spring and summer annually (Barlow and Kuhar, 2005; Sparks, 1979).

The females can produce up to 1000 eggs deposited in clusters up to 400 eggs. First instar larvae are able to move to different hosts by ballooning (Barlow and Kuhar, 2005). The most damage is

caused by the last larval instar, consuming more than three quarter of the leaf area fed during their whole larval phase (Sparks, 1979).

One of the herbivore's principal natural enemies is the generalist wasp *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) (Barlow and Kuhar, 2005; Sourakov and Mitchell, 2000). This parasitoid attacks first and second instar larvae of *S. frugiperda* (Ashley et al., 1982) by laying a single egg inside its host. Hence, parasitized larvae are retarded in their developmental time, show lethargic behavior and die before reaching the fourth instar (Ashley, 1983). The herbivore's reduced growth and feeding activity may benefit the plant as it should lead to less severe effects on seed production compared to plants attacked by unparasitized caterpillars (Hoballah et al., 2002; van Loon et al., 2000).

Tritrophic systems consisting of maize and other plants, *Spodoptera* larvae and their natural enemy *C. marginiventris* have been studied in detail ever since Turlings et al. (1991) showed that the plant is the main source of volatile attractants for parasitoids (D'Alessandro et al., 2006; Gouinguené et al., 2005; Röse et al., 1998). Surprisingly however, it remains vague which - and under which circumstances - the many herbivore-induced compounds would play a behavior-modifying role. Some volatiles for instance are innately attractive while others are not or have to be learned by classical conditioning. Studies using different approaches were able to restrict the innately attractive components to mainly polar compounds (D'Alessandro et al., 2009; D'Alessandro et al., 2006; D'Alessandro and Turlings, 2005). Interestingly some minor compounds, even in concentrations below GC detection thresholds seem to be very attractive to *C. marginiventris* (D'Alessandro et al., 2009; Gouinguené et al., 2005). These results suggest that analyses of the emitted blends without olfactometric assays of the parasitoid's behavior may lead to wrong conclusions concerning the potential effects of abiotic factors on the plants' indirect defense.

### 1.7. Hypotheses and Questions

Abiotic stresses modify the plant's physiology and signaling pathways. An altered plant physiology, in turn, may directly affect the feeding behavior and performance of herbivores and indirectly the fitness of parasitoids.

Abiotic factors such as UV radiation and heavy metal stress may directly affect JA dependent pathways regulating the expression of herbivore induced VOC whereas N deficiency affects the nutritional status of the plant potentially influencing JA level as well. If the quantity or composition of herbivore induced VOC is affected, this may change the plants' attractivity for parasitoids and thus the plants ability to defend itself indirectly.

The specific questions I addressed in this study were:

- What are the effects of ambient UV radiation, HM stress and N deficiency on the growth, physiology and production of non-volatile and induced volatile secondary compounds of soybean and maize?
- Are there plant mediated effects of abiotic stresses on the performance and food consumption of the herbivore *S. frugiperda*?
- Do the abiotic factors ambient UV radiation, heavy metal stress and N deficiency alter the induced VOC blend of soybean and maize to such an extend that the host searching behavior of *C. marginiventris* and subsequently the efficiency of the IID is altered?

# 2. Ambient ultraviolet radiation induces protective responses in soybean but does not attenuate indirect defense

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Ambient ultraviolet radiation does not alter induced VOC emission in soybean and thus host location of the parasitoid Cotesia marginiventris remains effective.

**Abstract**

We investigated the effects of ambient ultraviolet (UV) radiation on (i) the performance and chemistry of soybean plants, (ii) the performance of *Spodoptera frugiperda* and (iii) the foraging behavior of the herbivore's natural enemy *Cotesia marginiventris* which exploits herbivore induced plant volatiles (VOC) for host location. The accumulation of protective phenolics was faster in plants receiving ambient UV than in controls exposed to sun light lacking UV. Accordingly, isorhamnetin- and quercetin-based flavonoids were increased in UV exposed plants. No UV effects were found on the performance and feeding behavior of S. *frugiperda*. Herbivore-damaged plants emitted the same VOC when grown under ambient or attenuated UV for 5, 10 or 30 days. Consequently, *C. marginiventris* was attracted but did not discriminate between exposed and unexposed soybeans. In summary, ambient UV radiation affected soybean morphology and physiology but did not destabilize interactions between trophic levels.


Keywords: Indirect defense; Volatile organic compounds; Flavonoids; Parasitoid; Olfactometer; Host location behavior

## 2.1. Introduction

Feeding activity or egg deposition by herbivorous insects elicit a jasmonic acid (JA)-dependent signaling cascade within the affected plant tissue that leads to the biosynthesis and emission of numerous volatile organic compounds (VOC) deriving from at least three different pathways (D'Alessandro et al., 2006; Meiners and Hilker, 2000). These VOC guide natural enemies to their prey or host and are thus considered as a plant's indirect defense (Dicke, 1994). The ability to produce and release VOC can be affected by different biotic and abiotic factors. Fungal infection, for example, may reduce herbivore-induced VOC (Rostás et al., 2006) whereas nitrogen deficiency (Schmelz et al., 2003b), dry soil and light increase their emission rates (Gouinguené and Turlings, 2002). If the quantity or composition of the induced volatile blend is modified, then alterations in the host location efficiency of parasitoids or predators may be expected (Vuorinen et al., 2004b).

A single abiotic factor receiving enhanced attention during recent years is solar ultraviolet (UV) radiation (Ballaré et al., 2001; Julkunen-Tiitto et al., 2005). This is primarily due to the concern over stratospheric ozone depletion and the resultant increase in UV-B radiation but also because it has been recognized that UV radiation is an environmental factor that can vary for many other reasons and may thus influence ecological interactions (Paul and Gwynn-Jones, 2003). Although numerous studies exist on the impact of UV-B light on plants, its effect on the VOC-mediated attraction of natural enemies of herbivores has not been studied, yet.

The most consistent effect of UV light on plants is the induction of UV absorbing compounds such as phenolic acids and flavonoids (e.g. Izaguirre et al., 2007) that protect the tissue against the deleterious effects of this highly energetic radiation, e.g. on macromolecules like DNA (Bassman, 2004). However, flavonoids can also play ecological roles by acting as toxins (Caasi-Lit, 2005) or feeding stimulants (van Loon et al., 2002) for herbivorous insects. Furthermore, UV radiation may influence JA levels and lead to an overlap in gene expression caused by UV-B and herbivory (Stratmann, 2003). Therefore, we hypothesized that UV radiation could also enhance herbivore-induced VOC emission, possibly by priming terpene synthase genes. Surprisingly, little is known about how terpenoids and other VOC are affected by UV radiation and the existing evidence is inconsistent (Bassman, 2004). Supplemental UV-B was shown to increase levels of constitutive VOC in the oil glands of aromatic herbs like *Ocimum basilicum* or *Mentha piperita* in some cases but not in others (Johnson et al., 1999; Maffei and Scannerini, 2000). Also, isoprene emissions were greater in *Quercus gambelii* but not in *Mucuna pruriens* or *Acer platanoides* when grown under elevated UV-B (Harley et al., 1996). Increasing isoprenoid levels in response to UV radiation may help the plant to protect itself from deleterious reactive oxygen

species (Owen and Penuelas, 2005; Penuelas and Munne-Bosch, 2005). Considering the varying impacts of UV radiation on plant metabolism, it may seem obvious that the spectrum of UV effects on growth and feeding behavior of insect herbivores may also vary largely between plant-insect systems (reviewed in Ballaré et al., 2001; Paul et al., 1997; Roberts and Paul, 2006). Here we addressed the question whether soybean plants (*Glycine max* cv. London) exposed to full or attenuated ambient UV radiation show differences in leaf chemistry that would affect the extent of feeding and the development of the generalist pest species *Spodoptera frugiperda* (Lepidoptera, Noctuidae). Furthermore, we asked whether UV radiation modulates the quantity or composition of the VOC blend that is triggered by herbivore feeding and if this would change the attractiveness of the emitted blend to the parasitoid *Cotesia marginiventris* (Hymenoptera, Braconidae).

## 2.2. Materials and methods

### 2.2.1. Plant and insect material

Soybean seeds (*Glycine max* (L) Merr. cv. London), pre-inoculated with *Bradyrhizobium japonicum* (Fix & Fertig_, Saatbau Linz), were obtained from Saatbau Linz (Leonding, Austria). Seedlings were grown solitarily in plastic tubes (height 11.2 cm, diameter 4 cm) containing an unfertilized commercial soil mix (Einheitserde Typ P, Patzer GmbH & Co. KG, Sinntal-Jossa, Germany). All experimental plants were reared in a climate chamber with a L16:D8 photoperiod (photosynthetic active radiation (PAR): 140 mmol photons $m^{-2}$ $s^{-1}$ at 50 cm distance from lamps, measured with X12 Optometer, Gigahertz Optik, Puchheim, Germany) at 29/20 °C (light/dark) and 75% relative humidity to provide equal conditions and to exclude herbivores until subjected to UV treatment in the exposition tents. Eggs of *Spodoptera frugiperda* (Smith) were kindly provided by Bayer CropScience AG (Monheim, Germany) on a weekly basis. After hatching, larvae were reared in plastic boxes (19 × 9 × 5.5 cm) and provided with kidney bean-based artificial diet for noctuids (modified from King and Leppla, 1984). The insects were kept in a separate climate chamber with a L15:D9 photoperiod at 28/25 °C (light/dark) and 75% humidity. After 5 to 6 days (second larval stage, $L_2$) caterpillars were used either for parasitoid rearing or for induction of volatiles in soybean plants. A colony of *Cotesia marginiventris* (Cresson) was maintained in the laboratory. For rearing, about 45 *S. frugiperda* larvae were offered to three mated *C. marginiventris* females in a plastic box (20 × 20 × 5.5 cm). After 24 h wasps were removed and herbivore larvae were kept in the boxes until the emergence of the wasp cocoons. The cocoons were removed from the herbivore boxes and transferred to rearing cages (Bugdorm

I, Megaview Science Education Services Co. Ltd, Taichung, Taiwan). The cages were checked daily for eclosed imagines. Adult parasitoids were provided with water and honey.

2.2.2. Exposure to UV radiation

Two UV exposition tents, one equipped with a Teflon foil (Novofol, Siegsdorf, Germany; VIS + UV treatment) permeable to the whole spectrum of solar radiation, the other one with a polyester foil (‘‘LEE 226 UV’’, FFL-Rieger, Munich; VIS treatment) excluding radiation below 380 nm (UV-A and UV-B), were situated outdoors at the Botanical Garden of the University of Würzburg, Germany (49° 45’ N, 9° 55’ E). Tents had a ground area of 3 × 1 m with the long axis being east-west oriented. Roof height was 1.8 m at the north axis and 1.2 m at the south axis. The open northern wall was shielded with the appropriate foil (mounted at 45°, 20 cm from the top of the tent) to prevent diffuse radiation but to allow for ventilation. For further details on UV tents refer to Kolb et al. (2001) and Reifenrath and Müller (2007). To check for any alterations in radiation permeability of the foils due to aging, the transmission of the foils was measured weekly with a UNICAM UV4 spectrometer (ATI Unicam). Spectral irradiance inside the tents was measured with an Optronic OL 754 Portable High Accuracy UV-Visible Spectroradiometer (Optronic, Orlando, FL, USA). Additionally, relative humidity and temperature inside the tents were measured with Tinytag Ultra data loggers (Gemini Data Loggers Ltd, Chichester, UK) in 30 min intervals during the whole exposure season.

2.2.3. Effects of UV radiation on plant performance, chlorophyll fluorescence and flavonoid induction

To assess the effects of UV radiation on the performance of soybean, 8- day-old plants (VE stage, McWilliams et al., 1999) were transferred from a UV-free climate chamber to the exposition tents. Plants were allowed to grow for 10 weeks under VIS + UV and VIS conditions, respectively, until reaching the late R5 stage. Total plant height, number of nods, number of lateral saplings, pod numbers and seed numbers were measured. For all other experiments seedlings were grown in the climate chamber for 14 days (V3 stage) and then transferred to the UV exposition tents for 5 days. Only fluorescence measurements were conducted for 8 days to assess the accumulation of UV-B screening pigments such as flavonoids and hydrocinnamic acids in the leaf epidermis of the experimental plants (Bilger et al., 2001). Pigment induction was measured as percentage UV shield, which is defined as $100 \times [1 - F(UV)/F(BL)]$ with F(UV) = dark-level fluorescence yield at 375 nm excitation, F(BL) = dark-level fluorescence

yield at 470 nm excitation. Measurements were carried out inside exposition tents with a non-invasive portable UV-A-PAM (Walz Mess -u. Regeltechnik, Effeltrich, Germany) on the first trifoliate leaf of six plants per tent. The same leaves were assessed in situ at 09:00 h on a daily basis. Flavonoid induction in the first trifoliate leaf was assessed from VIS + UV and VIS-treated soybeans after 5 days of exposure. At this time point maximum difference was found between the UV shields of both treatments (Fig. 2). Using a cork-borer (diameter 18 mm), leaf discs were cut out from plants growing in the same cohort as experimental plants used for volatile collection or behavioral assays. The leaf discs were frozen in liquid nitrogen and lyophilized for later analyses of flavonoid contents. The lyophilized samples were ground in a mixer mill (Retsch MM301, Retsch GmbH, Haan, Germany) at 13.4 Hz for 15 s. Then flavonoids were extracted three times with 500 ml methanol (v/v 80%; Roth, Karlsruhe, Germany). Chlorophyll was removed from the combined extracts using 500 ml petroleum ether (Fluka, Taufkirchen, Germany) and 20 ml catechine (5 mM; Phytoplan, Heidelberg, Germany) was added as internal standard. After complete evaporation of the solvent, the pellet was resuspended in 250 ml methanol (100%) and filtered (mesh 0.2 mm). The samples were stored at -20 °C. Quantitative and qualitative analyses of the flavonoid composition was conducted by HPLC (Series 1100, Hewlett Packard, Waldbronn, Germany) on a RP-18 column (SUPELCOSIL LC-18, length 25 cm, inner diameter 4.6 mm, film 5 mm, Supelco, Taufkirchen, Germany) at 25 °C. Injection volume was 50 ml. A stepwise increase of 8% to 100% acetonitrile in 0.1% formic acid which was held for 5 min before a step return to 8% acetonitrile was used as solvent gradient. The overall separation time was 60 min. Spectroscopic detection was between 190 and 500 nm. Data were analyzed with ChemStation for LC 3D (Agilent Technologies, Santa Clara, CA, USA). For identification, the retention times and spectra of the respective compounds were compared to spectra and retention times of authentic standards at a detection wavelength of 254 nm.

2.2.4. Plant-mediated UV effects on herbivore performance and feeding behavior

A performance test was conducted to assess whether UV light had any indirect effects on a generalist herbivore due to alterations in the nutritional quality of the treated soybean leaves. Neonate larvae of *S. frugiperda* were weighed and kept individually in Petri dishes (diameter 8.5 cm) with moistened filter paper in a climate chamber as described above (Section 2.1). They were fed ad libitum with fresh cut leaflets of the first trifoliate soybean leaves from plants exposed for 5 days to VIS + UV or VIS conditions. Each plant was harvested only once. Weight increases of larvae were measured from days 5 to 6 (larval stage $L_2$) and days 10 to 11 ($L_3$). In

addition, pupal and adult weight as well as developmental time was recorded. In addition, a no-choice feeding trial with $L_2$ larvae was performed to see whether larvae compensate for potentially lower quality food due to UV-exposition by ingesting larger quantities of leaf tissue. For this, $L_2$ larvae were individually placed in Petri dishes (diameter 8.5 cm) and fed with one soybean leaflet (first trifoliate leaf) from plants exposed for 5 days to VIS + UV or VIS conditions, respectively. After 24 h the areas fed were measured as described in Rostás et al. (2006).

2.2.5. Plant-mediated UV effects on VOC emission and parasitoid behavior

The effect of ambient UV light on the emission of herbivore-induced volatiles and consequently on the behavior of the parasitoid was investigated in a six-arm olfactometer (for details see Turlings et al., 2004). Five-day-exposed soybean plants were individually placed into the cup of an odor source vessel of the six-arm olfactometer. The soil was covered with aluminum foil to prevent the herbivore larvae from falling into the gap between planting tube and odor source vessel. Twenty-five *S. frugiperda* larvae ($L_2$) were placed on each plant and allowed to feed overnight (approximately 16 h). On the following day, volatile collections and behavioral assays were carried out simultaneously from 09:00 to 12:00 h. Ten fluorescent lamps (PAR inside the odor source vessels: 130 µmol photons $m^{-2}$ $s^{-1}$ at 30 cm distance from the lamps) were switched on three hours before testing the wasps. Odor source vessels containing a plant that had received either VIS + UV or VIS radiation were placed vis-à-vis in the olfactometer. The other four vessels remained empty as controls. After connecting the vessels to the air delivery and the olfactometer, the air stream was allowed to stabilize for 10 min. The flow rate was 1.2 L $min^{-1}$ for incoming air and 0.6 L $min^{-1}$ for air going out to the behavioral arena or the volatile traps, respectively.

Mated 3–5-day-old females of *C. marginiventris* were used in the behavioral assays. Wasps had no oviposition or VOC experience to allow for observing their innate behavior. Wasps were released in the olfactometer in groups of six as they do not interfere with each others choices (Turlings et al., 2004). After 30 min the choices made by the parasitoids were recorded and the group was replaced by a new one. Five groups of wasps were tested on the same day. One day with five releases was considered as one replicate. Eight replicate days were carried out with a new pair of plants and new wasps each day. Volatiles emanating from soybeans were collected with SuperQ traps as described previously (Rostás and Eggert, 2008). After each experimental day the glass and Teflon parts of the olfactometer were cleaned with deionized water and rinsed with ethanol (v/v 70%), acetone and hexane. After evaporation of the solvents, the glass parts

were placed in an oven at 200 °C for 1 h. The trapped volatiles were eluted with 150 ml methylene chloride, two internal standards (n-octane and nonyl acetate, Sigma-Aldrich, Taufkirchen, Germany, 400 ng each in 20 ml methylene chloride) were added and the samples were stored at -80 °C. The qualitative and quantitative volatile composition of each sample was analyzed on an Agilent Technologies 6890N Network GC System coupled with a 5973 Network Mass Selective Detector. Three microliters of each sample were injected with an automated injection system in pulsed splitless mode. The column was an Agilent 19091-s933 HP-1 capillary column (length 30 m, diameter 0.25 mm, film thickness 0.25 µm). The oven was held at 35 °C for 3 min and then increased with 8 °C $min^{-1}$ to a final temperature of 230 °C which was held for 10 min. Helium (1.5 ml $min^{-1}$) was used as carrier gas. Compounds were identified using MSD ChemStation (Agilent Technologies) software with the Wiley 275 mass spectrum library and by using the software MassFinder3/Terpenoids library (Hochmuth Scientific Software, Hamburg, Germany). Identities were further confirmed by co-injection of authentic standards (Sigma-Aldrich). Quantification was obtained by comparing the area of the compounds to the area of the internal standards.

#### 2.2.6. Statistical analyses

Performance parameters of *S. frugiperda* were compared with an ANCOVA using initial weight as covariable and measured performance parameters as variables. Plant performance parameters, larval feeding in the no-choice assay, environmental conditions in the tents and differences in volatile composition of the treated plants were analyzed with a Student t-test for independent samples. Differences in flavonoid composition were analyzed with a Mann-Whitney U-test. The analyses were conducted using STATISTICA 7.1 (StatSoft, Tulsa, USA). Behavior of the wasps was analyzed with a log-linear model as described in Turlings et al. (2004) using the software package R (http://www.r-project.org/).

### 2.3. Results

#### 2.3.1. Exposure conditions

The transmittance spectra of the foils used for VIS + UV and VIS treatment showed clear differences in the UV region: the polyester foil (VIS) excluded wavelengths larger than 380 nm and thus nearly all UV irradiance, whereas the Teflon foil (VIS + UV) was permeable to wavelengths down to 290 nm (Fig. 1). Inside the exposition tents, irradiance above 400 nm was only slightly different between both treatments while virtually no UV light reached the plants in

the VIS tent (Fig. 1, insert). The transmission of the foils remained constant during the whole exposition time. Temperature inside the tents ranged between 2.8 °C and 41.0 °C (mean 19.8 °C, 95% confidence interval 19.6 °C to 20.0 °C) for the VIS + UV and between 3.1 °C and 41.5 °C (mean 19.7 °C, 95% confidence interval 19.5 °C to 19.9 °C) for the VIS tent. Relative humidity inside the tents ranged between 26.7% and 100% (mean 73.7%, 95 % confidence interval 73.2% to 74.3%) for the VIS + UV and between 28.2% and 100% (mean72.7%, 95% confidence interval 72.2% to 73.0%) for the VIS tent. None of the measured environmental conditions were significantly different between the tents (Student's t-test, t = -0.74 and -0.10, $p > 0.5$, $n_{VIS} = 4924$, $n_{VIS+UV} = 4541$, $n_{VIS} = 4619$, $n_{VIS+UV} = 4418$ for temperature and relative humidity, respectively).

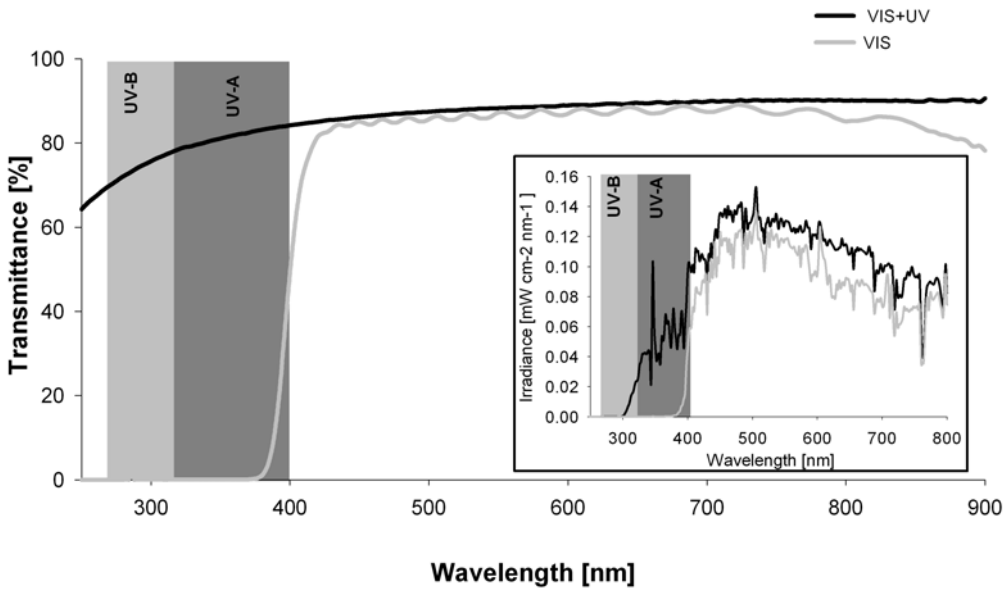


**Figure 1**: Transmittance spectra [%] of foils used for UV attenuation in the field. The insert shows spectral irradiance [mW $cm^{-2}$ $nm^{-1}$] inside exposition tents. VIS+UV = full ambient radiation, Teflon foil; VIS = UV attenuated ambient radiation, LEE 226UV Polyethylene foil. Irradiance was measured on June 20th, 2005 under cloudless sky at noon. Range of UV-A and UV-B radiation is indicated by grey and dark grey area respectively.

2.3.2. Effects of UV radiation on plant performance and leaf phenolics

Plants exposed to VIS + UV or VIS conditions for 10 weeks showed a significant difference in total height. Soybeans grown under full ambient radiation were 4 cm smaller on average. Numbers of nods, lateral saplings, pods and seeds were not significantly affected (Table 1).

**Table 1**: Growth parameters of soybean plants exposed to full ambient (VIS+UV) or UV attenuated (VIS) radiation for 10 weeks (until late R5 stage).

| Parameter | VIS+UV | VIS | Statistics |
|---|---|---|---|
| Total height [cm] | 67.6 ± 1.1 | 71.6 ± 1.3 | $p < 0.05$; $t = -2.090$ |
| Number of nods | 9.7 ± 0.2 | 9.7 ± 0.2 | $p > 0.1$; $t = 0.107$ |
| Number of lateral saplings | 5.1 ± 0.3 | 5.3 ± 0.3 | $p > 0.1$; $t = -0.514$ |
| Number of pods | 21.1 ± 0.9 | 20.1 ± 0.7 | $p > 0.1$; $t = 0.879$ |
| Number of seeds | 36.4 ± 1.7 | 33.1 ± 1.4 | $p > 0.1$; $t = 1.539$ |

Values are mean ± SE. $n = 30$. Statistics are indicated for a *t*-test for independent samples

UV-A-PAM measurements showed that exposition to both, VIS + UV and VIS conditions, gradually increased the concentration of UV reflecting compounds (UV shield). In plants exposed to VIS + UV conditions this increase was clearly faster compared to plants grown in tents that blocked UV light (Fig. 2). The largest difference in the UV shield between VIS + UV and VIS exposed plants was found on days four and five after exposition (17.5%). Therefore, plants exposed for 5 days were used for all other experiments except for the assessments of plant performance and the long term VOC measurements. After the sixth day of exposition, the amount of UV protecting compounds converged and remained at about 72% in both treatments.

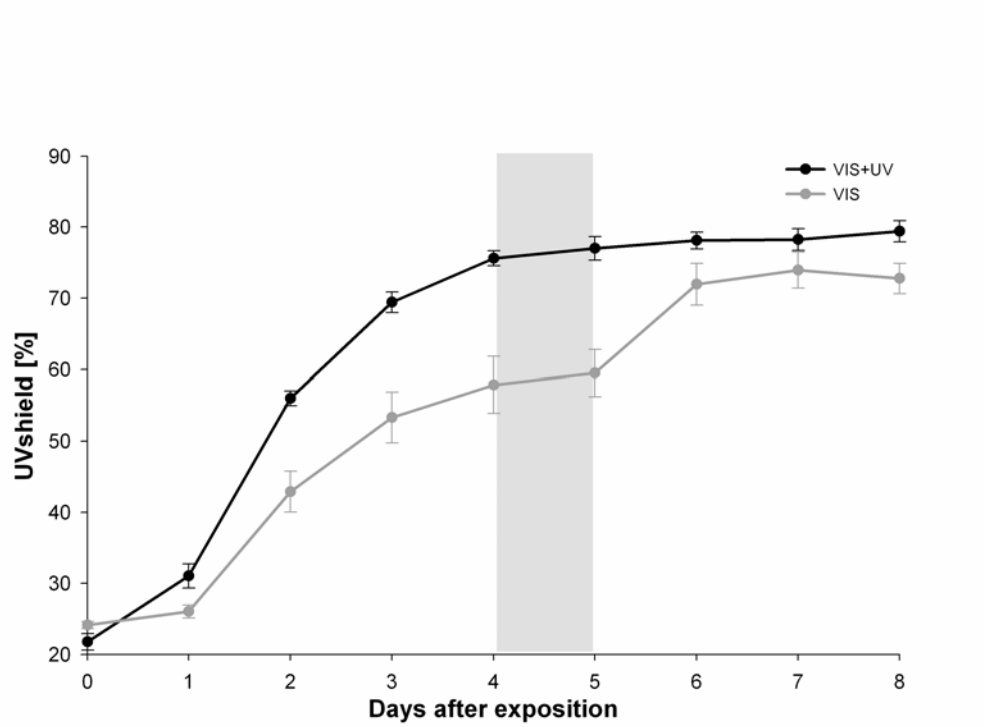


**Figure 2**: Kinetics of epidermal UV shield [%] buildup in soybean leaves receiving full (VIS+UV) or UV attenuated (VIS) solar radiation. The first trifoliate leaf was measured by UV-A-PAM at 9 a.m. on a daily base. Depicted are means (± SE, $n$ = 6). Maximum difference in UV shield is indicated by grey area.

Total flavonoid concentration in leaves exposed to VIS conditions (197.0 µmol $g^{-1}$DW ± 42.7, mean ± SE, $n$ = 6) was lower by 65% compared to plants receiving VIS + UV radiation (302.9 µmol $g^{-1}$DW ± 25.5, mean ± SE, $n$ = 6). However, this difference was not statistically significant (Mann-Whitney U-test, Z = 1.64, $p > 0.1$, $n$ = 6). Three of the analyzed flavonoids (Iso1, Q1, Q2; Fig. 3) were significantly increased in VIS + UV-treated plants (Mann-Whitney U-test, Z = -2.74, $p < 0.01$, $n$ = 6). Compounds Iso1, Iso2, Iso3 and Iso4 were identified as isorhamnetin-based flavonols, compounds K1 and K2 were kaempferol-based flavonols, compounds Q1 and Q2 quercetin-based flavonols, and peak DP appeared as double-peak in the HPLC chromatogram and consisted of a kaempferol- and an isorhamnetin-based flavonol. The relative content of kaempferol-based flavonols was significantly decreased in favor of quercetin-based flavonols in plants receiving full ambient radiation (Mann-Whitney U-test, Z = -2.74, $p < 0.01$, $n$ = 6). In this analysis peak DP was excluded, because the proportion of the components of the double-peak could not be clearly separated.

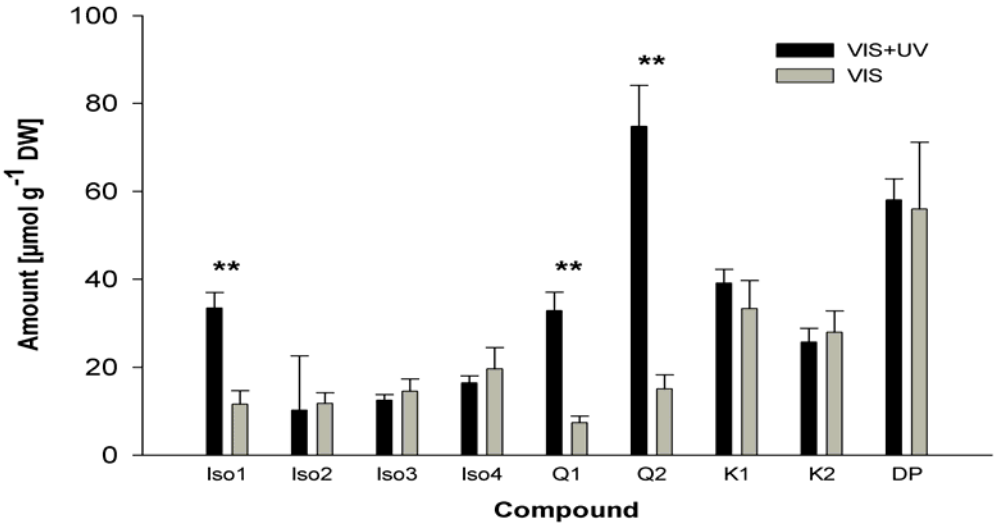


**Figure 3**: Composition of flavonoids in soybean leaves after exposure (5 d) to full (VIS+UV) and attenuated (VIS) solar radiation. Bars are mean values ± SE ($n$ = 6). Asterisks indicate statistically significant differences (** $p < 0.01$, Mann-Whitney-U-test). Iso = isorhamnetin-based flavonols, Q = quercetin-based flavonols, K = kaempferol-based flavonols. DP = unresolved Iso+K double-peak. Within each group compounds are arranged by retention times.

2.3.3. Plant-mediated UV effects on herbivore performance and behavior

Weight increases in larval stage 2 and 3, larval and pupal times as well as pupal and adult weights showed no significant differences (ANCOVA, $p > 0.05$, Table 2) between insects reared on VIS or VIS + UV-treated plants. Mortality until pupation ($\chi^2$ test, $\chi^2 = 0$, df = 1, $p = 1.0$) and until adult emergence ($\chi^2$ test, $\chi^2 = 1.12$, df = 1, $p > 0.1$) was not different between larvae reared on differently treated plants. In the no-choice test larvae did not consume significantly more tissue from UV exposed than from unexposed leaves (VIS + UV: 23 ± 4 mm$^2$, VIS: 15 ± 5 mm$^2$; Student's t-test, t = -1.40, $p > 0.1$, $n$ = 9–10).

**Table 2**: Performance parameters of *S. frugiperda* larvae reared on soybean plants exposed to ambient solar radiation lacking (VIS) or including (VIS+UV) the UV spectrum for 5 d.

| Parameter | VIS+UV | VIS | Statistics |
|---|---|---|---|
| Weight increase day 5 to day 6 [mg] | 18.3 ± 1.8 | 16.9 ± 1.6 | $n$ = 35/33; p > 0.5; F = 0.093 |
| Weight increase day 10 to day 11 [mg] | 76.4 ± 5.8 | 90.6 ± 5.6 | $n$ = 34/31; p > 0.05; F = 3.116 |
| Pupal weight [mg] | 162.7 ± 2.9 | 157.3 ± 3.8 | $n$ = 27/26; p > 0.1; F = 0.765 |
| Adult weight [mg] | 79.5 ± 1.9 | 77.7 ± 2.4 | $n$ = 27/22; p > 0.5; F = 0.169 |
| Larval time [d] | 18.2 ± 0.1 | 18.4 ± 0.1 | $n$ = 27/27; p > 0.1; F = 1.985 |
| Pupal time [d] | 8.1 ± 0.2 | 7.8 ± 0.2 | $n$ = 27/21; p > 0.5; F = 0.290 |

Values are means ± SE. Statistical parameters are indicated for an ANCOVA, $n$ = $\text{individuals}_{VIS+UV}$ / $\text{individuals}_{VIS}$.

2.3.4. Plant-mediated UV effects on VOC emission and parasitoid behavior

No emission of VOC was detected in undamaged soybean plants. This was the case in both UV treatments. Plants damaged by *S. frugiperda* larvae emitted 26 different volatile compounds into the headspace of which 20 could be identified (Table 3). The main constituents were (*E,E* )-α-farnesene (33% of total emission) and indole (18% and 17% of total emission in VIS and VIS + UV plants, respectively). However, there were no significant differences in the quantitative and qualitative composition of the induced volatiles between VIS + UV and VIS-treated soybean plants neither for the emission corrected for fresh weight nor for uncorrected values (Student's t-test, Table 3).

**Table 3**: VOC in the headspace of herbivore-damaged soybean plants. Plants were exposed to full ambient (VIS+UV) or UV attenuated (VIS) radiation for 5 d.

| Compound | c.e.[ng $g^{-1}$ FW $h^{-1}$] | | % of total emission | | Whole-plant emission [ng $h^{-1}$] | | Statistics for c.e. |
|---|---|---|---|---|---|---|---|
| | VIS + UV | VIS | VIS + UV | VIS | VIS + UV | VIS | |
| (*E,E*)-α-Farnesene | 202 ± 35.1 | 206 ± 33.1 | 33.0 | 33.3 | 533 ± 107.8 | 594 ± 1.4 | $p > 0.5$; $t = 0.077$ |
| Indole | 105 ± 16.9 | 114 ± 27.9 | 17.3 | 18.4 | 270 ± 47.6 | 326 ± 79.5 | $p > 0.5$; $t = 0.267$ |
| (*Z*)-3-Hexenyl acetate | 56 ± 8.6 | 52 ± 7.4 | 9.3 | 8.4 | 143 ± 23.5 | 148 ± 19.8 | $p > 0.5$; $t = -0.387$ |
| Methyl anthranilate | 19 ± 4.9 | 32 ± 15.2 | 3.1 | 5.2 | 51 ± 14.9 | 94 ± 79.5 | $p > 0.1$; $t = 0.838$ |
| (*Z*)-3-Hexenol | 25 ± 4.9 | 30 ± 7.2 | 4.1 | 4.9 | 61 ± 10.4 | 82 ± 17.6 | $p > 0.5$; $t = 0.667$ |
| (*E*)-β-Ocimene | 18 ± 2.1 | 20 ± 4.8 | 2.9 | 3.3 | 47 ± 7.1 | 57 ± 9.6 | $p > 0.5$; $t = 0.583$ |
| (*Z*)-3-Hexenyl-α-methyl butyrate | 13 ± 4.4 | 11 ± 3.1 | 2.1 | 1.7 | 34 ± 11.7 | 28 ± 6.7 | $p > 0.5$; $t = -0.390$ |
| (*Z*)-3-Hexenyl propionate | 9 ± 1.4 | 8 ± 1.4 | 1.4 | 1.3 | 22 ± 3.9 | 22 ± 4.0 | $p > 0.5$; $t = -0.276$ |
| (*Z*)-3-Hexenal | 7 ± 1.2 | 7 ± 1.5 | 1.1 | 1.1 | 17 ± 3.3 | 20 ± 3.7 | $p > 0.1$; $t = 0.193$ |
| Benzenacetonitrile | 6 ± 2.4 | 7 ± 2.4 | 1.0 | 1.1 | 16 ± 6.8 | 19 ± 6.8 | $p > 0.5$; $t = 0.185$ |
| (*Z*)-Jasmone | 7 ± 1.1 | 7 ± 1.1 | 1.2 | 1.1 | 19 ± 3.6 | 19 ± 2.9 | $p > 0.5$; $t = -0.537$ |
| Methyljasmonate | 2 ± 0.5 | 3 ± 1.2 | 0.4 | 0.5 | 6 ± 1.2 | 8 ± 3.3 | $p > 0.5$; $t = 0.376$ |
| (*Z*)-3-Hexenyl isobutyrate | 3 ± 1.1 | 3 ± 0.7 | 0.5 | 0.4 | 8 ± 2.9 | 7 ± 1.8 | $p > 0.5$; $t = -0.315$ |
| β-Bergamotene | 2 ± 0.4 | 2 ± 0.5 | 0.3 | 0.3 | 5 ± 1.2 | 6 ± 1.4 | $p > 0.5$ ; $t = 0.462$ |
| (*E*)-Caryophyllene | 1 ± 0.2 | 1 ± 0.2 | 0.2 | 0.2 | 4 ± 0.6 | 4 ± 0.7 | $p > 0.5$; $t = -0.058$ |
| Benzenacetaldehyde | 1 ± 0.3 | 1 ± 0.5 | 0.2 | 0.2 | 3 ± 0.7 | 3 ± 1.1 | $p > 0.5$; $t = 0.113$ |
| Germacrene-D | 1 ± 0.1 | 1 ± 0.1 | 0.1 | 0.1 | 2 ± 0.3 | 2 ± 0.4 | $p > 0.5$; $t = -0.198$ |
| Tridecatetraene | 1 ± 0.3 | 1 ± 0.3 | 0.2 | 0.1 | 3 ± 0.9 | 2 ± 0.7 | $p > 0.5$; $t = -0.326$ |
| α-Humulene | 1 ± 0.2 | 1 ± 0.1 | 0.2 | 0.1 | 2 ± 0.5 | 2 ± 0.5 | $p > 0.5$; $t = -0.536$ |
| Benzaldehyde | 1 ± 0.1 | 1 ± 0.1 | 0.1 | 0.1 | 1 ± 0.2 | 2 ± 0.4 | $p > 0.5$; $t = 0.353$ |

Compounds were identified according to their retention times, library mass spectra and by coelution with commercial standards. c.e. (corrected emission) is emission corrected according to fresh weight of the plants. Statistics and % refer to corrected emission. No significant differences were found (Student's *t*-test for independent samples, $n = 8$). Means ± SE are given.

Furthermore, plants exposed for 10 days (Student's t-test, $p > 0.05$ for each compound, $n = 6$) and 30 days (Student's t-test, $p > 0.05$ for each compound, $n = 6$), respectively, did not vary in the quantitative and qualitative composition of their volatile blends, as well. Only overall emission decreased from exposition day 5 to exposition day 30 (Fig. 4). This age dependent decrease was statistically significant between 10 and 30 days VIS + UV exposed plants (Student's t-test, t = 3.415, $p < 0.01$, $n = 6$) and between 5 and 30 day exposed plants for both treatments (Student's t-test, VIS + UV: t = 3.597, $p < 0.01$; VIS: t = 2.637, $p < 0.05$, $n_{5\ days} = 8$, $n_{30\ days} = 6$).

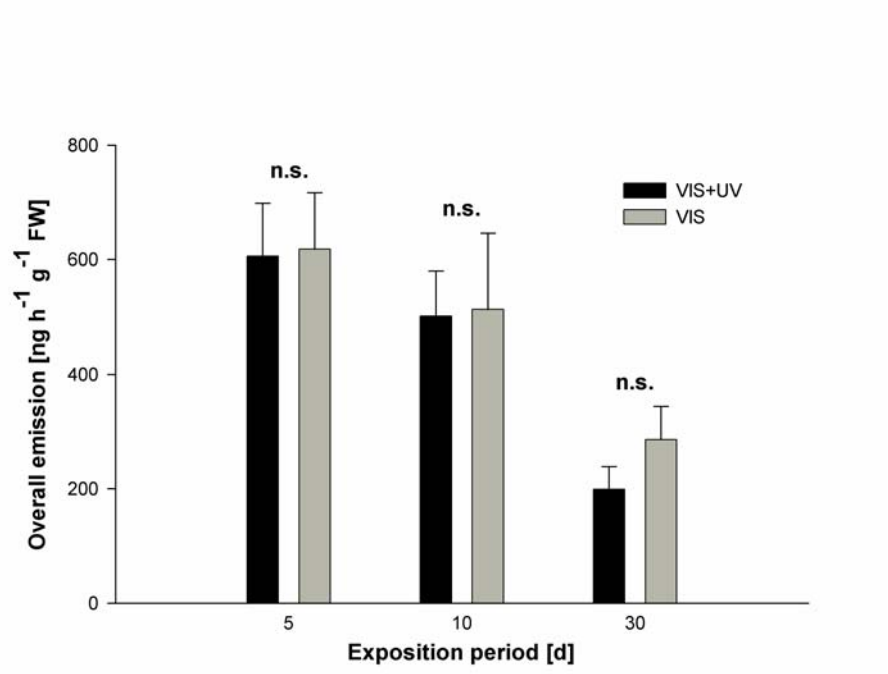


**Figure 4**: Overall VOC emission of *S. frugiperda* damaged plants per gram fresh weight and hour. Bars are mean values ± SE ($n = 8$ on day five and 6 on day 10 and 30). No significant difference between VIS+UV and VIS exposed plants within one exposition period was found (Student's *t*-test) Asterisks indicate statistically significant differences between treatments of different exposition periods (Student's *t*-test).

Naïve females of *C. marginiventris* were highly attracted by the herbivore-induced volatile blends of VIS + UV or VIS-treated plants when compared to clean air. But wasps had no preference for either one of the offered plant odors (log-linear- model, $p > 0.05$, $n = 8$, Fig. 5).

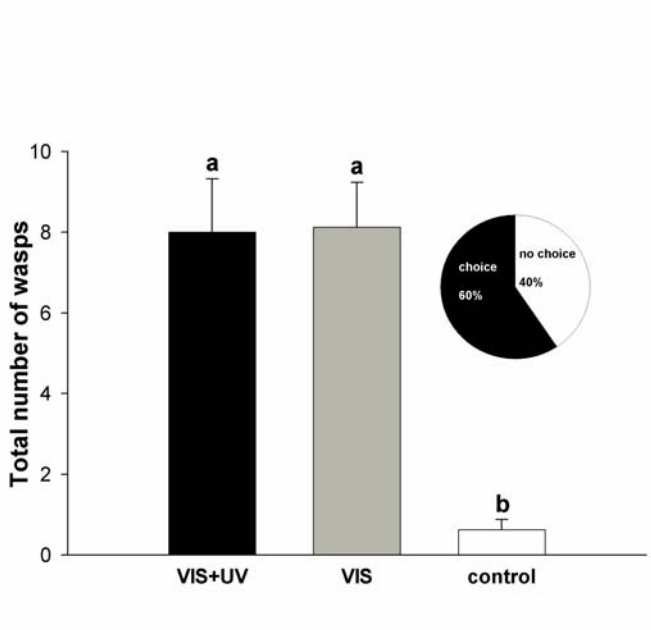


**Figure 5**: Response of naïve *C. marginiventris* females to herbivore-induced volatile blends emitted by soybean plants exposed to full (VIS+UV) or UV attenuated (VIS) solar radiation. control = clean air. Bars represent mean numbers (± SE) of wasps making a choice in the six-arm-olfactometer. Total number of wasps choosing the four controls was divided by four. Different letters indicate statistically significant differences**.** Log-linear model fitted to quasipoisson distribution; $n = 8$ independent experiments with 30 wasps.

### 2.4. Discussion

Ambient UV radiation exerted a clear effect on the growth and the secondary chemistry of *G. max.* After 10 weeks of field exposition, soybean plants receiving full ambient radiation were significantly smaller compared to plants exposed to radiation that almost lacked UV light. This observation was in accordance with results from *Triticum aestivum* (Yuan et al., 1998), and soybean (Yuan et al., 2002) in studies using additional UV-B radiation or for *Cucumis sativus*, *Colobanthus quitensis* and *Deschampsia antarctica* in studies using UV exclusion filters (Day et al., 1999; Krizek et al., 1997). A possible mechanism for reduced growth could be the shortening of the internodes caused by reduced cell wall extensibility (Ros and Tevini, 1995). Exposition to VIS + UV conditions also resulted in a faster increase in the UV shields of plants receiving full solar radiation compared to VIS exposed plants within the first 6 days (Fig. 2). The difference was most likely caused by a stronger accumulation of phenolic compounds. The general notion that leaf phenolics are induced to serve as photo-protectives was confirmed by a meta-analysis of plant field studies using enhanced UV radiation (Searles et al., 2001) as well as studies assessing

the responses to solar UV light (e.g. Izaguirre et al., 2007). In soybean, increases in total leaf phenolics exposed to ambient UV radiation were previously demonstrated by Mazza et al. (2000). On the other hand, screening of the total flavonoid contents in 20 Chinese soybean cultivars in a field study using UV-B lamps revealed that seven cultivars had increased total flavonoid levels while five showed decreased levels. No changes were observed in eight cultivars (Zu et al., 2003). Since alterations in the levels of individual flavonoids were not taken into account, UV-B could have had an impact on certain compounds without increasing the total level. This was the case in our study: three of the analyzed flavonoids showed a significant increase in plants receiving full ambient radiation (Fig. 3). Of these, two compounds were quercetin-based flavonols, resulting in a shift in the relative flavonol content in favor of the quercetin glycosides and at the expense of kaempferol glycosides. As quercetin flavonols are known to have an improved ability as free radical scavengers due to the additional ortho-dihydroxyl group in the B-ring (Harborne and Williams, 2000) compared to kaempferol flavonols, it might be of advantage for the plants to invest more in quercetin flavonols under UV stress. Despite differences in leaf phenolics between VIS and VIS + UV exposed soybeans, *S. frugiperda* larvae were not affected in their performance parameters (Table 2). The noctuid moth *S. frugiperda* is a generalist herbivore with a wide range of host plants such as maize, soybean, barley, clover or cotton (Sparks, 1979). Thus, the larvae can tolerate large differences in host plant chemistry and one may speculate that the variability caused by UV radiation may be too subtle to affect this generalist. UV mediated effects could be stronger in specialists. However, so far there is no specific pattern discernible with respect to host plant specialization. For example, Caputo et al. (2006) found no impact on the larval performance of the crucifer-specialist *Plutella xylostella* when fed UV-treated *Arabidopsis thaliana* while Zavala et al. (2001) reported a worse performance of the Fabaceae-feeding moth *Anticarsia gemmatalis* on soybean. Effects of UV on plants and subsequently on higher trophic levels seem to be very specific for the investigated species and even for different populations of the same species on both trophic levels (Lindroth et al., 2000). We hypothesized that UV could induce or alter the production of JA-mediated VOC and thus influence the host searching behavior of the parasitoid *C. marginiventris*. However, in our study, we found no effects of UV light on the qualitative and quantitative composition of herbivore-induced volatiles in soybeans. This was true for soybeans exposed for 5, 10 or30 days, respectively (Table 3, Fig. 4). We conclude that in our experiments the induction of photo-protective phenolics was sufficiently effective to prevent any UV effects on VOC synthesis, e.g. through the generation of reactive oxygen species leading to JA accumulation. These results corroborate the findings of Turtola et al. (2006), who found

unaltered (constitutive) terpene compositions in *Pinus sylvestris* and *Picea abies* after exposition to enhanced UV radiation. In accordance with the unaltered VOC blends, parasitoid wasps showed no significant preference for herbivore-damaged plants that had received either VIS + UV or VIS light treatments (Fig. 5). The olfactometer experiments were carried out with plants exposed for 5 days since the largest divergence in the UV shields were found at this time point. VOC analyses of plants exposed for 10 or 30 days do not suggest a different outcome of this plant-parasitoid interaction.

### 2.5. Conclusions

Ambient UV radiation affected the morphology and the composition of secondary compounds such as flavonoids in soybeans. However, this did not change the plants' suitability as a host for *S. frugiperda*. Caterpillar-induced VOC emission after UV exposition remained unaffected and hence parasitoid wasps were attracted by VIS + UV and VIS-treated plants in the same manner. Our results suggest that the induced indirect defense of soybean plants against herbivores remains effective and stable when subjected to altered UV conditions.

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# 3. Nitrogen Deficiency Affects Bottom-Up Cascade Without Disrupting Indirect Plant Defense

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**Abstract**

Nitrogen (N) is an important macronutrient for plants and insects alike, and the availability of this critical element may considerably modify bottom-up effects in tritrophic systems. By using hydroponically cultured *Glycine max*, we investigated the impact of N deficiency on plant growth, photosynthetic efficiency, primary metabolism, and herbivore-induced volatile (VOC) emission. Cascading effects of N deficiency on higher trophic levels were assessed by measuring the performances of the herbivore *Spodoptera frugiperda* and its parasitoid *Cotesia marginiventris*. In addition, we studied the volatile-guided foraging behavior of *C. marginiventris* to explore whether nutrient stress affects the plant's indirect defense. Our results show that photosynthetic efficiency, leaf N, and soluble protein content were significantly reduced in N deficient plants whereas root biomass was increased. Nitrogen starved plants emitted the same range of herbivore-induced VOCs as control plants, but quantitative changes occurred in the release of the main compound and two other volatiles. Herbivore growth and the performance of parasitoids developing inside the affected hosts were attenuated when caterpillars fed on N deficient plants. The behavioral response of *C. marginiventris* to induced VOCs from N deficient hosts, however, remained unaffected. In summary, N stress had strong bottom-up effects over three trophic levels, but the plant's indirect defense remained intact.

Key Words: Glycine max Induced defense Volatile organic compounds Nitrogen Biological control Parasitoids

### 3.1. Introduction

In terrestrial ecosystems, nitrogen (N) is an important macronutrient for plants. Due to soil properties, N availability can be patchy and may vary even on a small scale (Keddy, 2007). Plants show plastic responses to N deficiency by profoundly reprogramming N and carbon (C) metabolism (Lou and Baldwin, 2004; Scheible et al., 2004). In an effort to acquire the missing nutrients more efficiently, plants resort to altered biomass allocation between shoot and root and enhanced root branching. Deficiency also leads to sugar and starch accumulation in leaves, and exerts negative feedback on photosynthesis. Eventually, impeded uptake will lead to reduced leaf N content and a higher C/N ratio (Hermans et al., 2006). Ecologically, a shift towards more C and less N can cause significant changes in bottom-up interactions between primary producers and subsequent trophic levels. Plant N content may affect either herbivore development directly (Berner et al., 2005; Coley et al., 2006; Fischer and Fiedler, 2000; Scriber, 1977), or deficiency effects can cascade up to higher trophic levels, thus altering top-down influences. Soil conditions may indirectly alter the abundance or performance of parasitoids and predators. For instance, ladybird beetles (*Aiolocaria hexaspilota*) feeding on willow leaf beetles (*Plagiodera versicolora*) had a higher adult mass and shorter developmental time when their prey was reared on leaves with high N content (Kagata et al., 2005). A plant's nutritional quality is determined not only by the amounts of primary compounds such as proteins or carbohydrates but also by the levels of secondary metabolites. Both factors are intertwined as N availability may affect the synthesis of constitutive and induced defensive secondary compounds. Depending on the metabolites and plant species in question, N availability may lead to changing levels of secondary compounds (e.g Chen et al., 2008b; Cipollini et al., 2002; Dudt and Shure, 1994; Hemming and Lindroth, 1999; Lou and Baldwin, 2004; Stout et al., 1998). As a response to feeding or egg deposition by herbivores, plants release volatile organic compounds (VOC), which comprise mainly fatty acid derivatives, terpenoids, phenyl propanoids, and benzenoids. Within the ecosystem, these metabolites can have multiple functions, but primarily they are known as signals that guide natural enemies to their herbivorous prey or host (Dicke, 2009; Heil, 2008; Holopainen, 2004). The production and release of VOCs may vary considerably depending on the plant's nutritional status. Maize seedlings, for example, show decreased emissions of induced plant VOCs when N-P-K fertilization was reduced (Gouinguené and Turlings, 2002). Manipulating N availability alone, however, resulted in enhanced levels of induced VOCs in maize (Schmelz et al., 2003b) and cotton (Chen et al., 2008b) but not in *Nicotiana attenuata* at low levels of N (Lou and Baldwin, 2004). Concentrations of the phytohormone jasmonic acid (JA) correlated negatively

with N availability and positively with VOC induction, thus, it was suggested that changes in JA provide a mechanism to regulate the magnitude of plant defense responses (Chen et al., 2008b). A blend of VOCs that varies in the composition or quantity of its components due to abiotic factors may constitute a signal with altered information content and may potentially modify the host finding behavior of natural enemies (Rostás and Turlings, 2008; Turlings and Wäckers, 2004). Several studies have explored the effects of abiotic factors, such as light (Gouinguené and Turlings, 2002; Maeda et al., 2000), humidity (Gouinguené and Turlings, 2002), carbon dioxide (Vuorinen et al., 2004b), UV radiation (Blande et al., 2009; Winter and Rostas, 2008), ozone (Vuorinen et al., 2004a), or nutrient supply (Gouinguené and Turlings, 2002) on VOC induction. Only some of these also have tested whether altered VOC blends affect parasitoid or predator attraction (Blande et al., 2009; Maeda et al., 2000; Vuorinen et al., 2004a; Vuorinen et al., 2004b; Winter and Rostas, 2008). However, behavioral experiments are necessary to understand whether a given abiotic factor has the potential to disrupt the facultative mutualism between plants and natural enemies. Changes in the release rate of certain compounds do not automatically translate into differential host searching behavior (Rostás et al., 2006), while stronger or weaker attraction may not always be reflected by detectable changes in the measured VOCs of an induced plant (D'Alessandro et al., 2009; Gouinguené et al., 2005; Rostás and Turlings, 2008). Soybean plants (*Glycine max*) demand high amounts of N, and a large proportion of it is acquired from N-fixing rhizobacteria. This makes soybean generally less dependent on soil N, but nevertheless, deficiency may occur in patches where appropriate symbionts are lacking or whenever plants and bacteria fail to establish good root nodulation. Unfavorable environmental conditions or fungicide application has been shown to reduce strongly root nodulation and thus nitrogen acquisition (Roth, 2009; Zilli et al., 2009). Here, we addressed the question, whether N deficiency would result in significant bottom-up effects in a tritrophic system consisting of soybean, the herbivore *Spodoptera frugiperda*, and its larval parasitoid *Cotesia marginiventris*. In addition to direct effects on the growth, development, survival, and longevity of plants and insects, we focused on the impact of low N availability on the quantity and quality of herbivore induced plant VOC and the attractiveness of the blends for host searching parasitoids.

## 3.2. Methods and Materials

### 3.2.1. Plant and Insect Material

Soybean seeds (*Glycine max* (L) Merr. cv. London) were obtained from Saatbau Linz (Leonding, Austria). Seedlings were grown in plastic trays (30×20×4.5 cm, Wiesauplast, Wiesau, Germany) containing silica sand for 14–16 d and then subjected to N treatments. Further rearing conditions of plants and insects are described in Winter and Rostas (2008).

### 3.2.2. Nitrogen Treatments

After 14–16 d, plants (V 1 stage, McWilliams et al., 1999) were removed carefully from the sand, and roots were rinsed with deionized water. All seedlings then were transferred to black plastic containers (30.5×20.3×13.3 cm, Rotilabo® Drehstapelwanne, Carl Roth, Karlsruhe, Germany) containing 5.6 l of hydroponic solution, aerated with a membrane pump. Fifteen plants were grown in each container with a distance of 7 cm between each individual. The hydroponic solution was exchanged every 3–4 d. To maintain appropriate salt concentrations and pH of the solution, electric conductivity (EC) and pH were regularly controlled with a combined pH/EC tester (Combo 2, Carl Roth, Karlsruhe, Germany). EC was adjusted to 2.2 mS, pH was kept at 5.9–6.1. Plants were exposed for 5 d to a modified Hoagland solution (Hoagland and Arnon, 1938). For plants growing in nitrogen deficient solution (–N treatment), $KNO_3$ was replaced with $K_2SO_4$ (Carl Roth, Karlsruhe, Germany), and $Ca(NO_3)_2$ with $CaCl_2$ (AppliChem, Darmstadt, Germany) in equivalent concentrations.

### 3.2.3. Effects of Nitrogen Deficiency on Plant Growth and Physiology

The effect of N deficiency on growth and physiology of soybean was assessed by exposing plants to the respective N treatments for 5 d. Then, shoots and roots of six plants per treatment were freeze-dried for 48 h and weighed to calculate shoot-to-root-ratios. Eight plants per treatment grown in the same cohort were used to determine C/N-ratios and concentrations of soluble proteins in the leaves. For this, leaf discs were cut out with a cork borer (diam 17.8 mm) from unifoliate leaves, freeze-dried, weighed, and ground. Half of the material was used to analyze total C and N content by quantitative decomposition of substances by oxidative combustion (CHN-O-Rapid, Heraeus, Hanau, Germany). The other half was extracted 3 × with 500 µl deionized water. Extracts were combined, and soluble protein content was determined

with Bradford reagent (Sigma-Aldrich, Seelze, Germany) using bovine serum albumin (1.4 mg $ml^{-1}$ in water) as standard. Samples were arranged in a 96 well plate, and absorbance was measured with a photometer (Multiskan EX, Thermo Labsystems, Vantaa, Finland) at 595 nm. To assess the effects of N limitation on the photosynthetic efficiency of soybean, the adaxial leaf side of the first trifoliate leaf of 5 plants per treatment was examined with a PAM-2000 fluorometer (Walz Mess–u. Regeltechnik, Effeltrich, Germany). Maximum photochemical yield of photosystem II (PSII) was measured in dark-adapted leaves as the ratio of variable ($F_V$) to maximal ($F_M$) chlorophyll fluorescence at room temperature with $F_V/F_M = (F_M - F_0)/F_M$ (Schreiber et al., 1986). Minimum fluorescence ($F_0$) was excited at 655 nm and 600 Hz modulation frequency, and maximum fluorescence ($F_M$) was measured with 100 kHz modulation frequency. The $F_M$ was elicited by saturating pulses of 0.8 s duration from a built-in halogen lamp.

### 3.2.4. Plant-Mediated Effects of Nitrogen Deficiency on Herbivore Growth, Developmental Time, Survival and Feeding Behavior

An herbivore performance test was conducted to assess the effects of plant N limitation. Fifty neonate larvae of *Spodoptera frugiperda* were weighed and kept individually in Petri dishes (diam. 8.5 cm) with moistened filter paper in a climate chamber as described above. They were fed *ad libitum* with fresh cut leaflets of the first trifoliate soybean leaves from plants kept for 5 d in +N or –N solution. Each plant was harvested only once. Weight increases of larvae were measured between day 5 and 6 ($L_2$) and day 10 and 11 ($L_3$). In addition, pupal and adult weights, as well as developmental times were recorded. Two feeding trials were performed to see whether larvae ($L_2$) compensated for potentially lower food quality due to N limitation by ingesting larger quantities of leaf tissue (no-choice assay), or by choosing food with potentially higher quality (choice assay). For the no-choice assay, 15 single larvae ($L_2$) were placed in Petri dishes (diam. 8.5 cm) and fed with one soybean leaflet (first trifoliate leaf) from plants grown for 5 d in +N or –N solution, respectively. For the choice assay, 12 single larvae ($L_2$) were placed in Petri dishes (diam. 8.5 cm) and allowed to choose between two soybean leaflets of the first trifoliate leaf, one from plants grown for 5 d in +N solution, the other leaflet from a plant grown in –N solution. After 24 h, leaf consumption was measured by scanning the leaves and calculating the removed areas as described in Rostás et al. (2006). For C/N analyses, another cohort of 8 neonate larvae was treated as described for the performance test. After 6 and 15 d, respectively, the larvae were

starved for 1 hr, frozen, freeze dried, and ground. Total C and N content was analyzed as described above.

### 3.2.5. Herbivore-Mediated Effects of Plant Nitrogen Deficiency on Parasitoid Growth, Developmental Time and Longevity

The developmental time, growth, and longevity of the parasitoid *Cotesia marginiventris* developing inside *S. frugiperda* was measured to assess host-mediated effects of plant N limitation. Three-day old *S. frugiperda* larvae were fed with freshly cut soybean leaves of the respective N treatment for 2 d. The larvae then were offered in six groups of 5 to a 4–6 d old mated *C. marginiventris* female in a Petri dish (diam. 5.5 cm). After parasitism of 15 larvae per treatment was observed, caterpillars were kept separately in Petri dishes (diam. 8.5 cm) with moist filter paper and fed *ad libitum* with freshly cut leaves from +N or –N plants. Food was exchanged at least every 2nd day. Emerging parasitoid cocoons were transferred to individual Petri dishes (diam. 5.5 cm) with dry filter paper. Eclosed parasitoids were provided with water only. Developmental time, pupal weight at 24 hr post emergence, and longevity of the adult parasitoids were recorded.

### 3.2.6. Plant-Mediated Nitrogen Effects on VOC Emission and Parasitoid Behavior

The effects of N limitation on the emission of herbivore-induced volatiles and consequently on the behavior of the parasitoid was investigated in a six-arm- olfactometer (for details see Turlings et al., 2004). Soybean plants exposed for five days were placed individually into the cup of an odor source vessel of the six-arm-olfactometer and provided with approximately 50 ml of the accordant hydroponic solution. The cup was covered with two semicircular polycarbonate plugs that had an opening in the center to hold the plant in an upright position and to prevent larvae from falling into the solution. Twenty-five *S. frugiperda* larvae ($L_2$) were placed on each plant and were allowed to feed overnight (approx. 16 hr). On the following day, volatile collections and behavioral assays were carried out simultaneously from 9:00 AM till 12:00 AM. Ten fluorescent lamps (PAR inside odor source vessels: 130 µmol photons $m^{-2}$ $sec^{-1}$ at 30 cm distance from lamps) were switched on 3 hr before testing the wasps. Odor source vessels containing a plant that had received either +N or –N treatment were placed vis-à-vis in the olfactometer. The other four vessels remained empty as controls. After connecting the vessels to the air delivery and the olfactometer, the air stream was allowed to stabilize for 10 min. The flow

rate was 1.2 l $min^{-1}$ for incoming air and 0.6 l $min^{-1}$ for air going out to the behavioral arena or the volatile traps, respectively. Mated 3–5 d old females of *C. marginiventris* were used in the behavioral assays. Wasps had no oviposition experience prior to the experiment. All wasps were tested in groups of 6, as they do not interfere with each other's choices (Turlings et al., 2004). After 30 min, the choices made by the parasitoids were recorded, and the group was replaced by a new one. Five groups of wasps were tested on the same day. One day with 5 releases was considered as one replicate. Six replicate days were carried out with a new pair of plants and new wasps each day. Volatiles emanating from soybeans were collected with SuperQ traps as described previously (Rostás and Eggert, 2008). After each experimental day, the glass and teflon parts of the olfactometer were cleaned with deionized water and rinsed with ethanol (v/v 70%), acetone, and hexane. After evaporation of the solvents, all glass parts were placed in an oven at 200°C for 1 hr. Trapped volatiles were eluted with 150 µl methylene chloride. Two internal standards (n-octane and nonyl acetate, Sigma-Aldrich, Taufkirchen, Germany, 400 ng each in 20 µl methylene chloride), were added, and the samples were stored at −80°C. The qualitative and quantitative volatile composition of each sample was analyzed on an Agilent Technologies 6890N Network GC System coupled with a 5973 Network Mass Selective Detector. Three µl of each sample were injected with an automated injection system in pulsed splitless mode. The column was an Agilent 19091-s933 HP-1 capillary column (length 30 m, diam 0.25 mm, film thickness 0.25 µm). The oven was held at 35°C for 3 min and then increased with 8°C $min^{-1}$ to a final temperature of 230°C that was held for 10 min. Helium (1.5 ml $min^{-1}$) was used as carrier gas. Compounds were identified using MSD ChemStation (Agilent Technologies) software with the Wiley 275 mass spectrum library and by using the software MassFinder3/Terpenoids library (Hochmuth Scientific Software, Hamburg, Germany). Identities were confirmed further by co-injection of authentic standards (Sigma-Aldrich, Taufkirchen, Germany). Quantification was obtained by comparing the area of the compounds to the areas of the internal standards.

### 3.2.7. Statistical Analyses

Plant parameters were analyzed with a Mann-Whitney-U-test. Values of plant C/N ratios and soluble protein contents were Bonferroni corrected prior to the analyses. Performance parameters of *S. frugiperda* and *C. marginiventris* were compared with ANCOVA using initial weight of *S. frugiperda* as covariable and measured performance parameters as variables. Herbivore mortality was analyzed with a $\chi^2$-test. Plant photosynthetic efficiency, larval feeding in the no-choice

assay, larval C/N ratios, and differences in VOC compositions were analyzed with Student's t-tests for independent samples. Only VOCs occurring in at least 50% of all samples were analyzed statistically. Larval feeding in the choice assay was assessed with a t-test for dependent samples. The analyses were conducted using the STATISTICA 7.1 software package (StatSoft, Tulsa, OK, USA). For the six-arm-olfactometer, the entity computing a repetition in the statistical analysis corresponds to the response of a group of 6 wasps released, which was shown to follow a multinomial distribution (Ricard and Davison, 2007). As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasi-likelihood functions to compensate for the overdispersion of parasitoids within the olfactometer. The model was fitted by maximum quasi-likelihood estimation in the software package R (http://www.R-project.org), and its adequacy was assessed through likelihood ratio statistics and examination of residuals (Turlings et al., 2004).

### 3.3. Results

3.3.1. Effects of Nitrogen Limitation on Plant Morphology and Physiology

Plants exposed to –N conditions for 5 d showed a significantly decreased shoot-to-root ratio due to increased root biomass (dry weight) compared to +N exposed plants (Table 1). In leaves of –N plants, the amounts of soluble proteins were reduced significantly whereas the C/N ratio was increased due to a lower N content (Table 1). Maximum photochemical efficiency of PS II, measured as $F_V/F_M$, was reduced significantly in –N compared to +N treated plants (Table 1).

**Table 1**: Morphological and physiological parameters of soybean exposed to different N treatments

| Parameter | +N[a] | -N[b] | Statistics[c] |
|---|---|---|---|
| Shoot/root ratio | 9.84 ± 1.74 | 3.73 ± 0.44 | $N = 6$, $Z = 2.88$, $\mathbf{P^d < 0.005}$ |
| Soluble leaf protein content (mg $g^{-1}$ DW) | 57.74 ± 6.02 | 37.36 ± 2.78 | $N = 8$, $Z = 2.42$, $\mathbf{P^d < 0.05}$ |
| Leaf C/N ratio | 10.19 ± 0.40 | 16.26 ± 1.16 | $N = 8$, $Z = -3.05$, $\mathbf{P^d < 0.005}$ |
| Maximum photochemical efficiency of PS II ($F_v/F_m$) | 0.806 ± 0.003 | 0.788 ± 0.006 | $N = 5$, $t = 2.87$, $\mathbf{P^d < 0.05}$ |

[a] Mean values ± SE for plants exposed to full Hoagland solution for five days.

[b] Mean values ± SE for plants exposed to N deficient Hoagland solution for five days.

[c] Statistical parameters are indicated for Mann-Whitney-*U*-tests and Student's *t*-test ($F_V/F_M$).

[d] P-values in bold indicate statistically significant differences.

3.3.2. Plant-Mediated Nitrogen Effects on Herbivore Growth, Developmental Time, Survival, and Behavior

Herbivore larvae fed with leaves from N deficient plants gained significantly less biomass during their development, which resulted in significantly lower pupal and adult weights (Table 2). The C/N ratios in these larvae were reduced compared to larvae fed with +N treated plants. However, developmental time (Table 2), as well as larval mortality ($\chi^2$ test, $\chi^2$=0.08, *df*=1, *P*>0.5) and successful adult emergence ($\chi^2$ test, $\chi^2$=0.35, *df*=1, *P*>0.5), were not influenced by plant N limitation.

**Table 2**: Performance parameters of *Spodoptera frugiperda* reared on soybean exposed to different N treatments

| Parameter | +N[a] | -N[b] | Statistics[c] |
|---|---|---|---|
| Weight increase day 5/6 [mg] | 3.04 ± 0.29 | 2.12 ± 0.25 | $N^{d}$ = 21/24, $F$ = 5.28, $\boldsymbol{P^{e} < 0.01}$ |
| Weight increase day 10/11 [mg] | 34.17 ± 4.52 | 21.63 ± 2.84 | $N^{d}$ = 19/22, $F$ = 5.19, $\boldsymbol{P^{e} < 0.05}$ |
| Pupal weight [mg] | 138.88 ± 5.43 | 113.86 ± 5.20 | $N^{d}$ = 12/11, $F$ = 9.2, $\boldsymbol{P^{e} < 0.01}$ |
| Adult weight [mg] | 63.76 ± 2.08 | 47.84 ± 5.15 | $N^{d}$ = 7/9, $F$ = 5.67, $\boldsymbol{P^{e} < 0.05}$ |
| Larval C/N (day15) | 3.52 ± 0.39 | 4.16 ± 0.25 | $N^{d}$ =6/8, $t$ = –0.369, $\boldsymbol{P^{e} < 0.005}$ |
| Larval time [d] | 21.08 ± 0.75 | 22.36 ± 0.81 | $N^{d}$ = 12/11, $F$ = 0.79, $P > 0.1$ |
| Pupal time [d] | 8.88 ± 0.23 | 8.30 ± 0.21 | $N^{d}$ = 8/10, $F$ = 2.27, $P > 0.1$ |

[a] Mean values ± SE for plants exposed to full Hoagland solution for five days.

[b] Mean values ± SE for plants exposed to N deficient Hoagland solution for five days.

[c] Statistical parameters are indicated for ANCOVA and Student's *t*-test (Larval C/N)

[d] $N$ = $\text{individuals}_{+N}/\text{individuals}_{-N}$.

[e] P-values in bold indicate statistically significant differences.

In choice and no-choice tests, the larvae did not consume significantly more leaf tissue from –N treated than from +N treated plants (Fig. 1).

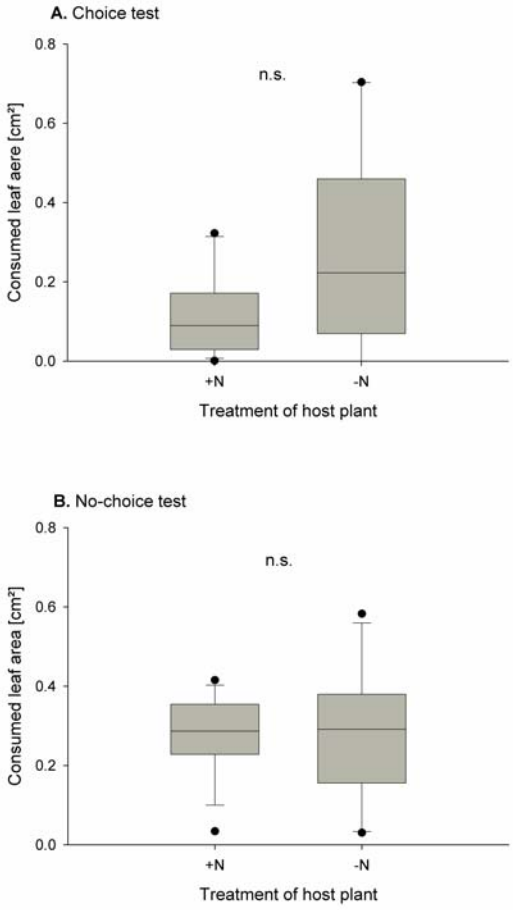


**Figure 1**: Leaf consumption by second instar *Spodoptera frugiperda* larvae measured as leaf area removed in 24 hours. Soybean plants were exposed to either full (+N) or N deficient (-N) Hoagland solution for five days. Box-plots show median (line), 25%-75% percentiles (box), 10%-90% percentiles (whisker) and outliers (dots). (**A**) Choice test: Students *t*-test for dependent samples ($t$ = -2.14 $P > 0.05$, $N = 12$). (**B**) No-choice test: Students *t*-test for independent samples ($t$=0.00, $P > 0.5$, $N_{+N} = 15$, $N_{-N} = 14$).

3.3.3. Plant-Mediated Nitrogen Effects on Parasitoid Growth, Developmental Time, and Longevity

Individuals of *C. marginiventris* reared in caterpillars of *S. frugiperda*, which in turn were fed with leaves from –N treated soybeans, had significantly lower pupal weights. Developmental time and longevity did not differ between wasps reared in herbivore larvae fed with +N or –N treated leaves (Table 3).

**Table 3**: Performance parameters of *Cotesia marginiventris* reared inside *Spodoptera frugiperda*

| Parameter | +N[a] | -N[b] | Statistics[c] |
|---|---|---|---|
| Pupal weight [mg] | 2.18 ± 0.05 | 1.73 ± 0.09 | $N^d$ = 14/12, $F$ = 19.52, $\mathbf{P^e < 0.001}$ |
| Larval time [d] | 8.1 ± 0.1 | 8.4 ± 0.09 | $N^d$ = 15/12, $F$ = 1.02, $P > 0.1$ |
| Pupal time [d] | 5.2 ± 0.2 | 5.0 ± 0.0 | $N^d$ = 9/8, $F$ = 1.01, $P > 0.1$ |
| Longevity [d] | 14.6 ± 0.3 | 14.6 ± 0.6 | $N^d$ = 7/7, $F$ = 0.08, $P > 0.5$ |

[a] Mean values ± SE for caterpillars fed with plants exposed to full Hoagland solution for five days.

[b] Mean values ± SE for caterpillars fed with plants exposed to N deficient Hoagland solution for five days.

[c] Statistical parameters are indicated for an ANCOVA

[d] $N$ = individuals$_{+N}$/individuals$_{-N}$.

[e] P-values in bold indicate statistically significant differences.

3.3.4. Plant-Mediated Nitrogen Effects on VOC Emission and Parasitoid Behavior

In both N treatments, only trace amounts of (*E,E*)-α-farnesene could be detected from undamaged soybean plants. In contrast, plants damaged by *S. frugiperda* larvae released 22 different compounds of which 18 were identified (Fig. 2). The main constituents were (*E,E*)-α-farnesene (20% and 41% of total emission for +N and –N plants, respectively) and indole (24% and 12% of total emission for +N and –N plants, respectively) when emission was corrected for plant fresh weight. While the same compounds were emitted by +N and –N treated soybean plants, three substances were released in significantly different amounts. The release rates of the sesquiterpenes *β*-bergamotene and (*E,E*)-α-farnesene were approx. five times (t=−2.51. *df*=10, *P*<0.05) and three times (t=−2.64. *df*=18, *P*<0.05) higher in –N plants than in +N plants. In contrast, a 50% decrease was observed in the emission of (*Z*)-3-hexenyl-α-methylbutyrate (t=2.30. *df*= 10, *P*<0.05). All other volatiles were released in similar amounts. The statistical values given here refer to emission rates corrected for plant fresh weight, but the same pattern was obtained for uncorrected values (Student's-t-test). Differences in the quantities of the three volatiles did not cause a significant shift in overall emission.

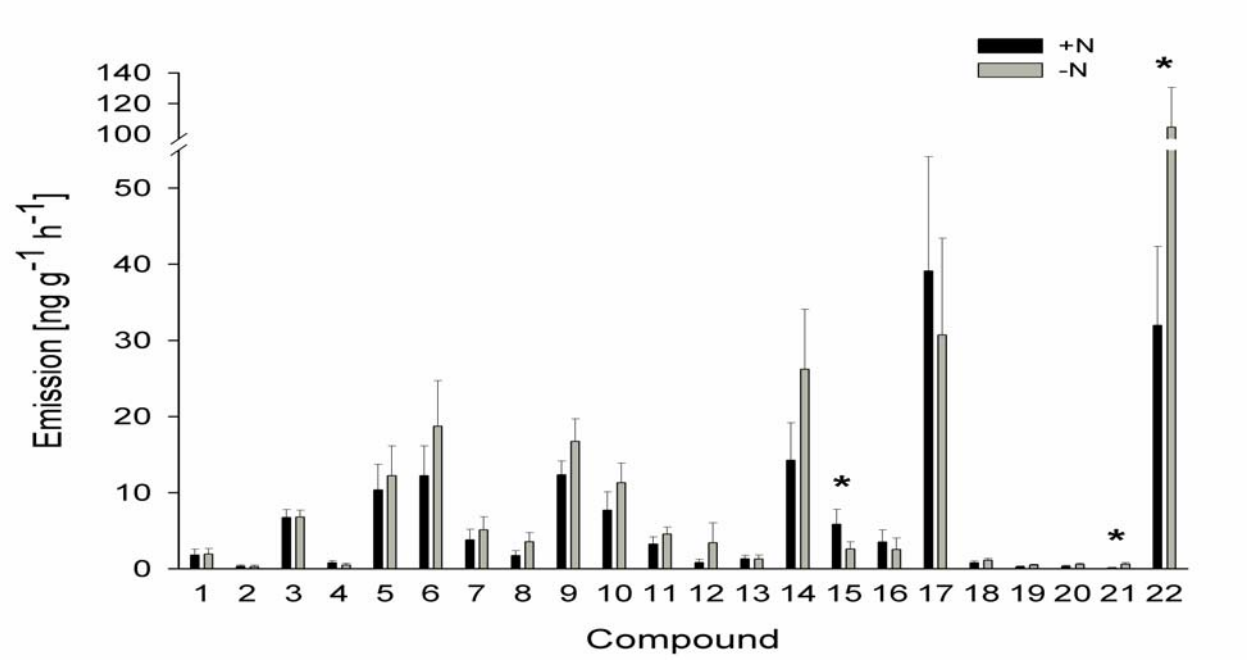


**Figure 2**: Plant VOC emission in response to herbivory and full (+N) or deficient (-N) supply of N for five days. Bars indicate mean values, whiskers are ± SE. Asterisks indicate statistically significant differences (*$P$ < 0.05, Student's $t$-test, $n$ = 10). Compounds were identified according to their retention times, library mass spectra and by coelution with commercial standards and are arranged by retention times in the figure.

1) n. i. 2) (*Z*)-3-Hexenal 3) n. i. 4) n. i. 5) (*Z*)-3-Hexenol 6) n. i. 7) α-Pinene 9) (*Z*)-3-Hexenyl acetate 10) (*E*)-β-Ocimene 11) (*Z*)-3-Hexenyl propionate 12) Benzeneacetonitrile 13) (*Z*)-3-Hexenylisobutyrate 14) Methyl salicylate 15) (*Z*)-3-Hexenyl-α-methylbutyrate 16) n. i. 17) Indole 18) (*E*)-Caryophyllene 19) α-Humulene 20) Germacrene D 21) *β*-Bergamotene 22) (*E,E*)-α-Farnesene. n.i. = Compound not identified.

Naïve females of *C. marginiventris* were highly attracted by the herbivore-induced volatiles of both +N and –N-treated plants when compared to clean air. But wasps had no preference for either one of the offered plant odors (log-linear- model, $P$>0.05, $N$=6, Fig. 3)

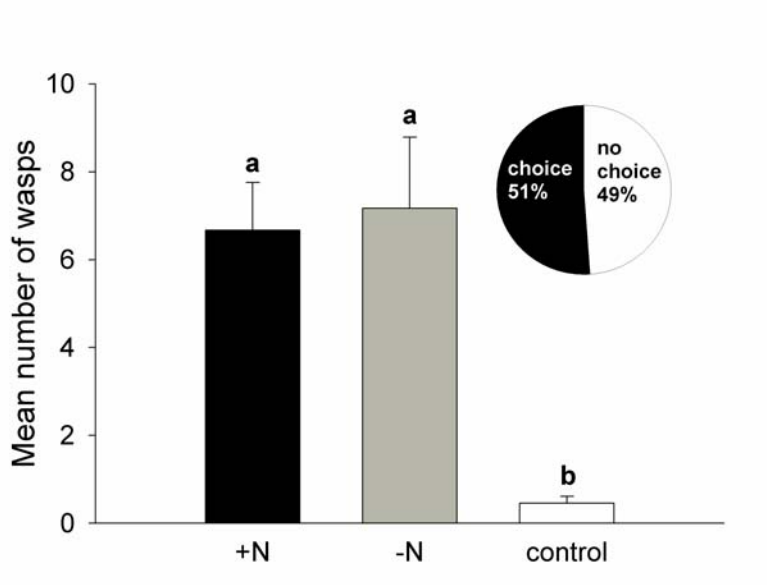


**Figure 3**: Response of naïve *Cotesia marginiventris* to herbivore induced volatiles of soybean. Plants were reared in either full (+N) or nitrogen deficient (-N) Hoagland solution. Control was clean air; total numbers of wasps choosing one of the four control arms were divided by four. Bars represent mean (+SE) numbers of wasps making a choice in the olfactometer. Different letters indicate statistically significant differences (Log-linear model fitted to quasipoisson distribution; $N$ = 6 independent experiments with 30 wasps each). Pie chart shows mean percentage of wasps making a choice.

### 3.4. Discussion

Deficiency in N severely affected the morphology and physiology of soybeans. In our study, plants lacking N increased their root biomass while shoot biomass remained unchanged (Table 1). Shifting the root-shoot-ratio is a well known response to low N availability and is considered to be an adaptation to suboptimal substrates which allows plants to more efficiently absorb nitrate from the soil (Hill et al., 2006; Schopfer and Brennicke, 2005). It has been proposed that changes in phytohormonal balances in conjunction with sugar signals orchestrate cell division and differentiation, thus leading to optimized root morphology (Hermans et al., 2006). We found that shortage in N supply negatively affected photosynthesis as indicated by the reduced maximum photochemical efficiency of PSII (Table 1). These results corroborate several studies that have used chlorophyll fluorescence to monitor the responses to N deprivation (Kumagai et al., 2007; Lu and Zhang, 2000). Deficiency downregulates open PSII reaction centers and leads to increased light induced non-photochemical quenching and enhanced susceptibility to photoinhibition (Lu and Zhang, 2000). A lack in chlorophyll content and

Ribulose-1, 5-bisphosphate-carboxylase/-oxygenase (RuBisCo), the most abundant protein in plants, is likely to also have contributed to a decline in photosynthesis. Chlorophyll and RuBisCo were not measured specifically, but leaves of soybeans exposed to N stress had lower leaf N levels, contained less protein (Table 1), and were lighter in color. Negative effects on the leaf's N and protein content correlated with poor growth of *S. frugiperda* larvae. Caterpillars fed with –N leaves gained less weight during their development and had reduced pupal and adult weights compared to larvae reared on +N leaves (Table 2). Similar results were reported from *Spodoptera exigua* developing on N deficient cotton (Chen et al., 2008a) or larvae of other Lepidoptera feeding on several host plants (Coley et al., 2006). However, studies exist where the correlation between food N content and herbivore growth is less consistent, implying that N is a limiting nutrient for larval development, but not the only one (Tabashnik, 1982). Clancy (1992) hypothesized that host plant N determines the amount of food ingested and thus affects the amount of other nutrients incorporated, resulting in an altered growth rate and survival rates of the herbivore caused by a deficiency of other nutrients than N.

Interestingly, despite poorer growth on –N leaves, *S. frugiperda* larvae did not prefer the superior food when both types of leaves were offered in a dual-choice assay, nor did they show any compensational feeding in a no-choice setup (Fig. 1). This contrasts with findings reported by Chen et al. (2008a) and Merkx-Jacques et al. (2008) who found that *S. exigua* larvae opted for leaves of high N content or a protein-biased diet, respectively. The incongruent feeding behavior of the two species may reflect their different feeding preferences. Larvae of *S. exigua*, for instance, are known to prefer leaves while *S. frugiperda* may often change to fruiting structures when available.

The impact of N deficiency on the first trophic level was transmitted indirectly to the third trophic level as the growth of *C. marginiventris* also was affected. Parasitoids had significantly lower pupal and adult weights when their hosts had fed on N-deprived soybean leaves (Table 3). Pupal weight often is correlated with fecundity and consequently with fitness (Bourchier, 1991). Thus, females of *C. marginiventris* that hatch from poor hosts may be expected to produce fewer eggs and have fewer offspring. Plant quality is crucial for the performance and fertility of parasitoids in many cases (Campan and Benrey, 2004; Caron et al., 2008; Sarfraz et al., 2008; Setamou et al., 2005). *Cotesia flavipes* had a higher mean progeny size on its host *Chilo partellus*, when this was reared on cultivated compared to wild gramineous plants (Setamou et al., 2005). Eventually, the effect of poor plant quality on parasitoid fitness depends on the species' life history. Gregarious, koinobiont parasitoids, for instance, should be affected more strongly than solitary parasitoids when choosing poor hosts. They spend a considerable amount

of time in examining their hosts and will lay their eggs in one or relatively few individuals (Brodeur and Boivin, 2004). In contrast, females of the generalist, solitary endoparasitoid *C. marginiventris* allocate their offspring to many caterpillars by feeding on different host plants and thus reducing the fitness costs imposed by parasitizing an inferior host. Nevertheless, fitness costs could be substantial if a larger patch is affected by N deficiency and if wasps forage exclusively within this patch.

The host foraging behavior of parasitoid wasps is influenced by herbivore-induced plant VOCs, which are used as signals to locate those particular plants that are infested by herbivores. Soybeans grown in N deficient hydroponic solution emitted the same spectrum of herbivore-induced VOCs as fertilized plants. However, quantitative changes were found for some VOCs. Low N availability led to more than three times higher emission rates of the main compound (*E,E*)-α-farnesene, and significantly increased the release of the sesquiterpene *β*-bergamotene. Emission of the green leaf volatile (*Z*)-3- hexenyl-α-methylbutyrate, however, was significantly reduced (Fig. 2). Despite these changes, total amounts of VOCs were not affected by N deficiency. Increased levels of constitutive mono- and sesquiterpenes have been found in N limited *Heterotheca subaxillaris* (Mihaliak and Lincoln, 1985). Looking at induced VOCs, higher levels were found also in N deficient, hydroponically cultivated maize, and in soil-grown cotton. In both plants, the accumulation of the phytohormone jasmonic acid (JA), an important factor in the signaling cascade leading to volatile biosynthesis, correlated negatively with N (Chen et al., 2008b; Schmelz et al., 2003b). It has been argued that N starved plants produce greater induced defense responses because N deficiency commonly leads to higher levels of leaf sugars and starch. This larger pool of nonstructural carbohydrates may be used for enhanced VOC biosynthesis (Schmelz et al., 2003b). Females of *Cotesia* spp. have been reported to respond in a dose-dependent manner to the total blend of herbivore-induced VOCs (NgiSong et al., 1996; Turlings et al., 2004). With the main compound (*E,E*)-α-farnesene being emitted in higher amounts by N deficient soybeans, a preference for this odor could have been expected. Naïve *C. marginiventris* females, however, did not differentiate between the blends of stressed and fertilized plants (Fig. 3). Our results thus confirm observations by Chen et al. (2008b) from cage experiments with cotton plants. The authors found that *C. marginiventris* parasitized the same numbers of caterpillars irrespective of the plants' N levels and concluded that the parasitoids did not differentiate between the herbivore induced VOC blends. Several reasons for this behavior are conceivable. First, the VOCs that were emitted in higher quantities were not the relevant key compounds that induce wasp attraction. This notion is supported by an elegant experiment using transgenic *Arabidopsis thaliana* that overexpressed the maize terpene synthase

gene TPS10. In this study, Schnee et al. (2006) demonstrated that several sesquiterpenes, among them (*E*)-β-farnesene or (*E*)-α-bergamotene, were not attractive to naïve *C. marginiventris*. Moreover, the wasps responded only if they had experienced these compounds during oviposition. Further evidence comes from studies that tested specific fractions of the induced odor blend. D'Alessandro and Turlings (2005) confirmed that a blend lacking most sesquiterpenes was as attractive to naïve and experienced *C. marginiventris* as the full mix. The same holds true for a blend that lacked VOCs from the shikimic acid pathway, while on the other hand, wasps responded strongly to unknown compounds in quantities that were to low to be detected (D'Alessandro et al., 2009; D'Alessandro et al., 2006; Rostás and Turlings, 2008). In fact, our knowledge on which VOCs innately trigger attraction in parasitoids is fairly rudimentary and needs further testing. Moreover, in nature, such cues are rarely isolated but always occur within the context of background odor, that may mask or enhance the odors of the target plant (Mumm and Hilker, 2005; Schroeder and Hilker, 2008). In summary, N deprivation had a strong negative impact on the whole tritrophic system, attenuating the performance of soybean plants, herbivorous caterpillars, and the parasitoid larvae that developed inside *S. frugiperda*. Despite such adverse effects on the plant's physiology and some alterations in VOC emission, the signal that indicates the presence of potential hosts obviously remained unchanged as the wasps' host searching behavior was not affected. These results suggest that the induced indirect defense against herbivores remains stable and effective even under low N conditions. On the parasitoid's side, reduced fitness due to low N availability cannot be ruled out, but might be negligible if only few plants within a patch are affected. From the plant's perspective and from a biocontrol point of view, this should be beneficial, as abiotic stress in this case does not promote higher susceptibility to another biotic stress factor.

# 4. Heavy metal stress primes for herbivore induced volatiles without affecting induced indirect defense of maize

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in preparation

Abstract

Heavy metal (HM) stress is supposed to interact with various plant biochemical pathways including those important for the induced indirect defense by affecting the production of reactive oxygen species and subsequently of jasmonic acid. *Zea maize* was cultured hydroponically to manipulate HM stress and investigate the effects of excess Cu and Cd on growth, photosynthetic efficiency, uptake of HM and other metal ions into the plant, kinetics of jasmonic acid and herbivore induced volatile (VOC) emission. To assess direct and indirect HM-effects on higher trophic levels, growth and feeding behavior of the herbivore *Spodoptera frugiperda* and the volatile guided host searching behavior of its parasitoid *Cotesia marginiventris* were studied. Excess HM led to high concentrations of the respective metals in the plants' roots. The HM significantly affected uptake of other metal ions into the plants. High concentrations of the HM negatively affected photosynthesis, aerial growth and to some extend root biomass of the plants. High concentrations of Cu had a transient priming effect on herbivore induced JA followed by an increased VOC emission caused by three green leaf volatiles and four terpenes. Treatment of the host plants with high HM concentrations negatively affected growth of the herbivore but not its food choice. Despite quantitatively and qualitatively affected volatile emission the host searching behavior of *C. marginiventris* remained unaffected.

## 4.1. Introduction

Soil pollution with heavy metals is an increasing problem despite improved filter techniques and use of new materials. Heavy metals can enter the soil from natural sources like igneous rocks but the main sources of pollution are anthropogenic, e.g. agricultural and industrial sewage (Orcutt and Nilsen, 2000; Sharma and Agrawal, 2005), mining or combustion processes (www.eea.europa.eu/publications/92-9157-202-0/3.3.pdf). The heavy metal problematic is stressed by (yet) anticipated rising pollution. In countries of the European Union for example an increase of emission of cadmium by 26 % and of copper by 8 % until the year 2010 is expected (http://www.eea.europa.eu/publications/92-9157-202-0/3.3.pdf).

Here we studied the effects of two different heavy metals, first copper (Cu), which is even essential for plants` physiology and can be tolerated in relatively high concentrations (Wintz et al., 2002) and secondly cadmium (Cd), a highly toxic metal (e.g (Popova et al., 2009; Wang et al., 2009). Moreover, we focus on heavy metals` effects on a maize-based tritrophic system.

The aforementioned metals can have a wide range of effects on plants. Cu leads to a decrease in chlorophyll content, root and leaf biomass as well as changes in enzyme activities and an inhibition of photosynthesis in maize when applied above a certain threshold (Mocquot et al., 1996; Tanyolac et al., 2007). Maize plants exposed to Cd exceeding a specific threshold show a reduction in growth of shoot and roots as well as altered root architecture, decreased nutrient uptake (Pál et al., 2006), diminished chlorophyll content and altered chloroplast ultrastructure (Rascio et al., 1993).

To retain excess Cu in the roots is one way of preventing the shoot from being affected, realized in maize plants. As a result, root cell division and elongation is reduced (Ouzounidou et al., 1995). Regarding Cu, maize could be termed a shoot-excluder (Florijn and Van Beusichem, 1993). Hence, maize can control the uptake of Cu to the aerial parts at least to a certain extend. Cd on the other hand is evenly distributed between root and leaves of maize plants (Lozano-Rodríguez et al., 1997), thus there seems to be no exclusion on root level.

Heavy metals entering the plant have been shown to create reactive oxygen species (ROS) in plant tissue either simply by a chemical reaction (Mithöfer et al., 2004) in the case of Cu or indirectly by an inhibited Calvin cycle in the case of Cd (Pál et al., 2006). ROS are also built up in plant tissue during herbivore attack due to membrane depolarization or hyperpolarization activating $Ca^{2+}$-permeable channels (Maffei et al., 2006) as well as during pathogen attack due to an NADPH-dependent enzyme system (Lamb and Dixon, 1997). In case of herbivory these ROS induce the synthesis of oxylipins, among others Jasmonic Acid (JA) (Mithöfer et al., 2004). JA

again may activate the plants´ induced indirect defense (IID) against herbivore attack (Schmelz et al., 2003a). IID enables the plant to cope with herbivores by attracting herbivores' enemies such as parasitoids and predators. Following herbivore feeding or egg deposition numerous volatile organic compounds (VOC) deriving from different pathways are synthesized and emitted from the affected plant (D'Alessandro et al., 2006; Meiners and Hilker, 2000). Natural enemies of the herbivore use these VOC for host finding (Dicke, 1994). Alterations in quantity or quality of the induced blend may modify the enemies' host finding ability (Rostás and Turlings, 2008; Turlings et al., 2004). Heavy metal stress may thus affect the plants` ability to produce and emit VOC by affecting JA-dependent signaling pathways subsequently resulting in an altered indirect defense.

On the other hand heavy metals may not only alter IID systems but plants may use them as a direct defense against herbivores (reviewed in (Boyd, 2007). Coleman et al. (2005) showed a direct toxicity of different metals including Cd and Cu to *Plutella xylostella* even at relatively low concentrations using an artificial diet. *Spodoptera exigua* larvae were found to be affected negatively by Ni in different species of *Streptanthus* depending on the leaf-concentration of the metal (Boyd and Moar, 1999). *Trichoplusia ni* reared on broccoli grown in sewage sludge containing high amounts of Cu and Cd showed higher mortality and prolonged developmental time than larvae reared on control plants (Larsen et al., 1994).

As described above, excess of heavy metal is an increasing problem and heavy metal stress may affect all trophic levels and their interactions but primarily direct effects of excess heavy metals on plants and herbivores are studied yet. Studies on heavy metal effects on the VOC-mediated behavior of parasitoids and thus the IID of plants are scarce but essential for the understanding of heavy metal pollution effects in complex ecosystems.

Our aim was, first to investigate direct bottom-up effects of Cu and Cd excess on the physiology and morphology of the important crop plant maize and subsequently on *S. frugiperda*, a major maize pest particularly in American countries. Additionally, we studied the effects of heavy metals on the induced VOC of maize and the consequential attractiveness of the blend to the parasitoid wasp *Cotesia marginiventris* in olfactometer experiments.

## 4.2. Materials and methods

### 4.2.1. Plant and insect material

Maize seeds (*Zea mays* (L). cv. Lambada) were obtained from BayWa (Munich, Germany). Seedlings were soaked in water for one day and afterwards grown in plastic trays

(30×20×4.5 cm, Wiesauplast, Wiesau, Germany) containing expanded clay (Lamstedt Ton, 2 – 4 mm particle size) for 5 days. Further rearing conditions of plants and insects were described in Winter and Rostas (2008).

### 4.2.2. Exposure to heavy metal treatments

After 5 days, plants (5 – 6 cm height) were carefully removed from the expanded clay and transferred to black plastic containers (30.5×20.3×13.3 cm, Rotilabo® Drehstapelwanne, Carl Roth, Karlsruhe, Germany) containing 5.6 l modified Hoagland solution (Hoagland and Arnon, 1938), aerated with an membrane aquarium pump (Elite 802, Hagen aquaristic equipment, Hohn, Germany). Plants were held by their seeds 3 cm above solution surface in a plastic cover sheet with 15 holes in three rows. Distance between plants was 7 cm. The solution was completely changed at least every fourth day. To maintain appropriate salt concentration and pH of the solution, electric conductivity (EC) and pH was controlled with a combined ph/ EC tester (Combo 2, Carl Roth, Karlsruhe, Germany) at least after changing of the solution. EC was adjusted to 2.2 mS and pH to 5.9-6.1.

After 2 days of acclimatization to the hydroponic system, plants were exposed for 3 – 7 days to different heavy metal concentrations depending on the experiment. We used copper and cadmium with two different concentrations each and a control without additional heavy metals. Cadmium was added at 5 µM (cadmium low CdL) and 50 µM (cadmium high CdH) as dissolved $CdCl_2 \times 2.5\ H_2O$ (Sigma-Aldrich, Seelze, Germany), copper at 10 µM (copper low CuL) and 80 µM (copper high CuH) as dissolved $CuSO_4 \times 5\ H_2O$ (Carl Roth, Karlsruhe, Germany) additionally to the copper already present in the Hoagland solution.

### 4.2.3. Effects of heavy metal stress on plant growth and physiology

The effect of heavy metal stress on the growth and physiology of maize was assessed by exposing plants to the respective treatments for 7 days unless otherwise noted. During this time growth of shoots and leaves were measured daily. After 7 days the roots were freeze-dried for 48 hours and weighed.

To assess the effects of heavy metal stress on the photosynthetic efficiency of maize, the adaxial leaf side of the primary and secondary leaf was examined with a PAM-2000 fluorometer (Walz Mess –u. Regeltechnik, Effeltrich, Germany). Maximum photochemical yield of PSII was measured in dark-adapted leaves as variable chlorophyll fluorescence at room temperature

[$Fv/Fm = (F_M - F_0)/F_M$] (Schreiber et al., 1986). Minimum fluorescence ($F_0$) was excited at 655 nm and 600 Hz modulation frequency, and maximum fluorescence ($F_M$) was measured with 100 kHz modulation frequency. The $F_M$ was elicited by saturating pulses of 0.8 s duration from a built-in halogen lamp. The plants were measured prior to heavy metal exposition, the day after start of exposition and in the following every second day at four points per leaf plus the newly grown leaf material for the secondary leaf.

Metal concentration in the plants was measured with inductively coupled plasma – atomic emission spectrometry (ICP-AES). Plants exposed for 5 days to the respective treatments were divided into leaf, shoot and root and freeze dried for 48 hours. Prior to the analysis, the material was ground in a mixer mill (Retsch MM301, Retsch GmbH, Haan, Germany). 30 to 100 mg were digested with 1 ml nitric acid (v/v 65 %) for 10 h at 160 °C. Afterwards 9 ml ultrapure water was added and the sample solution was gently mixed. Samples were analyzed in an ICP-sequential atomic emission spectrometer JY 70 PLUS (Division d'Instruments S.A., Jonin Yvon, France) with a fixed cross flow nebulizer. The concentrations of cadmium (wavelength 228.8 nm), copper (327.296 nm), iron (259.94 nm), zinc (213.856 nm) and magnesium (279.079 nm) were determined threefold for each sample to calculate a mean concentration per sample.

Concentration of the phytohormone jasmonic acid (JA) was determined with a modified vapor phase extraction method (Mishina and Zeier, 2006; Schmelz et al., 2004). After 3 days of exposure to the different treatment plants were separately transferred to plastic tubes (height 11.2 cm, diameter 4 cm) containing 120 ml of the respective hydroponic solution. Plant defensive reactions were induced by six larvae of *S. frugiperda* (second larval stage, $L_2$) placed in two clip cages (16mm inner diameter, 11 mm depth, Moore et al., 2003) at the oldest leaf. Controls were treated with empty cages. Clip cage, larvae and frass were removed and samples were taken from the treated leaf at induction time (without clip cages) 3 h, 6 h and 24 h after start of induction. Samples were weighed to approximately 200 mg, transferred to 2-ml-cups, frozen in liquid nitrogen and ground in a precooled mixer mill. Further sample preparation and analysis was done according to Mishina and Zeier (2006).

### 4.2.4. Plant-mediated effects of heavy metal stress on herbivore growth and feeding behavior

Larvae of *S. frugiperda* were fed with plant material exposed to either 50 µM cadmium or 80 µM copper for 5 days or control pants to asses possible effects of heavy metal treatment on their growth and food choice.

To assess effects on larval growth, neonate larvae of *S. frugiperda* were weighed and kept individually in petri dishes (diam. 8.5 cm) with moistened filter paper in a climate chamber as described above (4.2.1. *Plant and insect material*). They were fed with fresh cut longitudinally halved secondary leaves of maize plants from the respective treatment each time they were weighed. Each plant was harvested only once. Weight increases of larvae were measured every second day until day five and frass was collected. After 5 days the larvae were weighed again and transferred individually to 2-ml-vials. After 2 days without feeding, frass and larvae were separated, frozen and freeze dried for 48 h. Afterwards metal concentrations in the larvae and the frass were determined as described above (4.2.3. *Effects of heavy metal stress on plant growth and physiology*).

A choice assay with $L_2$ larvae was performed to see if larvae compensate for potentially lower food quality due to heavy metal stress by choosing food with potentially higher quality.

Single larvae ($L_2$) were placed in petri dishes (diam. 8.5 cm) and allowed to choose between two 5 cm long leaf parts of the secondary leaf, one from plants grown for 5 days in control solution without additional heavy metal, the other from a plant grown in either 50 µM cadmium or 80 µM copper treated solution.

After 24 hours it was noted at which of the leaves the larva had fed.

### 4.2.5. Plant-mediated heavy metal effects on VOC emission

The effect of heavy metal stress on the emission of herbivore-induced volatiles was investigated in a modified (after Turlings et al., 2004) push-and-pull system with a flow rate of 1.2 l $min^{-1}$ for incoming air and 0.6 l $min^{-1}$ for air going out to volatile traps. Three-day-exposed maize plants were individually transferred to plastic tubes (height 11.2 cm, diameter 4 cm) containing 110 ml of the respective hydroponic solution. The tubes were placed into the cup of an odor source vessel of the olfactometer and covered with PET-foil (Toppits, Minden, Germany) to prevent larvae from falling into the solution and to hold the plant in an upright position. The top opening of the vessel was plugged with cotton to allow the excessive air to escape. 15 *S. frugiperda* larvae ($L_2$) were placed on each plant and allowed to feed for three days. Volatile collections were carried out from 9:00 AM till 12:00 AM and from 12:00 A.M till 3:00 P.M. at all three days. Four fluorescent lamps (PAR inside odor source vessels: 70 µmol photons $m^{-2}$ $s^{-1}$ at 30 cm distance from lamps) illuminated the plants from 7:00 A.M. till 9:00 P.M.

Volatiles emanating from maize plants were collected with SuperQ traps as described previously (Rostás and Eggert, 2008). After each experimental day the glass parts of the olfactometer were

cleaned with deionized water and rinsed with ethanol (v/v 70%), acetone and hexane. After evaporation of the solvents, the glass parts were placed in an oven at 200°C for 1 h.
The trapped volatiles were eluted with 150 µl methylene chloride, two internal standards (*n*-octane and nonyl acetate, Sigma-Aldrich, Taufkirchen, Germany, 200 ng each in 10 µl methylene chloride) were added and the samples were stored at -80°C. The qualitative and quantitative volatile composition of each sample was analyzed on an Agilent Technologies 6890N Network GC System coupled with a 5973 Network Mass Selective Detector. Three µl of each sample were injected with an automated injection system in pulsed splitless mode. The column was an Agilent 19091-s933 HP-1 capillary column (length 30 m, diameter 0.25 mm, film thickness 0.25 µm). The oven was held at 35°C for 3 min and then increased with 8°C $min^{-1}$ to a final temperature of 230°C which was held for 10 min. Helium (1.5 ml $min^{-1}$) was used as carrier gas. Compounds were identified using MSD ChemStation (Agilent Technologies) software with the Wiley 275 mass spectrum library and by using the software MassFinder3/Terpenoids library (Hochmuth Scientific Software, Hamburg, Germany). Identities were further confirmed by co-injection of authentic standards (Sigma-Aldrich, Taufkirchen, Germany). Quantification was obtained by comparing the area of the compounds to the area of the internal standards.

4.2.6. Parasitoid behavior

The effect of heavy metal stress on the emission of herbivore-induced volatiles and consequently on the behavior of the parasitoid was investigated in a six-arm-olfactometer (for details see Turlings et al., 2004). Plants were prepared as described in 4.2.4. *Plant-mediated heavy metal effects on VOC emission*. On the third day of larval feeding, the odor source vessels were connected with the central choice chamber via Teflon tubes to allow simultaneous behavioral assays and volatile collection from 9:00 AM till 12:00 AM. Ten fluorescent lamps (PAR inside odor source vessels: 130 µmol photons $m^{-2}$ $s^{-1}$ at 30 cm distance from lamps) were switched on 3 h before testing the wasps. Odor source vessels containing a plant that had received either CuH or control treatment were placed *vis-à-vis* in the olfactometer. The other four vessels remained empty as controls. After connecting the vessels to the air delivery and the olfactometer, the air stream was allowed to stabilize for 10 min. The flow rate was 1.2 l $min^{-1}$ for incoming air and 0.6 l $min^{-1}$ for air going out to the behavioral arena or the volatile traps, respectively.
Mated 3-5-day-old females of *C. marginiventris* were used in the behavioral assays.
Wasps had no oviposition experience prior to the experiment. All wasps were tested in groups of six as they do not interfere with each other's choices (Turlings et al., 2004). After 30 min the

choices made by the parasitoids were recorded and the group was replaced by a new one. Five groups of wasps were tested on the same day.

One day with five releases was considered as one replicate. Six to eight replicate days were carried out with a new pair of plants and new wasps each day. Volatiles emanating from maize plants were collected with SuperQ traps as described previously (Rostás and Eggert, 2008). After each experimental day the glass and Teflon parts of the olfactometer were cleaned with deionized water and rinsed with ethanol (v/v 70%), acetone and hexane. After evaporation of the solvents, the glass parts were placed in an oven at 200°C for 1 h.

#### 4.2.7. Statistical analyses

Plant leaf water content, root dry weight and metal concentration in the shoots were compared with an ANOVA. Kinetics of the induced total volatile emission was analyzed with a Repeated Measures ANOVA. Plant growth, $F_v/F_m$, root-to-shoot ratio of the metals, kinetics of JA and the single components of the induced VOC were analyzed with a Kruskal-Wallis-Test. Growth of the herbivore was analyzed with a Student's-*t*-test and the food-choice of the larvae was analyzed with a sign-test.

The analyses were conducted using STATISTICA 7.1 (StatSoft, Tulsa, USA). In case of the volatiles, only compounds occurring in at least 3 of the 6 samples were statistically analyzed.

For the six-arm-olfactometer the entity computing a repetition in the statistical analysis corresponds to the response of a group of 6 wasps released, which was shown to follow a multinomial distribution (Ricard and Davison, 2007). As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasi-likelihood functions to compensate for the overdispersion of parasitoids within the olfactometer (Turlings et al., 2004). The model was fitted by maximum quasi-likelihood estimation in the software package R (http://www.R-project.org), and its adequacy was assessed through likelihood ratio statistics and examination of residuals (Turlings et al., 2004).

If necessary, data were transformed to achieve test assumptions.

### 4.3. Results

#### 4.3.1. Plant physiology and morphology

The concentration of copper and cadmium were highest in roots of plants treated with high concentrations of the respective metal (Tab. 1, 2). Iron concentration was lowered significantly

only in the shoots of CdH treated plants. Zinc concentration was significantly reduced in the shoots of CuH and in the roots of all heavy metal treated plants with the lowest concentration found in the CuH group compared to control plants. Magnesium concentration was significantly decreased in the shoots of CuH treated plants compared to the control. In the roots magnesium concentration was significantly decreased in CuH and CdH treated plants and significantly increased in CuL treated plants. The root-to-shoot ratio of the metals followed the same trend as the absolute concentrations (Tab. 1, 2).

**Table 1**: Concentration (mg $kg_{DW}^{-1}$) of metals in the organs of maize plants after five days of exposure to standard (control) and different increased copper (CuL, CuH) and cadmium (CdL, CdH) concentrations.

| Metal | control | | CuL | | CuH | | CdL | | CdH | | Statistics |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Shoot | root | shoot | root | shoot | root | shoot | root | shoot | root | |
| Copper | $17.60\pm4.93^{A}$ | $118\pm11.76^{a}$ | $22.80\pm1.14^{AB}$ | $804\pm43.89^{b}$ | $33.54\pm6.13^{B}$ | $2593\pm266^{c}$ | $10.62\pm0.55^{AC}$ | $121\pm3.95^{a}$ | $7.30\pm0.89^{C}$ | $91.96\pm7.24^{a}$ | root: F=10.87, shoot: F=18.35 |
| Cadmium | $0.01\pm0.01^{A}$ | $0.10\pm0.01^{a}$ | $0.14\pm0.06^{A}$ | $0.76\pm0.25^{b}$ | $0.42\pm0.23^{A}$ | $1.20\pm0.31^{b}$ | $101\pm4.28^{B}$ | $908\pm87.04^{c}$ | $189\pm6.45^{C}$ | $1899\pm67.15^{c}$ | root log F=2.71, shoot F=9.53 |
| Iron | $80.28\pm11.92^{A}$ | $1038\pm22.40$ | $54.02\pm2.74^{A}$ | $1758\pm279$ | $55.13\pm11.82^{A}$ | $912\pm127$ | $58.19\pm4.75^{A}$ | $1620\pm351$ | $44.55\pm3.67^{B}$ | $1689\pm261$ | root: F=2.62, shoot: F=2.56 |
| Zinc | $66.44\pm3.27^{AB}$ | $97.94\pm4.11^{b}$ | $80.46\pm4.50^{B}$ | $67.30\pm5.32^{a}$ | $26.36\pm1.61^{C}$ | $42.31\pm3.00^{c}$ | $63.92\pm2.99^{A}$ | $63.65\pm2.06^{a}$ | $62.47\pm4.99^{A}$ | $69.97\pm3.36^{a}$ | root: F=28.35, shoot: F=29.95 |
| Magnesium | $2364\pm96^{A}$ | $5127\pm163^{a}$ | $2654\pm46^{AB}$ | $6034\pm98^{c}$ | $2303\pm82^{B}$ | $3355\pm200^{b}$ | $2721\pm94^{AC}$ | $5036\pm138^{a}$ | $2691\pm108^{AC}$ | $3491\pm318^{b}$ | root: F=35.55, shoot: F=5.02 |

Values are mean ± S.E. "Shoot" is the mean of leaf and shoot concentration. Statistical values are an ANOVA, n=5. Different capital letters indicate statistically significant differences with $p\leq0.05$ within a row for the shoot concentrations, different lower case letters for the root concentrations.

**Table 2**: Root-to-shoot-ratio of the analyzed metals in maize plants exposed for five days to standard (control) and different increased copper (CuL, CuH) and cadmium (CdL, CdH) concentrations.

| Metal | Control | CuL | CuH | CdL | CdH | Statistics |
|---|---|---|---|---|---|---|
| Copper | $7.48\pm1.28^{a}$ | $34.05\pm2.05^{bc}$ | $80.16\pm9.57^{b}$ | $10.52\pm0.46^{ac}$ | $11.33\pm0.39^{abc}$ | H=21.15 |
| Cadmium | $1.09\pm0.02^{a}$ | $1.53\pm0.17^{ab}$ | $1.57\pm0.09^{ab}$ | $8.89\pm0.50^{b}$ | $10.02\pm0.16^{b}$ | H=21.28 |
| Iron | $16.88\pm3.65$ | $32.37\pm5.12$ | $19.11\pm3.94$ | $27.20\pm4.50$ | $37.01\pm4.37$ | H=10.66 |
| Zinc | $1.48\pm0.09^{a}$ | $0.84\pm0.05^{bc}$ | $1.59\pm0.08^{ac}$ | $1.00\pm0.05^{ac}$ | $1.14\pm0.10^{ac}$ | H=18.10 |
| Magnesium | $2.09\pm0.14^{abc}$ | $2.28\pm0.04^{a}$ | $1.46\pm0.06^{b}$ | $1.85\pm0.02^{a}$ | $1.31\pm0.15^{c}$ | H=19.87 |

Values are mean ± S.E. Statistical values are a Kruskal-Wallis-test, n=5. Different letters indicate statistically significant differences with $p\leq0.05$ within a row.

The aerial parts of CuH and CdH exposed plants grow significantly less than control plants. Growth of CuL and CdL plants were not affected significantly. CuH showed the least cumulative accrescense after 5 days of heavy metal exposure (Tab. 3). Leaf water content did not differ significantly between the tested treatments. Root dry weight was lowest in CuH treated plants. Heavy metal treatment also leads to a decrease in photosynthesis as indicated by the decrease in maximum photochemical efficiency with the lowest levels found in CdH and CuH treated plants (Tab. 3).

**Table 3**: Morphological and physiologic parameters of maize plants exposed to standard (control) and different increased concentrations of copper (CuL, CuH) and cadmium (CdL, CdH) for 7 days in hydroponic culture.

| Parameter | control | CuL | CuH | CdL | CdH | Statistics |
|---|---|---|---|---|---|---|
| Cumulative accrescense (cm) | 23.92±0.37$^{a}$ | 23.30±0.54$^{a}$ | 15.88±0.78$^{b}$ | 21.35±0.49$^{ac}$ | 19.04±0.43$^{bc}$ | $n$=19-20; H=61.68 |
| Leaf water content (%) | 91.02±0.58 | n.d. | 89.03±0.66 | n.d. | 89.02±2.66 | $n$=10/4/4, F=1.55, p>0.05 |
| Maximum photochemical efficiency of PS II ($F_v/F_m$) | 0.77±0.00$^{a}$ | 0.75±0.00$^{ab}$ | 0.71±0.01$^{b}$ | 0.75±0.00$^{a}$ | 0.69±0.01$^{b}$ | $n$=10, H=37.15 |
| Root dry weight (g) | 44.41±2.97$^{a}$ | 51.70±4.03$^{a}$ | 17.44±1.52$^{b}$ | 45.31±3.87$^{a}$ | 42.80±3.15$^{a}$ | $n$=20; F=21.33, |

Values are mean ± S.E. $F_v/F_m$ was measured at the secondary leaf. Statistical values are indicated for a Kruskal-Wallis-test (Cumulative accrescense, $F_v/F_m$) and an ANOVA (Leaf water content, Root dry weight). Different letters indicate statistically significant differences with $p \leq 0.05$ within a row.

During 24 hours after application of the herbivores the concentration of JA increased in all heavy metal treated plants. 3 hours post induction (hpi), CuH treated plants had a significantly higher JA concentration compared to control plants not supplied with heavy metals (Kruskal-Wallis-test, $n$=7-9, H=14.09, $p<0.01$). 6 hpi the CuH treated plants showed a significantly increased JA concentration compared to all other heavy metal treatments (Kruskal-Wallis-test, $n$=7-9, H=14.85, $p<0.01$) but not compared to control plants. 24 hpi no significant differences in the JA concentrations could be detected (Kruskal-Wallis-test, $n$=7-9, H=3.22, $p<0.5$, Fig. 1). At the start of the herbivore treatment and in plants not damaged by herbivores no JA could be found.

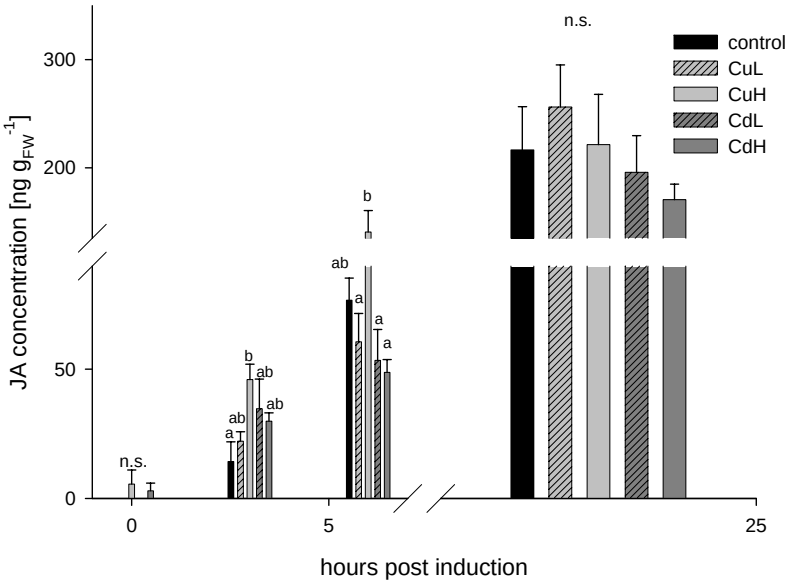


**Figure 1**: Kinetics of the induced jasmonic acid (JA) in maize plants exposed to standard (control) and different increased concentrations of copper (CuL, CuH) or cadmium (CdL, CdH) for three days at the start of induction. Build-up of JA was induced by feeding of six *Spodoptera frugiperda* larvae per plant at the oldest leaf for three, six or 24 hours respectively.
Mean values + S.E. of JA concentration (ng $g_{FW}^{-1}$) are depicted. Sample time point 0 equates "start of induction" in Fig. 2. Different letters indicate statistically significant differences with $p \leq 0.05$ within a sample point (Kruskal-Wallis-test, $n$=7, H=3.02).

In herbivore damaged maize plants we identified 17 different compounds. In plants not damaged by the herbivore we could detect almost no emission at all. Plants treated with high concentrations of heavy metals showed highest total emissions. At the start of induction (day 1) the control plants without heavy metal treatment showed the highest total emission (Repeated measures ANOVA, Fig. 2), 48 hours later (sample point day 3 morning) CuH treated plants showed a total emission significantly higher than the emission from the control plants (Repeated measures ANOVA, Fig. 2). At the last sample point, no significant differences in the total emissions were found (Repeated measures ANOVA, Fig. 2).

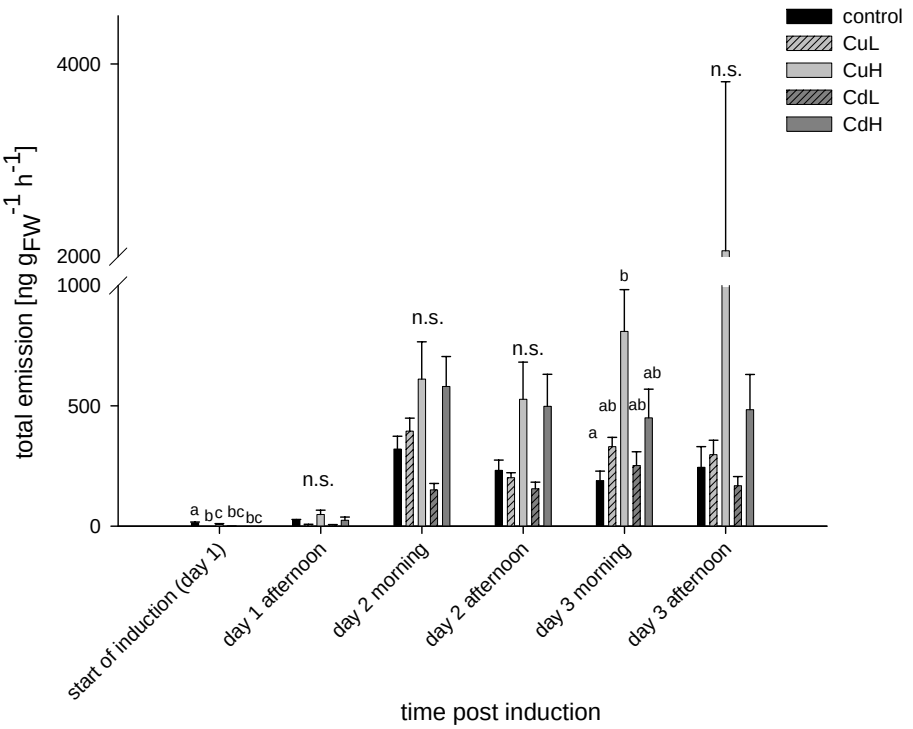


**Figure 2**: Kinetics of the *S. frugiperda* induced overall emission in maize plants exposed to standard (control) and different increased concentrations of copper (CuL, CuH) or cadmium (CdL, CdH) for three days at the start of induction.
Bar represent mean values + S.E. of emission per gram fresh weight and hour (ng $g_{FW}^{-1}$ $h^{-1}$). Different letters indicate statistically significant differences with $p \leq 0.05$ within a sample point (Repeated measures ANOVA, $n$=6, F=4.52).

At sample point "day 3 morning" we analyzed single compounds for their differences between the treatments. In the case of (*E*)-2-Hexenal, (*Z*)-3-Hexenol and (*Z*)-3-Hexenylacetat and of the terpenes Linalool, (*E*)-α-Bergamotene, (*E*)-β-Farnesene and *β*-Sesquiphellandrene the CuH treated plants showed increased emission compared to control plants (Fig. 3). Germacrene D was not found in CuH treated plants, Sesquiphellandrene was absent in control plants, Nerolidol was absent on CuL treated plants and *(E,E)*-4,8,12-Trimethyl-1,3,7,11-tridecatetraen (TMTT) was absent in CdL treated plants (Fig. 3).

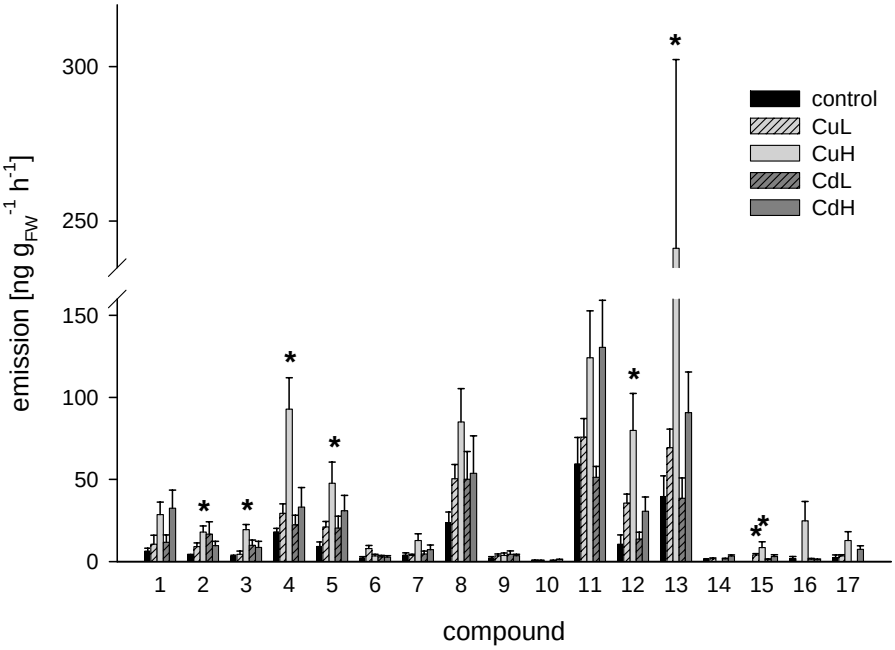


**Figure 3**: VOC in the headspace of herbivore-damaged maize plants. Plants were exposed to standard (control) and different increased concentrations of copper (CuL, CuH) or cadmium (CdL, CdH) for five days (equals "day 3 morning" in Fig. 2). Compounds were identified according to their retention times, library mass spectra and by coelution with commercial standards and are arranged by retention times in the figure.

1) (*Z*)-3-Hexenal 2) (*E*)-2-Hexenal 3) (*Z*)-3-Hexenol 4) (*Z*)-3-Hexenylacetate 5) Linalool 6) (*E*)-4,8-Dimethyl-1,3,7-nonatrien 7) Phenylethylester 8) Indole 9) Methylanthranilate 10) *α*-Copaene 11) (*E*)-Caryophyllene 12) (*E*)-α-Bergamotene 13) (*E*)-β-Farnesene 14) Germacrene D 15) *β*-Sesquiphellandrene 16) Nerolidole 17) *(E,E)*-4,8,12-Trimethyl-1,3,7,11-tridecatetraen (TMTT)

Bars are mean + S.E. of emission per gram fresh weight and hour (ng $g_{FW}^{-1}$ $h^{-1}$).

Asterisks indicate statistically significantly different (Kruskal-Wallis-test, n=6, *p<0.05) from emission of control plants.

4.3.2. Growth and feeding behavior of the herbivore

In the growth test the *S. frugiperda* larvae fed with CuH (Student's *t*-test, $t$=4.40, p<0.001, $n$=22, 23) and CdH (Student's *t*-test, $t$=8.39, p<0.001, $n$=21, 23) treated plants grow significantly slower than larvae fed with control plants. Between the larvae fed with heavy metal treated plants was no difference in growth (Student's *t*-test, $t$=0.49, p>0.5, $n$=23).

In the choice assay the larvae did not prefer one of the treatments significantly (CdH vs. control: sign test, Z=1.54, p>0.1, $n_{control}$=9, $n_{CdH}$=18; CuH vs. control: sign test, Z=1.49, p>0.1, $n_{control}$=10, $n_{CuH}$=19)

4.3.3. Parasitoid behavior

Mated naïve female *C. marginiventris* were highly attracted to induced blends of control and CuH treated maize plants compared to clean air. Despite the quantitative and qualitative differences in the blend between the tested plant treatments, wasps were equally attracted by both blends (log-linear model, *n*=9, p>0.05, Fig. 4).

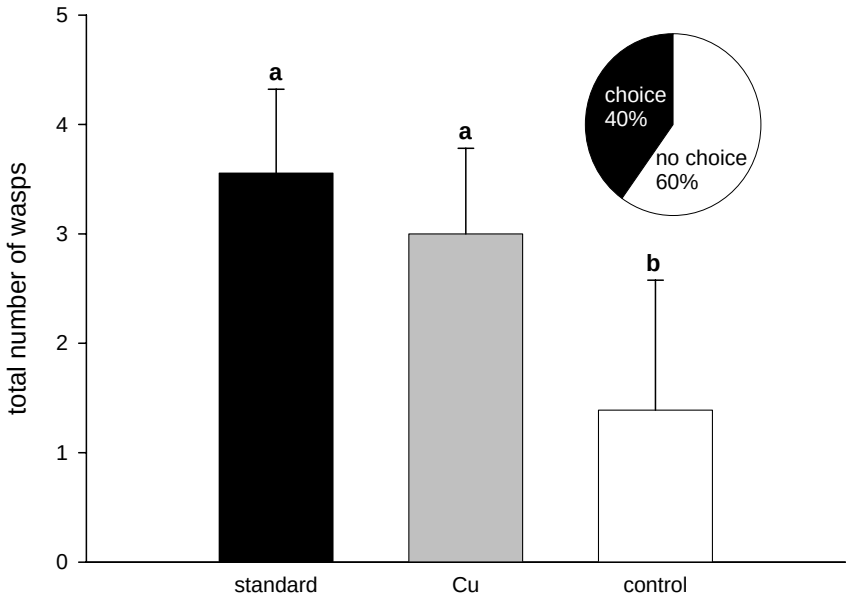


**Figure 4**: Response of naïve mated *Cotesia marginiventris* females to induced volatiles of hydroponically cultured maize plants treated with additionally 80 µM copper (Cu) or with standard solution (normal). Control=clean air. Bars represent mean numbers (+SE) of wasps making a choice in the six-arm olfactometer. Total number of wasps choosing the four controls was divided by four. Insert shows mean percentage of wasps making a choice. Different letters indicate statistically significant differences. Log-linear model fitted to quasipoisson distribution; *n*=9 independent experiments with 30 wasps each.

## 4.4. Discussion

Hydroponically cultured maize plants of the cultivar Lambada exposed to excess heavy metals allocated the two tested metals differently between shoot and root. The concentration of Copper was 34-fold (CuL treatment) to 80-fold (CuH treatment) higher in the roots than in the shoots in the Copper-treated plants (Tab. 2). In the Cadmium-treated plants the concentrations of Cadmium was 9-fold (CdL treatment) to 10-fold (CdH treatment) higher in the roots than in the shoots (Tab. 2). So maize of the cultivar Lambada seems to be a 'shoot-excluder' of Cu and Cd as defined in Florijn and Van Beusichem (1993). In the case of Cd, cross linking of Cd to carboxyl groups of the cell wall or interaction with thiol groups of the soluble proteins is proposed as a excluding mechanism (Lozano-Rodríguez et al., 1997). Cu may also be bound to the cell walls of the root cells to exclude excess ions from the plant's shoot (Orcutt and Nilsen, 2000; Woolhouse, 1983). In a range of plants, the root is the organ protecting the plant from an excess of heavy metal ions (Mazhoudi et al., 1997).

Despite the above described mechanisms of exclusion, considerable amounts of Cu and Cd reached the shoot of the plants (Tab. 1, 2). These increased heavy metal concentrations led to decreased growth of shoot and root (Tab. 3) of hydroponically cultured maize plants. This corroborated the results of Tanyolac et al. (2007) for two other maize cultivars and the results reviewed in Kahle (1993) for different tree species. The reduction in root growth was explained by decrease in cell elongation and cell division (Kahle, 1993), the reduction in shoot growth by direct toxic effects of the heavy metal or indirect effects like limitation of mineral and water acquisition (Nedjimi and Daoud, 2009). So the slightly decreased water content (Tab. 3) and the to some extend reduced amounts of mineral ions in the heavy metal treated plants (Tab. 1) in our study may be an explanation for the reduced growth of the aerial parts of the maize plants.

Iron was significantly reduced in the shoots of CdH treated plants, Zinc was significantly reduced in the shoots of CuH treated plants and Magnesium was significantly reduced in the shoots of CuH and CdH treated maize plants (Tab. 1). This negative interaction between heavy metals and uptake of essential nutrients is already described in a lot of studies (reviewed in Orcutt and Nilsen, 2000; Pál et al., 2006) and can be explained by competing uptake mechanisms (Pál et al., 2006).

To asses the effects of excess heavy metals on the physiology of maize plants we measured maximum photochemical efficiency of Photosystem II ($F_v/F_m$).

Heavy metals are known to affect plant photosynthesis (reviewed in Krupa and Baszynski, 1995; Sharma and Agrawal, 2005). In our study maize plants treated with high concentrations of heavy

metals showed the lowest $F_v/F_m$-values (Tab. 3) at the secondary leaf independent of the tested metal. Excess heavy metals can damage the proteins of the oxygen evolving complex and thus lead to a reduced quantum yield of PS II (reviewed for example in Di Toppi and Gabbrielli, 1999). This reduced photosynthetic efficiency and the consequential reduced gain of biomass and energy may be another reason for the above described altered plant growth.

The altered plant quality of maize treated with high concentrations of Cu or Cd had a significant negative effect on the growth of *S. frugiperda* larvae fed with the respective leaves. As described above Cd was transported to the leaves in considerable amounts (Tab. 1) and thus may have had a direct toxic effect on the herbivore as described for *Plutella xylostella* (Coleman et al., 2005). Cu was not transported to the leaves that much (Tab. 1, 2) hence the metal may have affected the *S. frugiperda* larvae indirectly by altering food plant quality as described by Görür (2007) for aphids.

To evaluate the effects of excess heavy metals on the induced defense of maize we analyzed the kinetics of JA and subsequently the herbivore induced volatiles (hiVOC). The mean basic level of JA (0 hour post induction h.p.i.) was almost not detectable (Fig. 1). During the next 24 hours the amount of herbivore induced JA increased in all treatments including the control without extra heavy metals (Fig. 1). In the Cu treatment the metal had a transient priming effect on JA as suggested by Maksymiec (2007) at the time point 3 h.p.i and 6 h.p.i., indicated by the increased JA concentrations of the CuH treated plants at these times (Fig. 1). At this time points the preexposure to the abiotic stress of excess copper leads to a stronger activation of the JA signaling cascade which is one definition of priming (Bruce et al., 2007). After 24 hours the concentration of JA was still increasing compared to the previous sampling points but not compared between the treatments (Fig. 1).

In Cd treated plants the JA concentration was affected only by herbivory as indicated by the increase of JA during 24 hours but no significant differences between the treatment and the control plants (Fig. 1). Schmelz et al. (2003a) described a similar kinetic of induced JA with increased levels after 4-6 h and a maximal JA concentration 8-13 h after start of herbivore feeding for maize without heavy metal treatment. In the case of the Cu treatment, the signaling cascade postulated by Mithöfer et al. (2004) seemed to be partly corroborated by our results. The excess of Cu leads to a production of reactive oxygen species (ROS, results not shown) and in the following to an increase of oxylipins, in our case for instance JA. In plants without herbivore feeding, no JA could be detected, so the metal alone had no inducing effect on JA production in our system, which is contrary to the postulation of Mithöfer et al. (2004). Thus we propose a

priming effect of the excess Cu on the signaling cascade with herbivory as the final activating stress.

Mithöfer et al. (2004) proposed an overlapping signaling pathway of the abiotic stress 'heavy metal' and the biotic stress 'herbivory' mediated by oxylipins. Herbivory can lead to the emission of a blend of VOC by the infested plant, e.g. in maize (Turlings and Tumlinson, 1992). Thus we tested the effects of excess Cu and Cd on the hiVOC of maize. We found a significant higher total emission caused by an increase of green leaf volatiles (GLV) on 'start of induction (day 1)' in the control plants (Fig. 2) compared to the other treatments. GLV are the product of the oxidative cleavage of plant cell membrane fatty acids and released immediately after wounding of the plant tissue (Fall et al., 1999; Turlings et al., 1998). In our case the herbivore may have avoided feeding at the metal treated plants initially thus creating the observed VOC pattern though the larvae did not show a preference in the choice test. Deterrence by plants containing high amounts of specific metals potentially leading to a delayed feeding was described earlier for *Pieris rapae* (Martens and Boyd, 1994; Pollard and Baker, 1997). At the sampling points 'day 1 afternoon' to 'day 3 afternoon' CuH treated plants tended to show a higher total emission than control plants with a significant difference at sampling point 'day 3 morning' (Fig. 2). This significant increase of the total emission was caused by an increase of the GLV (*E*)-2-Hexenal, (*Z*)-3-Hexenol and (*Z*)-3-Hexenylacetat, of the terpenes Linalool, (*E*)-α-Bergamotene, (*E*)-β-Farnesene and *β*-Sesquiphellandrene, thus the exposure to higher amounts of Cu did not only change the quantity but also the quality of the hiVOC as some of the compound were not found in all treatments (Fig. 3).

In our study the excess of the essential heavy metal copper leads to a transient increase of herbivore induced JA compared to control plants without the excess metal. This increased JA concentration in turn caused a higher emission of hiVOC of the maize plants in the heavy metal treated plants. Thus we propose a priming effect of the abiotic stress 'copper' on the biotic stress 'herbivory' in the sense of priming as defined in Bruce et al. (2007). To our knowledge this is the first study showing an priming effect of an preceding abiotic stress on hiVOC.

The altered VOC blend may change the ability of the plant to defend themselves indirectly (Vuorinen et al., 2004b) hence we tested the behavior of naïve mated *C. marginiventris* females to the blend of CuH treated plants in comparison with untreated plants in the six-arm-olfactometer. The behavioral experiments were carried out with plants exposed for 3 days to CuH or control treatment because we found the largest divergence in total emission (Fig. 2) and emission of single compounds (Fig. 3) for this combination. Despite the remarkably altered blend, the parasitoids did not prefer one of the treatments (Fig. 4). Analyses of VOC emissions

from plants treated with CuL, CdL or CdH did not suggest a different behavior of the parasitoids to these plants.

# 5. Conclusions

In this study the stability of a tritrophic system consisting of soybean or maize, the herbivorous larvae of *Spodoptera frugiperda* and the parasitoid wasp *Cotesia marginiventris* under different abiotic stresses was investigated. Main focus were the effects of the abiotic stress on the induced indirect defense of the plants.

Each type of abiotic stresses the plants were subjected to had considerable effects on the plants' morphology and physiology in this study.

In chapter 2 soybeans exposed to ambient UV radiation showed reduced growth, compared to plants receiving ambient radiation that lacked the UV part of the spectrum. This was likely due to reduced cell wall extensibility. The plants' seed production was not affected. UV exposed plants also increased the levels of UV–protecting compounds such as flavonoids and other phenolics in their leaves, which act as free radical scavengers. These are effective measures to prevent the damage of macromolecules by the energy-rich radiation.

In the case of nitrogen (N) deficiency, affected soybean plants increased their investment in root biomass, thus enabling a more efficient uptake of the lacking nutrient (chapter 3). Despite these efforts to compensate for N deficiency, leaf N as well as soluble leaf protein content was reduced and photosynthesis in the affected plants was impaired in N deficient plants.

Strong effects on growth and functions were also apparent in plants stressed by heavy metals. Excess copper (Cu) and cadmium (Cd) was retained in the roots of exposed maize plants but considerable amounts of Cd reached the shoot. Exposure to both heavy metals led to reduced growth of roots, presumably as a result of altered cell division and elongation. Reduced shoot growth may have been caused by direct toxic and/or indirect effects in the case of Cd, while only indirect effects were held responsible for the effect in the case of Cu (chapter 4). Heavy metals also competed for uptake with other metals like iron, zinc and magnesium as reduced concentrations of these micronutrients were found in heavy metal exposed plants. This "secondary nutrient deficiency" and the direct toxicity of the heavy metals may have led to the reduced photochemical efficiency of Photosystem II in the affected plants which in turn may also have contributed to the reduction in shoot growth.

The aforementioned abiotic factors caused significant alterations in the physiology and morphology of the studied plants. Therefore, the nutritional value of the plants for herbivores may have changed, as well. Depending on the abiotic stress agent in question, variable effects on the larvae of *S. frugiperda* were observed.

The shift in potentially herbivore-toxic leaf flavonoid composition, which was induced by UV radiation, had no effect on the performance of *S. frugiperda* larvae. Due to its generalistic lifestyle this herbivore species seems to be able to cope with the UV induced change in soybean leaf secondary chemistry (chapter 2).

In contrast, N limited plants were very poor hosts, as they negatively affected growth as well as pupal and adult weights of *S. frugiperda*, indicating an insufficient nutrition of the larvae feeding on these plants. This nutritional deficiency was corroborated by a reduced N content of the larvae themselves. Surprisingly, the larvae seemed to be unable to counterbalance N deficiency by altering their feeding behavior. When given the choice between leaves from N deficient and healthy soybeans, caterpillars did not prefer one over the other, nor did they compensate for lower food quality by feeding more leaf material of the deficient leaves in a no-choice bioassay. The herbivore larvae used as hosts by the parasitoid *C. marginiventris* transmitted the plant's N deficiency to the third trophic level as parasitoids reared inside herbivores that had been fed with N deficient soybean leaves, had significantly lower pupal weights.

Heavy metals also had a strong detrimental impact on the growth of herbivore larvae, suggesting either direct toxic effects in the case of Cd or indirect plant-mediated negative effects in the case of Cu. In this study the larvae did not refuse the heavy metal treated leaves in a choice assay as expected, again implying that caterpillars of S. frugiperda were limited in their ability to discriminate between stressed and healthy leaves.

Concerning the induced indirect defense (IID) of the tested plants as the main focus of this study, the effects on the plant's side depended on the specific stressor. Under all stressed or unstressed conditions, herbivory always induced the emission of volatile organic compounds (hiVOC), whereas almost no emission could be detected in undamaged plants.

In chapter 2 I found no significant difference between the hiVOC of plants receiving full ambient solar radiation and those receiving UV attenuated ambient solar radiation even after 30 days of exposure. Hence, the shift in photo-protective compounds seemed to be sufficient to prevent the plants from UV effects on metabolic or signaling pathways linked to hiVOC synthesis.

In soybean, deficient in the macronutrient N, the effects on hiVOC induction were more pronounced (chapter 3) than in UV manipulated plants. N deficient plants emitted qualitatively the same blend as fertilized plants but three of the detected compounds were affected significantly in their quantities. The two sesquiterpenes *β*-Bergamotene and (*E,E*)-α-Farnesene are emitted in higher amounts and (*Z*)-3-Hexenyl-α-methylbutyrate in lower amount. This could be explained by possible negative correlations of jasmonic acid (JA), a phytohormone important

in the signaling cascade of IID, and N availability. Another possible reason for the elevated emission of some compounds could be the increased availability of non-structural carbohydrates used for the production of hiVOC in N deficient plants.

In conjunction with the effects of heavy metal stress the kinetics of JA in maize was analyzed to get a more detailed view on the IID. Plants exposed to excess heavy metal concentrations but not damaged by the herbivore did not accumulate JA in detectable amounts, suggesting no hiVOC inducing effect of the heavy metals alone in our experimental setup. If herbivory occurred, excess Cu had a priming effect on JA shown by increased JA concentrations three and six hours post herbivore attack compared to the JA levels at these time points in plants exposed to herbivory only. Pre-exposure to an abiotic stress, in this case excess Cu, enhanced the plants' reaction to the second stress factor (herbivory), potentially by the production of reactive oxygen species. The resulting increase in JA levels correlated with an increased emission of hiVOC after three days of herbivory. Three $C_6$-based compounds and four terpenes were emitted in significantly higher amounts. Hence, high levels (80 µM) of Cu had primed the plant and enhanced the volatile response of maize to herbivory.

Excess Cd seemed to have no priming effect because plants exposed to Cd did not significantly differ from control plants in their kinetics of induced JA and hiVOC after herbivore attack. Thus, in this case herbivory alone had affected the production of hiVOC.

Parasitoids are known to respond to hiVOC in a dose-dependent manner, so a behavioral modification in case of an altered VOC blend was hypothesized. However, differences in the emission of hiVOC in soybean and maize due to abiotic stress factors did not lead to changes in the host seeking behavior displayed by the parasitoid *C. marginiventris*. The female parasitoids were well capable of distinguishing herbivore-damaged from undamaged plants but even considerable modifications of the herbivore induced blend due to N deficiency or excess Cu did not attenuate or strengthen the wasps' attraction to these blends in our six-arm-olfactometer setup. Several reasons for this outcome are conceivable. The affected compounds of the hiVOC blend are maybe not the relevant key compounds or the compounds that were emitted in elevated amounts mask these yet unknown key compounds.

These results also show the importance of behavioral experiments in IID studies. From the analysis of potentially behavior inducing cues alone it is not always possible to draw the correct conclusions concerning the behavior of the third trophic level and thus this level has to be included in the study.

From the parasitoids' point of view, none of the tested abiotic stress factors affected the plants in a way that impaired the wasps' ability to find host-infected plants. However, adverse effects on parasitoid fitness may be possible, if herbivores on stressed plants are parasitized as shown in the case of N limitation.

Even if the plant is subject to abiotic stresses that can affect the plants' morphology and physiology to a great extend, according to my results, the induced indirect defense of soybean and maize remains effective and stable, i.e. exposition to an abiotic stress did not enhance the susceptibility to a second biotic stress. This can also be beneficial for biocontrol of herbivore populations as they are regulated by parasitoids even under conditions that are suboptimal for plants.

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# 7. Summary

Plants exposed to herbivory may defend themselves by attracting the "enemies of their enemies", a phenomenon called induced indirect defense (IID). In this process, the *de novo* production and emission of volatile organic compounds (VOC) by the affected plant is activated via a jasmonic acid (JA) dependent signaling cascade. VOC can be very specific for the inducing herbivore as well as for the emitting plant. Carnivores as predatory mites and parasitoid wasps use these substances as prey- or host-finding cues. If the herbivore is parasitized successfully, its development is slowed and thus the damage of the plant is decreased.

Additional abiotic stress may modulate the plant's ability to produce and/or emit herbivore induced VOC.

Ultraviolet (UV) radiation can have multiple physiological effects on plants, amongst others the activation of the expression of genes that are also activated during anti-herbivore defense. To investigate UV effects, foils with different UV transmittance were used to manipulate ambient solar radiation. One foil was permeable for the whole solar spectrum including UV radiation whereas the other excluded radiation below a wavelength of 400 nm. Soybean exposed to UV increased concentrations of isorhamnetin- and quercetin-based flavonoids as effective photo-protective compounds in the leaves and showed a reduced growth compared to plants exposed to ambient radiation lacking UV. The altered chemical composition of the leaves had no effect on food choice and performance of herbivorous *Spodoptera frugiperda* larvae. Photo-protection by flavonoids seems to be efficient to prevent further UV effects on IID as plants of both treatments emitted the same blend of induced VOC and hence females of the parasitoid *Cotesia marginiventris* did not prefer plants from on of the treatments in the olfactometer.

Nitrogen is one important macronutrient for all trophic levels and thus deficiency of this nutrient was expected to affect IID of soybean profoundly. To manipulate N availability for soybean plants hydroponic culture was used. One treatment was cultured in a standard hydroponic solution whereas in the N deficiency treatment in the solution all salts containing N were replaced with N-free salts. In N deficient plants root biomass was increased to allow the plant to forage more efficiently for the nutrient. Despite this morphological adaptation, photosynthetic efficiency as well as leaf N and soluble protein content were reduced significantly in N deficient soybean. The N deficiency was passed on to the third trophic level as herbivores fed with the affected leaves had a reduced body N content on her part and showed a decreased growth but no feeding preference for the superior food. Parasitoids reared in such N deficient herbivores had significant lower pupal weight compared to parasitoids reared in hosts fed with fully fertilized

soybean. N deficient plants emitted a quantitatively altered herbivore induced blend. The two terpenes *β*-Bergamotene and (*E,E*)-α-Farnesene were emitted in higher amounts whereas (*Z*)-3-Hexenyl-α-methylbutyrate was emitted in significantly lower amount. Despite this quantitatively modified VOC blend the parasitoids host-searching behavior was not affected.

Heavy metals (HM) are proposed to affect various biochemical pathways in plants including defense pathways by production of reactive oxygen species (ROS) in the tissue. The ROS on its part may affect production and release of endogenous JA, an important messenger in defense signaling. In this study maize plants were grown hydroponically and exposed to different increased concentrations of copper and cadmium. Maize seems to be able to exclude the excess HM from the leaves because the HM were found mainly in the roots and only to a minor degree in the shoots of the plants. Despite this exclusion the HM significantly affected uptake of other metal ions into the plant. The excess of the HM in combination with the attenuated uptake of other ions led to a reduced growth of roots and shoots as well as to reduced photosynthetic efficiency. Thus the nutritional value of the plants for the herbivore was lowered either by direct toxic effects of the HM or indirectly by altering plant chemical composition. *S. frugiperda* larvae fed with leaves exposed to high HM concentrations showed a significantly reduced growth but they did prefer neither control nor HM treated plants in a food-choice assay. Cu had a transient priming effect on JA as pre-exposure to a high excess of Cu led to higher amounts of herbivore induced JA compared to control plants exposed only to standard concentration of Cu. As anticipated the increased JA was followed by an increase in herbivore induced VOC in high-Cu treated plants caused by a increase of the green leaf volatiles (*E*)-3-Hexenal, (*Z*)-3-Hexenol and (*Z*)-3-Hexenylacetat and the terpenes Linalool, (*E*)-α-Bergamotene, (*E*)-β-Farnesene, and *β*-Sesquiphellandrene. Despite these profound changes in herbivore induced VOC the parasitoids host searching behavior was not affected.

As described, the abiotic stresses UV, N deficiency and excess HM affected the morphology and physiology of soybean and maize, the performance of the herbivore *S. frugiperda* and even the performance of the parasitoid *C. marginiventris*. However the host searching behavior of the parasitoid was not affected even if the herbivore induced VOC blend was altered. Thus parasitoids seem to be a very reliable defender for plants and IID a very robust way of herbivore defense.

# 8. Zusammenfassung

Pflanzen, die Herbivorendruck ausgesetzt sind, können sich verteidigen, indem sie die „Feinde ihrer Feinde“ anlocken. Dieses Phänomen wird induzierte indirekte Verteidigung (englisch: induced indirect defense IID) genannt. Dabei wird die *de novo* Produktion und Abgabe von flüchtigen organischen Verbindungen (englisch: volatile organic compounds VOC) durch die betroffenen Pflanze über eine jasmonsäure (JA) -abhängige Signalkaskade aktiviert. Die VOC können sehr spezifisch sowohl für den auslösenden Herbivor als auch für die abgebende Pflanze sein. Karnivoren wie Raubmilben oder parasitoide Wespen nutzen diese Substanzen zur Beute- oder Wirtsfindung. Wurde der Herbivor erfolgreich parasitiert wird seine Entwicklung verlangsamt und damit der Schaden an der Pflanze verringert.

Ist die Pflanze außer Herbivorie noch zusätzlichem abiotischen Stress ausgesetzt, kann dieser die Fähigkeit der Pflanze zur Produktion und/oder Abgabe der herbivor-induzierten Duftstoffe beeinflussen.

Ultraviolette Strahlung (UV) kann verschiedenste physiologische Auswirkungen auf Pflanzen haben, darunter auch die Aktivierung der Expression von Genen, welche auch bei der Herbivorenabwehr aktiviert werden. Um solche möglichen UV-Effekte zu untersuchen, wurden Folien mit unterschiedlicher Durchlässigkeit für UV genutzt, um die natürliche Sonnenstrahlung zu manipulieren. Eine der Folien war durchlässig für das gesamt Spektrum des Sonnelichtes inklusive der UV-Strahlung, während die andere Folie Strahlung unterhalb einer Wellenlänge von 400 nm ausschloss. Sojapflanzen, die UV ausgesetzt waren, erhöhten die Konzentration von isorhamnetin- und quercetin-basierten Flavonoiden mit besonders effektiven Lichtschutzeigenschaften in ihren Blätter und zeigten außerdem ein reduziertes Längewachstum im Vergleich zu Pflanzen, die natürlicher Strahlung ohne UV-Anteil ausgesetzt waren. Die veränderte Blattchemie hatte jedoch keinen Einfluss auf Futterwahl und Entwicklung von herbivoren *Spodoptera frugiperda* Larven. Die Lichtschutzeigenschaften der Flavonoide verhinderten auch weitergehende UV-Effekte auf die IID, da Pflanzen beider Behandlungen das gleiche Gemisch induzierter VOC abgaben und daher Weibchen des Parasitoiden *Cotesia marginiventris* im Olfaktometer keine Bevorzugung für Pflanzen einer der beiden Behandlungen zeigten.

Stickstoff (N) ist ein wichtiges Makronährelement für alle trophischen Ebenen, daher wurde vermutet, dass ein Mangel dieses Elementes die induzierte indirekte Verteidigung von Soja tiefgreifend beeinflussen könnte. Um die Verfügbarkeit von N für Sojapflanzen gezielt zu manipulieren wurde ein Hydrokultursystem verwendet. Eine der Behandlungen wurde in einer

Standard-Hydrokulturlösung kultiviert während bei der Stickstoffmangelbehandlung die stickstoffhaltigen Salze in der Lösung durch stickstofffreie Salze ersetzt wurden. In N-Mangel-Pflanzen war die Wurzelbiomasse vergrößert, um eine effizientere Aufnahme des Nährstoffes zu ermöglichen. Trotz dieser morphologischen Anpassung waren Photosyntheseleistung, Blattstickstoffgehalt und Menge an löslichem Protein in N-mangel Soja signifikant verringert. Dieser Stickstoffmangel wurde bis zur dritten trophischen Ebene weitergegeben, da mit Stickstoffmangel-Blättern gefütterte Herbivore ihrerseits einen reduzierten Körperstickstoffgehalt und verringertes Wachstum zeigten, in Wahlexperimenten jedoch nicht höherwertiges Futter bevorzugten. Parasitoiden, welche in solchen N-magel Wirten gezüchtet wurden, erreichten ein geringeres Puppengwicht als Parasitoide, welche in Wirten gezüchtet wurden, die mit normal gedüngtem Soja gefüttert wurden. Pflanzen unter Sticktoffmangel emittierten einen qualitativ veränderten herbivor-induzierten Duft. Die beiden Terpene *β*-Bergamoten und (*E,E*)-α-Farnesen wurde in höheren Mengen abgegeben, während (*Z*)-3-Hexenyl-α-Methylbutyrat in geringerer Menge emittiert wurde. Trotz dieser quantitativen Änderung des Duftes war das Wirtsfindeverhalten der Parasitoiden nicht verändert.

Schwermetalle können verschiedenste biochemische Signal-u. Stoffwechselwege in Pflanzen beeinflussen, durch das Entstehen reaktiver Sauerstoffspezies (englisch: reactive oxygen species ROS) in Geweben auch Signalwege der pflanzlichen Verteidigung. Die ROS ihrerseits können die Produktion und Freisetzung endogener JA, ein wichtiger Signalstoff in der pflanzlichen Verteidigung, verändern. In dieser Studie wurden hydroponisch kultivierte Maispflanzen verschiedenen erhöhten Kupfer-u. Cadmiumkonzentrationen ausgesetzt. Mais scheint erhöhte Schwermetallmengen vom Spross ausschließen zu können, da die Metalle vor allem in den Wurzeln und nur in geringem Anteil im Spross wiedergefunden wurden. Trotz dieses Ausschlussmechanismuses beeinflussten die Schwermetalle die Aufnahme anderer Metallionen in die Pflanzen. Der Schwermetallüberschuss zusammen mit der eingeschränkten Aufnahme andere Ionen führte zu einem verringerten Wachstum von Wurzeln und Spross sowie verringerter Photosyntheseleistung. Der Futterwert der Pflanzen für die Herbivoren war daher entweder durch direkte toxische Eigenschaften der aufgenommenen Schwermetalle oder indirekt durch Änderung der chemischen Zusammensetzung der Pflanzen verringert. Wurden *S. frugiperda* Larven mit Maisblättern gefüttert, die hohen Schwermetallkonzentrationen ausgesetzt waren, zeigten sie ein signifikant verringertes Wachstum, bevorzugten in Futterwahltests jedoch weder Blätter von Kontrollpflanzen noch von schwermetallbehandelten Pflanzen. Da Pflanzen, die hohen Kupferkonzentrationen ausgesetzt waren höhere Mengen von herbivor-induzierter JA aufwiesen als Kontrollpflanzen, hatte Kupfer offenbar einen transienten Primingeffekt auf JA.

Wie erwartet folgte auf die erhöhte JA-Freisetzung eine erhöhte Emission herbivor-induzierter VOC in mit hohen Kupferkonzentrationen behandelten Pflanzen. Diese Steigerung der Emission war durch eine erhöhte Emission der Grünblattdüfte (*E*)-3-Hexenal, (*Z*)-3-Hexenol and (*Z*)-3-Hexenylacetat und der Terpene Linalool, (*E*)-α-Bergamoten, (*E*)-β-Farnesen, und *β*-Sesquiphellandren bedingt. Das Wirtsfindeverhalten der Parasitoiden blieb jedoch trotz der starken Veränderungen des herbivor-induzierten Pflanzenduftes unbeeinflusst.

Wie beschrieben haben die abiotischen Stressfaktoren UV-Strahlung, Stickstoffmangel und Schwermetallbelastung weitreichende Auswirkungen auf die Morphologie und Physiologie von Soja und Maispflanzen, die Larvalentwicklung des Herbivoren *S. frugiperda* und ebenso auf die Larvalentwicklung des Parasitoiden *C. marginiventris*. Das Wirtsfindeverhalten des Parasitoiden blieb jedoch trotz Änderungen in den herbivor-induzierten Duftstoffgemischen unbeeinflusst. Daher scheinen Parasitoide eine zuverlässige Verteidigung für Pflanzen und die induzierte indirekte Verteidigung eine gegen abiotischen Stress sehr robuste Art der Herbivorenabwehr darzustellen.

# 9. Appendix

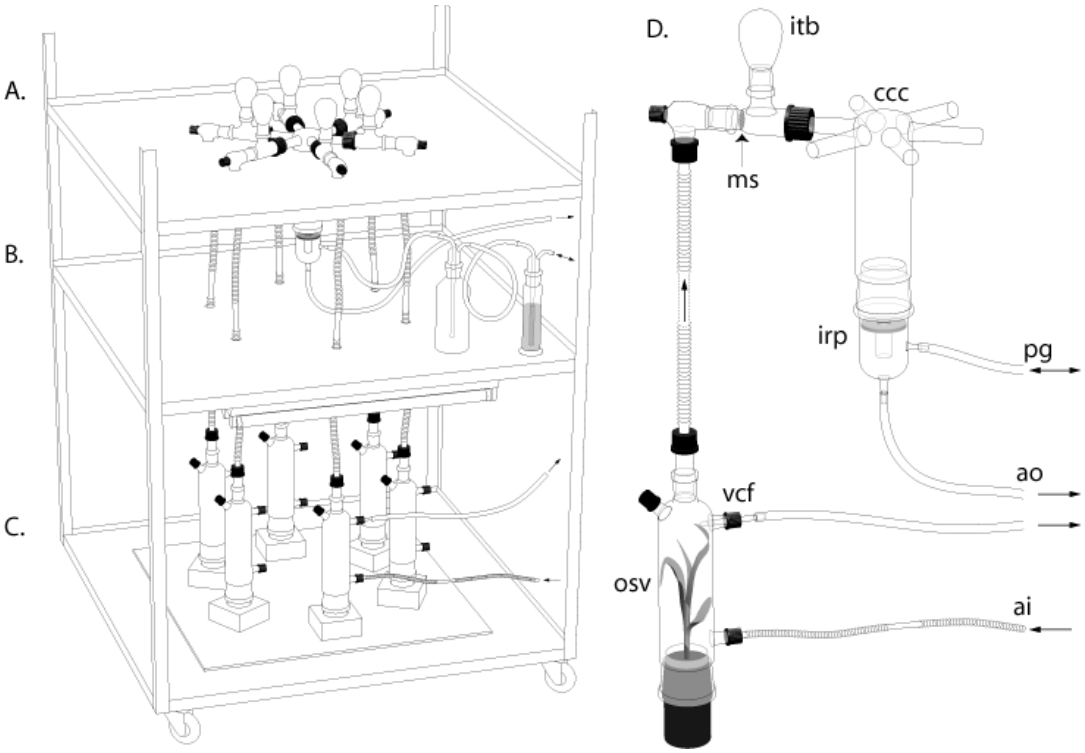


**Figure A1:** An overview of the six-arm olfactometer.

(A) Top shelf where the insects can make choice for one of six possible odours. (B) Middle shelf from where the insects are released. (C) Bottom shelf with the six odour sources (only for one of the chambers are all connecting tubes drawn). (D) Detailed depiction of the various parts and connections; (ai) air inlet, (osv) odour source vessel, (vcf) volatile collection filter, (ms) metal screen, (ccc) central choice chamber, (itb) insect trapping bulb, (irp) insect release point, (pg) pressure gauge, and (ao) air outlet. The corrugated tubes are made of Teflon and the exhaust tubes of Tygon. *Drawing by Dr. Thomas Degen.*

Source: http://www.unine.ch/zool/leae/olfacto_draw.html; www.thomas-degen.ch

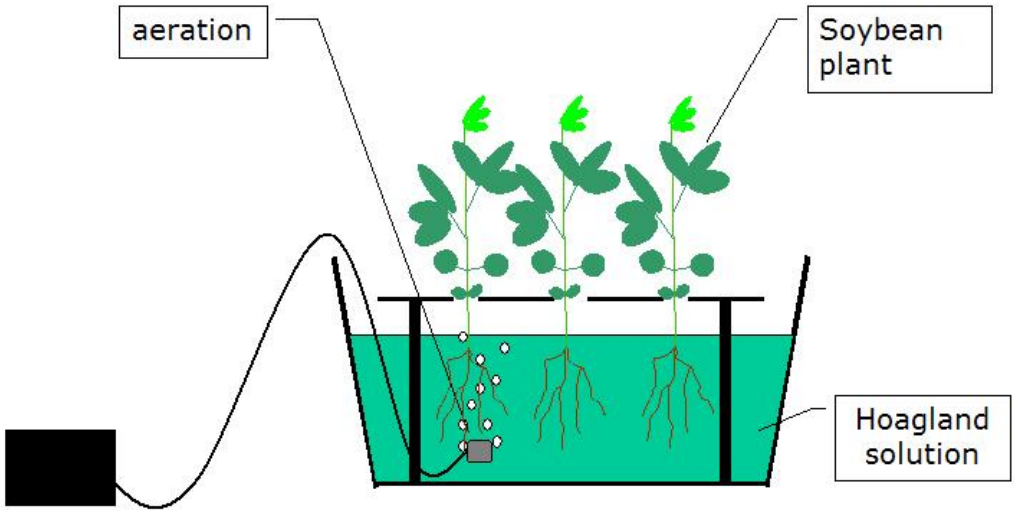


**Figure A2**: Diagram of the hydroponic system used to cultivate soybean or maize plants

**Table A1**: Composition of the hydroponic solutions used to manipulate nitrogen supply of soybean and heavy metal exposition of maize plants.

| Salt | Final Concentration | Provider |
|---|---|---|
| $KH_2PO_4$ | 1.0 mM | Carl Roth, Karlsruhe |
| $KNO_3$ | 6.0 mM | Sigma-Aldrich, Seelze |
| $Ca(NO_3)_2$ | 4.0 mM | Ferak, Berlin |
| $MgSO_4$ | 2.0 mM | AppliChem, Darmstadt |
| $FeSO_4$ | 22.4 µM | Carl Roth, Karlsruhe |
| Chelated with EDTA | 22.3 µM | |
| **Micronutrients** | | |
| $H_3BO_3$ | 4.62 µM | Ferak, Berlin |
| $MnCl_2 \times 4\ H_2O$ | 0.915 µM | Carl Roth, Karlsruhe |
| $ZnSO_4 \times 7\ H_2O$ | 0.077 µM | Carl Roth, Karlsruhe |
| $CuSO_4 \times 5\ H_2O$ | 0.032 µM | Carl Roth, Karlsruhe |
| $H_2MoO_4 \times\ H_2O$ | 0.012 µM | Merck, Darmstadt |

All providers are located in Germany.

**Table A2:** Proportion of N and C of individual plants, growing in a) N containing (+N) or b) N deficient (-N) hydroponic solution

a)

| Treatment | %N | %C |
|---|---|---|
| +N | 4.5 | 44.9 |
| +N | 3.9 | 44.1 |
| +N | 4.8 | 44.7 |
| +N | 4.6 | 43.0 |
| +N | 4.9 | 43.1 |
| +N | 3.6 | 43.7 |
| +N | 4.3 | 44.1 |
| +N | 4.2 | 44.6 |

b)

| Treatment | %N | %C |
|---|---|---|
| -N | 2.4 | 43.3 |
| -N | 2.7 | 44.2 |
| -N | 2.4 | 44.0 |
| -N | 3.8 | 43.5 |
| -N | 3.9 | 43.7 |
| -N | 2.4 | 44.0 |
| -N | 2.2 | 43.1 |
| -N | 2.7 | 44.0 |

**Table A3**: Weights of root and shoot of individual plants, growing in a) N containing (+N) or b) N deficient (-N) hydroponic solution

a)

| Treatment | Root weight (mg) | Shoot weight (mg) |
|---|---|---|
| +N | 22.5 | 213.1 |
| +N | 23.1 | 345.6 |
| +N | 59.5 | 419.0 |
| +N | 71.8 | 382.5 |
| +N | 16.2 | 238.1 |
| +N | 37.2 | 276.4 |

b)

| Treatment | Root weight (mg) | Shoot weight (mg) |
|---|---|---|
| -N | 95.7 | 392.6 |
| -N | 51.3 | 240.2 |
| -N | 49.4 | 243.3 |
| -N | 111.9 | 341.9 |
| -N | 163.9 | 334.1 |
| -N | 113.7 | 408.3 |

**Table A3:** Individual proportion of N and C of *S. frugiperda* larvae, fed for 15 days with soybean leaves growing in a) N containing (+N) or b) N deficient (-N) hydroponic solution

a)

| Treatment of foodplant | %N | %C |
|---|---|---|
| +N | 13.9 | 43.9 |
| +N | 12.4 | 44.3 |
| +N | 14.4 | 43.8 |
| +N | 11.6 | 45.3 |
| +N | 12.9 | 44.2 |
| +N | 10.9 | 43.7 |

b)

| Treatment of foodplant | %N | %C |
|---|---|---|
| -N | 9.6 | 43.1 |
| -N | 10.6 | 43.9 |
| -N | 11.6 | 42.5 |
| -N | 10.5 | 44.2 |
| -N | 10.8 | 43.3 |
| -N | 10.4 | 43.1 |
| -N | 9.8 | 41.9 |
| -N | 10.4 | 44.7 |

**Table A4**: Mean cumulative accrescence of heavy metal treated maize plants in cm.

| Day | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | | | | | | | | | | |
| C | 0.0 | 2.8 | 6.6 | 10.0 | 13.6 | 18.0 | 22.0 | 23.9 | 26.9 | 29.4 |
| CuL | 0.0 | 2.91 | 6.59 | 9.91 | 13.66 | 18.40 | 20.75 | 23.30 | 25.32 | 27.44 |
| CuH | 0.0 | 2.6 | 5.8 | 8.3 | 11.0 | 12.5 | 14.9 | 15.9 | 17.0 | 17.5 |
| CdL | 0.0 | 2.6 | 6.5 | 9.6 | 12.4 | 16.0 | 19.4 | 21.4 | 22.6 | 24.0 |
| CdH | 0.0 | 2.9 | 6.7 | 9.8 | 12.2 | 15.3 | 17.5 | 19.0 | 19.9 | 20.8 |

**Table A5**: Herbivore induced VOC emission in ng $g^{-1}_{FW}$ $h^{-1}$ of individual soybean plants exposed either to ambient radiation (VIS+UV) or to ambient radiation lacking UV-B (VIS).

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **VIS+UV-Plant1** | 7.89 | 49.30 | 0.60 | 60.08 | 1.80 | 22.93 | 11.96 | 8.96 | 5.24 | 26.69 | 130.03 | 16.25 | 7.75 | 1.78 | 1.77 | 1.07 | 2.14 | 255.32 | 1.42 | 4.57 |
| **VIS+UV-Plant2** | 8.12 | 23.21 | 0.35 | 67.09 | 0.28 | 13.18 | 5.81 | 4.46 | 2.96 | 12.08 | 54.44 | 6.53 | 7.40 | 1.46 | 0.31 | 1.10 | 0.82 | 119.55 | 0.81 | 1.57 |
| **VIS+UV-Plant3** | 1.10 | 5.16 | 0.34 | 23.75 | 0.91 | 10.14 | 7.34 | 2.53 | 2.30 | 6.03 | 48.87 | 8.83 | 4.91 | 0.43 | 0.00 | 0.40 | 1.34 | 166.00 | 0.74 | 1.00 |
| **VIS+UV-Plant4** | 1.58 | 12.27 | 0.37 | 32.53 | 0.31 | 19.99 | 6.45 | 3.87 | 2.27 | 10.50 | 78.38 | 13.44 | 5.29 | 1.62 | 0.63 | 1.02 | 1.02 | 187.02 | 0.49 | 1.20 |
| **VIS+UV-Plant5** | 8.13 | 16.70 | 0.00 | 68.67 | 2.53 | 16.69 | 4.56 | 0.00 | 0.00 | 10.87 | 132.18 | 17.46 | 4.85 | 1.50 | 1.91 | 1.11 | 2.10 | 163.42 | 0.00 | 2.25 |
| **VIS+UV-Plant6** | 8.53 | 35.51 | 1.42 | 91.47 | 1.61 | 28.65 | 16.57 | 21.27 | 9.04 | 35.74 | 194.02 | 44.77 | 14.67 | 2.02 | 1.47 | 0.78 | 4.33 | 426.30 | 3.00 | 4.56 |
| **VIS+UV-Plant7** | 8.39 | 26.17 | 0.58 | 74.65 | 0.00 | 15.77 | 9.48 | 1.46 | 0.00 | 0.00 | 87.41 | 8.20 | 8.14 | 1.12 | 0.87 | 0.80 | 1.24 | 163.21 | 0.76 | 1.73 |
| **VIS+UV-Plant8** | 9.76 | 28.30 | 0.52 | 31.01 | 1.13 | 15.74 | 6.18 | 5.22 | 1.81 | 0.00 | 115.02 | 36.49 | 6.02 | 0.88 | 0.83 | 0.69 | 1.13 | 135.09 | 0.58 | 2.51 |
| **VIS-plant1** | 7.84 | 49.02 | 0.58 | 59.74 | 2.79 | 35.47 | 11.89 | 8.91 | 5.21 | 26.54 | 128.28 | 16.16 | 7.70 | 1.23 | 0.78 | 0.79 | 2.13 | 253.86 | 0.84 | 3.73 |
| **VIS-plant2** | 8.06 | 22.04 | 0.52 | 69.37 | 0.00 | 10.61 | 6.94 | 2.49 | 2.13 | 6.53 | 44.36 | 3.66 | 6.26 | 1.13 | 0.57 | 0.65 | 0.89 | 114.73 | 0.72 | 0.98 |
| **VIS-plant3** | 3.50 | 2.45 | 0.67 | 21.53 | 0.00 | 6.51 | 5.14 | 1.48 | 1.59 | 3.64 | 45.91 | 7.75 | 3.34 | 0.34 | 0.00 | 0.31 | 0.58 | 109.47 | 0.28 | 0.50 |
| **VIS-plant4** | 1.23 | 18.08 | 1.05 | 66.35 | 0.57 | 28.85 | 9.25 | 7.15 | 3.05 | 11.18 | 123.78 | 28.43 | 6.26 | 1.87 | 0.56 | 1.13 | 3.13 | 252.05 | 0.58 | 2.73 |
| **VIS-plant5** | 16.11 | 48.42 | 0.34 | 77.53 | 0.00 | 20.17 | 7.33 | 0.00 | 0.00 | 8.08 | 77.76 | 13.22 | 5.44 | 1.13 | 1.13 | 0.93 | 1.40 | 188.13 | 0.00 | 0.00 |
| **VIS-plant6** | 6.48 | 20.50 | 0.91 | 55.67 | 2.09 | 26.37 | 10.82 | 20.47 | 4.44 | 20.27 | 174.35 | 53.37 | 10.55 | 1.59 | 0.76 | 0.82 | 4.06 | 361.93 | 2.32 | 4.87 |
| **VIS-plant7** | 5.45 | 20.46 | 0.00 | 25.61 | 0.59 | 8.68 | 0.00 | 2.08 | 0.00 | 0.00 | 45.83 | 5.78 | 2.13 | 1.31 | 1.19 | 0.83 | 0.84 | 100.08 | 0.61 | 0.58 |
| **VIS-plant8** | 7.83 | 61.86 | 0.62 | 38.46 | 3.03 | 26.48 | 12.47 | 10.19 | 4.01 | 8.80 | 269.76 | 130.52 | 10.61 | 2.10 | 0.88 | 1.31 | 3.36 | 265.31 | 1.39 | 9.81 |

**1**: (*Z*)-3-Hexenal, **2**: (*Z*)-3-Hexenol, **3**: Benzaldehyde, **4**: (*Z*)-3-Hexenylacetate, **5**: Benzenacetaldehyde, **6**: (*E*)-β-Ocimene, **7**: (*Z*)-3-Hexenylpropionate, **8**: Benzenacetonitrile, **9**: (*Z*)-3-Hexenyl Isobutyrate, **10**: (*Z*)-3-Hexenyl-α-Methylbutyrate, **11**: Indole, **12**: Methylanthranilate, **13**: (*Z*)-Jasmone, **14**: (*E*)-Caryophyllene, **15**: α-Humulene, **16**: Germacrene-D, **17**: β-Bergamotene, **18**: (*E,E*)-α-Farnesene, **19**: Tridecatetraene, **20**: Methyljasmonate

**Table A6**: Herbivore induced VOC emission in ng $g^{-1}_{FW}$ $h^{-1}$ of individual soybean plants. Plants were cultivated hydroponically either in the presence (+N) or absence of N (-N).

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **+N plant1** | 4.50 | 1.21 | 3.94 | 1.68 | 0.60 | 28.99 | 10.42 | 18.53 | 15.99 | 9.85 | 4.05 | 3.16 | 22.28 | 11.01 | 9.96 | 107.32 | 2.22 | 0.68 | 0.90 | 0.30 | 70.07 |
| **+N plant2** | 3.64 | 1.32 | 5.36 | 2.33 | 12.44 | 29.29 | 10.32 | 21.12 | 24.12 | 4.52 | 1.81 | 2.92 | 49.22 | 14.35 | 14.25 | 133.38 | 1.56 | 0.75 | 0.83 | 0.49 | 97.17 |
| **+N plant3** | 1.25 | 0.76 | 2.11 | 1.25 | 0.39 | 13.61 | 4.36 | 6.61 | 9.75 | 4.72 | 1.28 | 1.42 | 18.63 | 6.46 | 4.15 | 54.66 | 0.93 | 0.37 | 0.51 | 0.13 | 36.39 |
| **+N plant4** | 1.21 | 0.00 | 5.60 | 0.33 | 0.00 | 0.00 | 0.00 | 4.59 | 0.73 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 2.31 |
| **+N plant5** | 7.25 | 0.00 | 7.44 | 2.15 | 7.10 | 1.81 | 0.64 | 9.98 | 4.23 | 3.20 | 0.00 | 1.40 | 1.05 | 7.09 | 0.00 | 9.62 | 0.16 | 0.22 | 0.00 | 0.00 | 14.80 |
| **+N plant6** | 0.00 | 0.00 | 6.04 | 0.00 | 0.00 | 0.47 | 0.00 | 9.56 | 1.07 | 1.15 | 0.00 | 0.00 | 1.98 | 0.00 | 0.00 | 2.89 | 0.17 | 0.00 | 0.00 | 0.00 | 5.36 |
| **+N plant7** | 0.00 | 0.00 | 13.08 | 0.00 | 13.74 | 0.00 | 0.00 | 9.57 | 1.58 | 1.63 | 1.07 | 0.79 | 1.32 | 0.00 | 0.00 | 0.00 | 0.24 | 0.14 | 0.00 | 0.00 | 2.25 |
| **+N plant8** | 0.00 | 0.00 | 6.89 | 0.00 | 25.35 | 16.83 | 4.22 | 14.64 | 8.39 | 0.00 | 0.00 | 0.00 | 21.85 | 3.23 | 1.62 | 47.38 | 0.89 | 0.28 | 0.52 | 0.23 | 51.21 |
| **+N plant9** | 0.00 | 0.00 | 5.56 | 0.00 | 28.83 | 26.27 | 8.04 | 19.42 | 9.53 | 4.47 | 0.00 | 3.51 | 21.50 | 16.31 | 5.18 | 29.66 | 1.46 | 0.31 | 0.50 | 0.13 | 35.92 |
| **+N plant10** | 0.00 | 0.00 | 11.41 | 0.00 | 15.09 | 4.87 | 0.00 | 9.66 | 1.60 | 2.97 | 0.00 | 0.00 | 4.45 | 0.00 | 0.00 | 6.14 | 0.30 | 0.00 | 0.05 | 0.00 | 4.14 |
| | | | | | | | | | | | | | | | | | | | | | |
| **-N plant1** | 4.89 | 0.00 | 7.08 | 0.00 | 0.00 | 33.61 | 9.57 | 17.97 | 18.97 | 6.67 | 0.00 | 0.00 | 32.59 | 4.81 | 0.00 | 22.60 | 1.47 | 0.58 | 0.67 | 0.51 | 177.11 |
| **-N plant2** | 4.77 | 2.12 | 4.62 | 1.79 | 18.16 | 47.71 | 13.18 | 34.67 | 28.24 | 10.35 | 3.73 | 2.94 | 74.77 | 8.53 | 15.13 | 136.10 | 2.23 | 1.08 | 1.15 | 2.00 | 233.09 |
| **-N plant3** | 2.79 | 0.57 | 3.86 | 1.78 | 2.20 | 16.55 | 4.01 | 12.95 | 9.78 | 5.26 | 0.00 | 0.86 | 51.56 | 1.57 | 1.22 | 49.14 | 0.92 | 0.49 | 0.54 | 0.51 | 93.83 |
| **-N plant4** | 1.80 | 0.00 | 8.89 | 0.58 | 3.45 | 0.00 | 0.00 | 11.90 | 3.30 | 0.84 | 0.00 | 0.00 | 1.61 | 0.00 | 0.00 | 0.00 | 0.56 | 0.32 | 0.38 | 0.00 | 18.53 |
| **-N plant5** | 5.12 | 0.00 | 5.22 | 0.72 | 9.35 | 1.76 | 0.00 | 8.91 | 7.69 | 2.88 | 0.00 | 0.00 | 14.05 | 1.67 | 0.00 | 16.45 | 0.38 | 0.25 | 0.38 | 0.37 | 78.41 |
| **-N plant6** | 0.00 | 0.00 | 11.20 | 0.00 | 10.08 | 4.08 | 0.88 | 13.12 | 4.95 | 2.37 | 1.42 | 0.00 | 8.82 | 0.00 | 0.00 | 9.85 | 0.96 | 0.45 | 0.32 | 0.18 | 38.75 |
| **-N plant7** | 0.00 | 0.00 | 4.39 | 0.00 | 9.41 | 6.22 | 1.30 | 11.82 | 6.72 | 2.27 | 0.87 | 1.56 | 7.82 | 0.00 | 0.00 | 7.51 | 0.68 | 0.31 | 0.39 | 0.00 | 32.09 |
| **-N plant8** | 0.00 | 0.00 | 11.13 | 0.00 | 41.92 | 48.00 | 12.96 | 26.84 | 15.37 | 6.41 | 0.00 | 4.47 | 50.75 | 4.38 | 3.11 | 39.86 | 2.77 | 0.92 | 1.44 | 1.51 | 198.73 |
| **-N plant9** | 0.00 | 0.00 | 7.74 | 0.00 | 21.73 | 25.47 | 8.76 | 25.30 | 15.67 | 6.38 | 26.57 | 3.04 | 13.15 | 5.04 | 5.84 | 20.48 | 0.76 | 0.48 | 0.57 | 1.14 | 164.38 |
| **-N plant10** | 0.00 | 0.00 | 3.81 | 0.00 | 5.90 | 3.90 | 0.37 | 3.82 | 2.35 | 2.20 | 1.67 | 0.00 | 7.02 | 0.00 | 0.00 | 5.28 | 0.54 | 0.00 | 0.00 | 0.00 | 11.27 |

**1**: n.i., **2**: (*Z*)-3-Hexenal, **3**: n.i., **4**: n.i., **5**: (*Z*)-3-Hexenol, **6**: n.i., **7**: α-Pinene, **8**: (*Z*)-3-Hexenylacetate, **9**: (*E*)-β-Ocimene, **10**: (*Z*)-3-Hexenylpropionate, **11**: Benzeneacetonitrile, **12**: (*Z*)-3-Hexenyl Isobutyrate, **13**: Methylsalicylate, **14**: (*Z*)-3-Hexenyl-α-Methylbutyrate, **15**: n.i., **16**: Indole, **17**: (*E*)-Caryophyllene, **18**: α-Humulene, **19**: Germacrene-D, **20**: β-Bergamotene, **21**: (*E,E*)-α-Farnesene
**n.i.:** Compound not identified

**Table A7**: Herbivore induced VOC emission in ng $g^{-1}_{FW}$ $h^{-1}$ of individual soybean plants. Plants were cultivated hydroponically either in the presence of standard (c) or an increased concentrations of copper (CuH, CuL) or cadmium (CdH, CdL).

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **control plant 1** | 5.20 | 2.68 | 3.96 | 16.66 | 2.51 | 1.31 | 1.43 | 13.44 | 0.61 | 0.17 | 28.63 | 5.98 | 10.15 | 0.52 | 0.00 | 0.00 | 0.00 |
| **control plant 2** | 0.00 | 3.56 | 4.89 | 19.86 | 3.57 | 2.09 | 1.71 | 23.52 | 0.83 | 0.37 | 16.60 | 4.81 | 6.85 | 0.66 | 0.00 | 0.00 | 0.00 |
| **control plant 3** | 6.96 | 4.77 | 2.72 | 20.12 | 6.77 | 3.65 | 2.40 | 26.97 | 1.91 | 0.37 | 34.34 | 11.42 | 22.61 | 0.97 | 0.00 | 0.00 | 0.28 |
| **control plant 4** | 7.66 | 1.72 | 3.01 | 17.97 | 18.69 | 5.01 | 10.71 | 50.09 | 6.26 | 0.69 | 118.08 | 1.90 | 77.97 | 1.82 | 0.00 | 1.84 | 7.72 |
| **control plant 5** | 11.99 | 6.21 | 3.93 | 24.60 | 16.62 | 0.00 | 2.82 | 25.20 | 1.78 | 1.67 | 71.77 | 1.44 | 48.55 | 2.45 | 0.00 | 7.75 | 6.85 |
| **control plant 6** | 0.00 | 2.84 | 1.51 | 9.10 | 6.88 | 1.15 | 4.03 | 3.20 | 1.07 | 0.75 | 87.39 | 37.92 | 71.17 | 1.96 | 0.00 | 0.86 | 0.00 |
| | | | | | | | | | | | | | | | | | |
| **CuL plant 1** | 0.00 | 5.13 | 0.00 | 38.96 | 12.12 | 5.15 | 1.59 | 34.47 | 0.00 | 1.58 | 130.33 | 54.67 | 101.88 | 3.69 | 7.13 | 0.00 | 0.00 |
| **CuL plant 2** | 9.07 | 10.27 | 0.00 | 20.88 | 22.05 | 11.07 | 5.02 | 52.79 | 3.89 | 0.68 | 64.66 | 40.21 | 85.64 | 0.00 | 4.61 | 0.00 | 4.76 |
| **CuL plant 3** | 0.00 | 17.66 | 4.69 | 36.18 | 26.40 | 14.23 | 5.27 | 62.75 | 4.14 | 0.86 | 63.36 | 44.04 | 88.51 | 2.10 | 5.13 | 0.00 | 4.35 |
| **CuL plant 4** | 25.16 | 8.19 | 7.96 | 26.75 | 14.83 | 2.03 | 2.93 | 58.60 | 2.33 | 0.00 | 67.22 | 25.86 | 41.28 | 1.27 | 2.18 | 0.00 | 3.39 |
| **CuL plant 5** | 0.00 | 1.38 | 0.97 | 6.86 | 17.89 | 5.98 | 1.60 | 17.17 | 1.62 | 0.00 | 53.49 | 17.62 | 31.94 | 1.43 | 1.96 | 0.00 | 3.13 |
| **CuL plant 6** | 29.15 | 11.82 | 12.44 | 46.23 | 33.53 | 8.82 | 5.49 | 76.75 | 8.43 | 0.00 | 75.77 | 30.90 | 66.79 | 1.48 | 2.70 | 0.00 | 4.60 |
| | | | | | | | | | | | | | | | | | |
| **CuH plant 1** | 0.00 | 8.06 | 10.92 | 59.99 | 9.67 | 1.53 | 2.93 | 21.58 | 0.00 | 0.00 | 26.79 | 16.84 | 52.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **CuH plant 2** | 26.82 | 22.11 | 21.82 | 81.38 | 41.03 | 4.61 | 20.72 | 88.86 | 0.00 | 0.00 | 163.51 | 130.66 | 332.38 | 0.00 | 16.48 | 17.20 | 0.00 |
| **CuH plant 3** | 39.57 | 16.81 | 29.52 | 176.10 | 29.58 | 4.02 | 11.43 | 95.50 | 4.07 | 0.00 | 94.75 | 51.24 | 140.75 | 0.00 | 7.16 | 17.33 | 11.48 |
| **CuH plant 4** | 31.90 | 26.63 | 16.42 | 69.04 | 52.11 | 0.00 | 3.97 | 122.82 | 5.79 | 0.00 | 164.69 | 46.50 | 249.74 | 0.00 | 0.00 | 80.45 | 21.81 |
| **CuH plant 5** | 18.86 | 6.54 | 11.90 | 51.89 | 50.74 | 5.33 | 9.47 | 33.40 | 2.60 | 0.00 | 76.94 | 72.27 | 195.70 | 0.00 | 6.48 | 5.75 | 9.97 |
| **CuH plant 6** | 54.26 | 27.57 | 25.95 | 118.59 | 103.35 | 5.08 | 28.32 | 148.50 | 9.74 | 0.00 | 218.11 | 161.84 | 476.85 | 0.00 | 20.83 | 27.60 | 33.68 |
| | | | | | | | | | | | | | | | | | |
| **CdL plant 1** | 12.57 | 6.60 | 4.74 | 17.34 | 6.80 | 2.80 | 1.00 | 18.13 | 0.00 | 1.06 | 68.97 | 10.03 | 27.78 | 2.28 | 1.19 | 0.80 | 0.00 |
| **CdL plant 2** | 19.19 | 8.07 | 5.08 | 23.19 | 5.17 | 0.43 | 0.49 | 17.75 | 0.87 | 0.40 | 45.13 | 7.86 | 21.98 | 1.36 | 1.46 | 0.63 | 0.00 |
| **CdL plant 3** | 10.48 | 2.45 | 1.84 | 5.23 | 7.68 | 3.41 | 0.71 | 25.71 | 0.88 | 0.85 | 55.66 | 8.51 | 23.04 | 2.24 | 1.16 | 0.37 | 0.00 |
| **CdL plant 4** | 28.11 | 12.28 | 11.93 | 48.21 | 50.44 | 6.59 | 10.91 | 122.38 | 12.59 | 0.91 | 70.07 | 34.05 | 98.61 | 2.17 | 2.71 | 2.24 | 0.00 |
| **CdL plant 5** | 0.00 | 17.99 | 11.61 | 15.52 | 29.95 | 1.43 | 6.98 | 44.08 | 4.17 | 0.39 | 29.50 | 8.13 | 20.68 | 0.93 | 0.00 | 2.96 | 0.00 |
| **CdL plant 6** | 0.00 | 52.74 | 23.72 | 24.52 | 22.02 | 2.33 | 7.29 | 72.89 | 7.90 | 0.33 | 38.31 | 13.34 | 39.40 | 1.01 | 1.10 | 1.79 | 0.00 |

**Table A7** continued

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **CdH plant 1** | 1.51 | 0.00 | 0.00 | 3.02 | 11.34 | 1.59 | 2.15 | 6.22 | 2.01 | 1.30 | 137.13 | 26.73 | 87.72 | 3.10 | 4.86 | 1.67 | 10.24 |
| **CdH plant 2** | 4.28 | 3.06 | 1.47 | 5.68 | 7.72 | 0.63 | 0.29 | 3.94 | 0.79 | 0.88 | 44.26 | 7.98 | 21.38 | 2.46 | 0.00 | 0.50 | 4.95 |
| **CdH plant 3** | 29.01 | 10.52 | 0.95 | 24.81 | 35.94 | 2.02 | 10.04 | 31.31 | 4.29 | 1.13 | 134.79 | 33.78 | 104.49 | 2.92 | 1.93 | 0.43 | 0.00 |
| **CdH plant 4** | 72.05 | 16.00 | 19.49 | 69.31 | 64.46 | 5.50 | 18.16 | 123.33 | 6.04 | 1.96 | 255.77 | 67.61 | 193.52 | 6.64 | 7.10 | 2.47 | 14.11 |
| **CdH plant 5** | 39.42 | 12.21 | 19.57 | 67.35 | 49.32 | 4.79 | 10.08 | 124.71 | 5.67 | 1.29 | 116.82 | 35.04 | 99.31 | 2.30 | 3.21 | 1.43 | 10.85 |
| **CdH plant 6** | 48.84 | 15.93 | 9.88 | 29.18 | 16.74 | 1.27 | 2.68 | 33.34 | 2.23 | 0.70 | 94.00 | 12.38 | 38.12 | 2.42 | 1.10 | 0.00 | 4.44 |

**1**: (*Z*)-3-Hexenal, **2**: *(E)*-2-Hexenal **3**: (*Z*)-3-Hexenol, **4**: (*Z*)-3-Hexenylacetate, **5**: Linalool, **6**: (*E*)-4,8-Dimethyl-1,3,7- nonatriene **7**: Phenylethylester, **8**: Indole, **9**: Methylanthranilate, **10**: α-Copaene, **11**: (*E*)-β-Caryophyllene, **12**: (*E*)-α-Bergamotene **13**: (*E*)-β-Farnesene, **14**: Germacrene-D, **15**: β-Sesquiphellandrene, **16**: Nerolidol, **17**: (*E,E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene

**Table A8**: Number of *C. marginiventris* deciding for the herbivore induced volatiles of soybean in the six-arm-olfactometer. Plants were exposed to ambient sunlight or sunlight lacking UV-B and placed vis-à-vis in the olfactometer. Control is the total number of wasps in the four empty olfactometer arms; olfactometer is the number of wasps staying in the central choice chamber (see Fig X). One release equals six wasps.

| Date | Release number | VIS+UV | VIS | control | olfactometer |
|---|---|---|---|---|---|
| 26.7.05 | **1** | 1 | 4 | 0 | 1 |
| | **2** | 0 | 1 | 4 | 1 |
| | **3** | 2 | 2 | 2 | 0 |
| | **4** | 1 | 1 | 1 | 3 |
| | **5** | 2 | 1 | 0 | 3 |
| | **6** | 0 | 0 | 2 | 4 |
| 28.07.05 | **1** | 1 | 3 | 0 | 2 |
| | **2** | 1 | 3 | 0 | 2 |
| | **3** | 1 | 0 | 0 | 5 |
| | **4** | 1 | 2 | 0 | 3 |
| | **5** | 0 | 3 | 0 | 3 |
| | **6** | 0 | 2 | 0 | 4 |
| 29.07.05 | **1** | 2 | 2 | 1 | 1 |
| | **2** | 5 | 0 | 0 | 1 |
| | **3** | 3 | 1 | 1 | 1 |
| | **4** | 2 | 0 | 0 | 4 |
| | **5** | 2 | 1 | 1 | 2 |
| | **6** | 0 | 0 | 0 | 0 |
| 10.08.05 | **1** | 1 | 4 | 0 | 1 |
| | **2** | 2 | 2 | 1 | 1 |
| | **3** | 1 | 0 | 1 | 4 |
| | **4** | 0 | 1 | 1 | 4 |
| | **5** | 1 | 1 | 0 | 4 |
| 12.08.05 | **1** | 1 | 0 | 1 | 4 |
| | **2** | 1 | 3 | 0 | 2 |
| | **3** | 0 | 3 | 1 | 2 |
| | **4** | 2 | 3 | 1 | 0 |
| | **5** | 2 | 2 | 0 | 2 |
| 15.08.05 | **1** | 1 | 1 | 0 | 1 |
| | **2** | 1 | 2 | 0 | 3 |
| | **3** | 2 | 2 | 0 | 2 |
| | **4** | 2 | 3 | 0 | 1 |
| | **5** | 5 | 1 | 0 | 0 |
| 17.08.05 | **1** | 4 | 1 | 0 | 1 |
| | **2** | 2 | 0 | 0 | 4 |
| | **3** | 2 | 1 | 1 | 0 |
| | **4** | 2 | 1 | 0 | 3 |
| | **5** | 2 | 1 | 0 | 3 |
| 06.09.05 | **1** | 2 | 0 | 1 | 3 |
| | **2** | 1 | 2 | 0 | 3 |
| | **3** | 1 | 2 | 0 | 3 |
| | **4** | 0 | 2 | 0 | 4 |
| | **5** | 2 | 1 | 0 | 3 |

**Table A9**: Number of *C. marginiventris* deciding for the herbivore induced volatiles of soybean in the six-arm-olfactometer. Plants were cultivated hydroponically either in the presence or absence of N and placed vis-à-vis in the olfactometer. Control is the total number of wasps in the four empty olfactometer arms; olfactometer is the number of wasps staying in the central choice chamber (see Fig X). One release equals six wasps.

| Date | Release number | +N | -N | control | olfactometer |
|---|---|---|---|---|---|
| 03.04.06 | **1** | 0 | 1 | 1 | 4 |
| | **2** | 1 | 3 | 0 | 2 |
| | **3** | 0 | 6 | 0 | 0 |
| | **4** | 4 | 2 | 0 | 0 |
| | **5** | 3 | 1 | 0 | 2 |
| 05.04.06 | **1** | 1 | 0 | 2 | 3 |
| | **2** | 0 | 1 | 0 | 5 |
| | **3** | 0 | 0 | 0 | 6 |
| | **4** | 0 | 1 | 1 | 4 |
| | **5** | 1 | 1 | 0 | 4 |
| | **6** | 0 | 4 | 1 | 1 |
| 17.04.06 | **1** | 0 | 2 | 1 | 3 |
| | **2** | 2 | 1 | 0 | 3 |
| | **3** | 1 | 2 | 0 | 3 |
| | **4** | 2 | 2 | 0 | 2 |
| | **5** | 2 | 3 | 0 | 1 |
| 24.04.06 | **1** | 2 | 0 | 1 | 3 |
| | **2** | 0 | 3 | 0 | 3 |
| | **3** | 1 | 1 | 0 | 4 |
| | **4** | 2 | 2 | 0 | 2 |
| | **5** | 2 | 1 | 1 | 2 |
| 26.04.06 | **1** | 0 | 0 | 2 | 3 |
| | **2** | 1 | 2 | 0 | 3 |
| | **3** | 2 | 1 | 0 | 3 |
| | **4** | 1 | 1 | 0 | 4 |
| | **5** | 2 | 0 | 0 | 4 |
| 01.05.06 | **1** | 1 | 0 | 0 | 5 |
| | **2** | 2 | 0 | 0 | 4 |
| | **3** | 2 | 0 | 0 | 4 |
| | **4** | 3 | 1 | 0 | 2 |
| | **5** | 2 | 1 | 0 | 3 |

**Table A10**: Number of *C. marginiventris* deciding for the herbivore induced volatiles of maize in the six-arm-olfactometer. Plants were cultivated hydroponically either in the presence of standard (c) or an increased concentrations of copper (CuH) and placed vis-à-vis in the olfactometer. Control is the total number of wasps in the four empty olfactometer arms; olfactometer is the number of wasps staying in the central choice chamber (see Fig X). One release equals six wasps.

| Date | Release number | c | CuH | control | olfactometer |
|---|---|---|---|---|---|
| 09.04.08 | **1** | 0 | 1 | 0 | 5 |
| | **2** | 2 | 0 | 3 | 1 |
| | **3** | 1 | 0 | 0 | 5 |
| | **4** | 1 | 0 | 0 | 5 |
| | **5** | 1 | 1 | 0 | 4 |
| 11.04.08 | **1** | 2 | 0 | 1 | 3 |
| | **2** | 2 | 0 | 0 | 4 |
| | **3** | 2 | 1 | 0 | 3 |
| | **4** | 0 | 2 | 0 | 4 |
| | **5** | 2 | 0 | 1 | 3 |
| 16.04.08 | **1** | 0 | 1 | 0 | 5 |
| | **2** | 0 | 0 | 0 | 6 |
| | **3** | 1 | 0 | 0 | 5 |
| | **4** | 1 | 1 | 0 | 4 |
| | **5** | 0 | 0 | 5 | 1 |
| 23.04.08 | **1** | 1 | 1 | 0 | 4 |
| | **2** | 0 | 2 | 1 | 3 |
| | **3** | 0 | 2 | 1 | 3 |
| | **4** | 1 | 1 | 2 | 2 |
| | **5** | 1 | 1 | 2 | 2 |
| 30.04.08 | **1** | 0 | 0 | 0 | 6 |
| | **2** | 1 | 3 | 1 | 1 |
| | **3** | 1 | 0 | 1 | 4 |
| | **4** | 0 | 1 | 3 | 2 |
| | **5** | 3 | 1 | 0 | 2 |
| 02.05.08 | **1** | 0 | 0 | 2 | 4 |
| | **2** | 0 | 0 | 4 | 2 |
| | **3** | 0 | 0 | 2 | 4 |
| | **4** | 0 | 0 | 0 | 6 |
| | **5** | 0 | 0 | 4 | 2 |
| 09.05.08 | **1** | 0 | 0 | 2 | 4 |
| | **2** | 0 | 0 | 3 | 3 |
| | **3** | 0 | 2 | 1 | 3 |
| | **4** | 2 | 1 | 0 | 3 |
| | **5** | 1 | 2 | 0 | 3 |
| 28.05.08 | **1** | 0 | 0 | 1 | 5 |
| | **2** | 2 | 0 | 1 | 3 |
| | **3** | 0 | 0 | 1 | 5 |
| | **4** | 0 | 0 | 2 | 4 |
| | **5** | 2 | 0 | 1 | 3 |
| 30.05.08 | **1** | 0 | 1 | 0 | 5 |
| | **2** | 0 | 2 | 0 | 4 |
| | **3** | 0 | 0 | 0 | 6 |
| | **4** | 1 | 0 | 2 | 3 |
| | **5** | 1 | 0 | 2 | 3 |

# 10. Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und dabei keine anderen als die hier angegebenen Quellen und Hilfsmittel verwendet habe.

Ferner erkläre ich, dass ich diese Arbeit weder einer anderen Prüfungsbehörde vorgelegt, noch anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Ich erkläre, dass ich bisher keine akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg, den

(Thorsten Winter)

# 11. Curriculum vitae

von Thorsten Ralf Winter

| | |
|---|---|
| Geburtsdatum, -ort: | 7 Juli 1978, Suhl |

**Studium**

| | |
|---|---|
| Ab Oktober 2009 | wissenschaftliche Hilfskraft im Büro für Öffentlichkeitsarbeit des Rudolf-Virchow-Zentrums Würzburg |
| Ab Juni 2005 | Promotion am Lehrstuhl für Botanik 2 der Bayerischen Julius – Maximilians – Universität Würzburg, Titel „Induced indirect defense in soybean and maize: Effects of ultraviolet radiation, nitrogen availability and heavy metal stress“, betreut von Prof. Dr. M Riederer und Dr. M. Rostás |
| Mai 2004 – April 2005 | wissenschaftliche Hilfskraft am Zentrum für Infektionsforschung der Universität Würzburg, AG PD Dr. Ute Hentschel |
| April 2003 – März 2004 | Diplomarbeit, Thema: "Kolonieverteilung und Koloniestruktur von *Dorylus (Dichthadia) laevigatus* (Hymenoptera:Formicidae:Dorylinae) im Tawau-Hills-Park (Sabah/Malaysia)", betreut von Prof. Dr. K. E. Linsenmair |
| September 2002 – November 2002 | Freilandarbeiten für die Diplomarbeit im Tawau-Hills-Park, Sabah/Malaysia |
| Juli 2001 – September 2004 | studentische Hilfskraft am Lehrstuhl für Verhaltensphysiologie und Soziobiologie der Universität Würzburg |
| März 2001 – Mai 2001 | Fortgeschrittenenpraktikum im Kinabalu National Park, Sabah/Malaysia |
| November 1999 – April 2003 | Studium der Biologie an der Bayerischen Julius – Maximilians – Universität Würzburg, Abschluss Diplom |
| November 1997 – September 1999 | Studium der Biologie an der Bayerischen Julius – Maximilians – Universität Würzburg, Abschluss Vordiplom |

**Schulische Ausbildung**

| | |
|---|---|
| September 1991 – Juni 1997 | Hennebergisches Gymnasium Georg Ernst Schleusingen, Abschluss Abitur |

# 12. Publications and Conference contributions

Publications

Thorsten R. Winter, Lena Borkowski, Katharina Kaiser, Michael Rostás (in preparation): Heavy metal stress primes for herbivore induced volatiles without affecting induced indirect defense of maize

Thorsten R. Winter, Rostás, M. (2010): Nitrogen Deficiency affects Bottom-Up Cascade without Disrupting Indirect Plant Defense. Chem. Ecol. 36: 642–651

Thorsten R. Winter, Rostás, M. (2008): Ambient ultraviolet radiation induces protective responses in soybean but does not attenuate indirect defense. Environ. Pollut. 155: 290–297

Berghoff SM, Gadau J, Winter T, Linsenmair KE, Maschwitz U (2003): Sociobiology of hypogaeic army ants: characterization of two sympatric Dorylus species on Borneo and their colony conflicts. Insectes Soc. 50: 139–147

Conference contributions

Thorsten R. Winter, Michael Rostàs (2008): *Effects of nitrogen manipulation on tritrophic interactions in soybean – responses of experienced parasitoids*. Workshop on Multitrophic Interactions, Goettingen, 6.–7. März 2008 (**Poster**)

Winter, T. R., Rostàs, M. (2007*): Effects of nitrogen availability on tritrophic interactions in soybean*. 23. Jahrestreffen der ISCE, Jena, 22-26 Juli 2007 (**Poster**)

Thorsten Winter and Michael Rostás (2006): *Tritrophic interactions in soybean: effects of ambient UV radiation*. 22. Jahrestreffen der ISCE, Barcelona, Spanien, 15.–19. Juli 2007 (**Vortrag**).

Thorsten Winter and Michael Rostás (2006): *Ambient UV-B affects plant physiology but doesn't disturb tritrophic interactions*. biology06, Muséum d'histoire naturelle – Conservatoire et Jardin botaniques Ville de Genève Université de Genève (**Poster**)

Thorsten R. Winter, Stefanie M. Berghoff, Jürgen Gadau (2004): *Colony density and inter and intracolonial relationship of the army ant Dorylus (Dichthadia) laevigatus (Dorylinae: Formicidae: Hymenoptera) in Borneo* in Abstractband, 97. Jahresversammlung der Deutschen Zoologischen Gesellschaft vom 31. Mai – 4. Juni, Universtiät Rostock, S. 129 (**Poster**)

Winter, TR, Berghoff, SM, Gadau, J (2004): *Colony density, distribution and intercolonial relationship of the army ants Dorylus (Dichthadia) laevigatus and Dorylus (Alaopone) cf. vishnui in Borneo* in: Society for Tropical ecology 17th annual conference "Biodiversity and dynamics in tropical ecosystems" (Axmacher JC and Golland T eds.) S.238 (**Poster**)

Winter, TR, Berghoff, SM, Gadau, J (2003): *Colony density, distribution and intercolonial relationship of the army ants Dorylus (Dichthadia) laevigatus and Dorylus (Alaopone) cf. vishnui in Borneo* in: International Union for the Study of Social Insects, 18th meeting of the German speaking section in Regensburg S. 46 (**Poster**)

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