

# Influence of herbivore-induced changes in host plants on reproductive behaviours in *Spodoptera littoralis*

Herbivore-plant interactions

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Cover: A larva of *Spodoptera littoralis* on cotton plant leaf surface.  
(Photo: P. Anderson)

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### Abstract

Insect herbivores orient towards host plants using sensory cues and olfaction plays a major role, especially in nocturnal herbivores, during selection of host plants suitable for feeding, mating and oviposition. Plants defend themselves from herbivore feeding by producing volatiles as well as non-volatiles chemical compounds. Volatile compounds produced in response to feeding damage by herbivores are commonly referred to as herbivore-induced plant volatiles (HIPVs). Emissions of HIPVs are ecologically important as they can increase plant resistance by repelling herbivores and by attracting the natural enemies of the herbivores. We observed a significant reduction in mating when male and female *Spodoptera littoralis* moths were allowed to mate in the presence of damaged cotton (*Gossypium hirsutum*) plants. Male activation and attraction towards female sex pheromone was reduced in the presence herbivore-damaged cotton plants. Similarly, females kept individually with damaged cotton plants spent less time in calling compared to females on undamaged plants. These results provide first evidence that herbivore-induced changes in host plants can affect calling and mating behaviours of an insect herbivore.

In behavioural studies, in the laboratory as well as in the field, we showed that HIPV emissions from damaged cotton plant neighbours provide resistance to undamaged plants within both conspecific and heterospecific plant patches. Furthermore we found that associational resistance through HIPVs is unidirectional and is highly specific among the tested plant species. Undamaged cotton and alfalfa (*Medicago sativa*) plants in patches with damaged cotton plant neighbours received fewer eggs, whereas we found no associational resistance when damaged alfalfa and clover (*Trifolium alexandrinum*) neighbours were present. Electrophysiological (GC-EAD) studies showed that the antennae of the mated female *S. littoralis* moths detected 18 compounds among the headspace collections of HIPVs from damaged cotton plants. Behavioural studies showed that a blend of seven *de novo* synthesized volatile compounds among GC-EAD active compounds were sufficient to repel ovipositing *S. littoralis*. Our results suggest that *de novo* synthesized volatile compounds provide signalling cues to ovipositing female moths that the plants are under herbivore attack and can be used as reliable cues to avoid plants of low food quality and to reduce risk for competition and predation. Our studies show that HIPVs can have large effect on both male and female reproductive behaviours and that it can affect ecological interactions.

**Keywords:** Herbivory, *Spodoptera littoralis*, olfaction, *Gossypium hirsutum*, oviposition, HIPVs, associational resistance, plant-herbivore interactions, repellent,

gas-chromatography electroantennographic detection (GC-EAD), plant defence, signal reliability, sexual communication

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## Dedication

To all my Teachers

To all my Friends and Family members

To my beloved Parents (May your souls rest in peace. Amin)

وَالْعَصْرِ (103:1) إِنَّ الْإِنْسَانَ لَفِي خُسْرٍ (103:2) إِلَّا الَّذِينَ آمَنُوا وَعَمِلُوا الصَّالِحَاتِ وَتَوَاصَوْا بِالْحَقِّ  
وَتَوَاصَوْا بِالصَّبْرِ (103:3)

*By time! (103:1) Indeed, mankind is in loss (103:2) Except for those who have faith and do righteous deeds, and counsel each other to hold on to truth and counsel each other to be steadfast (103:3).*

- Al-Qur'an

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## List of Publications

- I Ali Zakir, Medhat M Sadek, Marie Bengtsson, Bill S Hansson, Peter Witzgall, Peter Anderson. Herbivore-induced plant volatiles provide associational resistance against an ovipositing herbivore (submitted).
- II Ali Zakir, Marie Bengtsson, Medhat M Sadek, Bill S Hansson, Peter Witzgall, Peter Anderson. Herbivore-induced *de novo* synthesized volatile compounds in cotton repel oviposition in the moth *Spodoptera littoralis* (submitted).
- III Ali Zakir, Mohamed Khallaf, Bill S Hansson, Peter Witzgall, Peter Anderson. Herbivore-induced changes in host plant modulate reproductive behaviours in male *Spodoptera littoralis* moths (manuscript).
- IV Ali Zakir, Bill S Hansson, Peter Witzgall, Peter Anderson. Modulation of female calling behaviour in *Spodoptera littoralis* by herbivore damage induced changes in a host plant (manuscript).

# 1 Introduction

## 1.1 Host plant selection in herbivores

Green plants are a source of food and reproduction for herbivores. The diversity of potential threats to plants in nature is wide and quite impressive. Important herbivores are mammals, reptiles, amphibians, birds, mollusks, worms, arthropods, viruses, bacteria, fungi, and other microorganisms. Representing a significant part of life on earth, insect herbivores are a considerable threat to the plants (Schoonhoven *et al.*, 2005; Karban & Baldwin, 1997). Insect herbivores use various feeding strategies to obtain nutrients from aboveground (vegetative) and belowground (root) plant parts and inflict mechanical damage on plant tissues. The quantity and quality of injury varies greatly, depending on the feeding tactic. Approximately two thirds of all known herbivorous insect species are leaf-eating beetles (Coleoptera) or caterpillars (Lepidoptera) that cause damage with mouthparts designed for chewing, snipping, or tearing (Howe & Jander, 2008; Schoonhoven *et al.*, 2005).

Oviposition behaviour is an important factor for the fitness of the herbivores as the selection of suitable a host plant is crucial for the growth and development of their offspring (Schoonhoven *et al.*, 2005). Since, in many herbivores, the newly emerged offspring is restricted to feed on the plants where they have hatched (Renwick & Chew, 1994; Renwick, 1989), it is important for adult insects to assess the quality of the plant i.e., the nutritional value, if the plant is already occupied by other insects or if the risk for predation is high (Bernays, 2001). Plants produce chemical cues, both volatile and non-volatile, that can be used as information for insects searching for a suitable host plant for feeding or oviposition (Hopkins *et al.*, 2009; Renwick, 1989). For many insect herbivores olfactory cues are very important and are used by the insect to orientate towards and accept a specific host plant within a plant patch (Bruce & Pickett, 2011).



Many insect herbivores also use volatile cues to locate mating partners and the chances of mating increases in association with host plants. Volatile emissions from host plants have been shown to synergize with the female sex pheromone and enhance male attraction (Landolt & Phillips, 1997; McNeil & Delisle, 1989). In addition, volatiles from non-host plant may also affect reproductive behaviours. In coleopterans, where males release pheromones to attract females and other males, non-host plant volatiles have been shown to have antagonistic effects on attraction behaviour (Allison *et al.*, 2004; Zhang & Schlyter, 2004). In general, the acceptance or rejection of a host plant suitable for mating and oviposition is based on the balance between positive stimuli, attractants and stimulants, and negative stimuli, repellents and deterrents (Renwick & Chew, 1994).

## 1.2 Plant resistance against insect herbivores

Plants use physical, physiological and chemical defensive traits to resist against herbivore attack. If defence strategy adopted by the host plant is effective or not depends, among other things, on the host selection behaviour of herbivores (van Dam *et al.*, 2001). The defensive traits assist plants to reduce herbivore attack by affecting host selection behaviour or by reducing the growth and development of offspring. Plants that have effective defence traits are likely to be better represented in the future generations than those that failed to resist against their attackers (Howe & Jander, 2008; Karban & Baldwin, 1997). Expression of defensive traits in plants can be constitutive i.e., always expressed in the plant and function independent of herbivore attack and develop under the continuous developmental program within the plant. Plant can also have an induced defence that is activated in response to herbivore attack and initiates production of secondary metabolites in the plant as a defence against the attackers. Both constitutive and induced defence can be direct, which means that they affect the herbivore directly, or indirectly, where the plants use other organisms as part of their defence. What type of defence a plant uses depends on the strategies adopted by the attackers (Stout *et al.*, 2006; Agrawal, 2005).

Physical factors including morphological and structural features e.g., leaf surface toughness, nectaries, thorns, spines serve as direct defence (Howe & Schaller, 2008). In comparison, food resources provided by the plant to attract natural enemies of the herbivores serve as indirect constitutive defence (Kessler & Heil, 2011). Similarly, induced defence can be direct; when secondary metabolites produced by the plants directly affect the herbivore preference or performance, or indirect; when natural enemies are attracted

towards induced plants and help plants to resist against further herbivore attack (Agrawal, 2001; Karban & Baldwin, 1997).

### 1.3 Associational resistance

Induced changes in plants may also have effects not only on the focal plant, but may also affect neighbouring plants. In this context, plant resistance in relation to herbivore preference depends on host plant's defensive characteristics (Karbon & Baldwin, 1997) and on the vegetational diversity around the host plant (Hambäck *et al.*, 2000; Andow, 1991). Thus, an individual plant's resistance or susceptibility to herbivores can be influenced by the surrounding plants (Jactel *et al.*, 2011; Barbosa *et al.*, 2009; Agrawal *et al.*, 2006; Hambäck *et al.*, 2000; Atsatt & O'Dowd, 1976).

Since Tahvanainen and Root (1972) introduced the term “associational resistance” (AR) in their study, several mechanisms of AR have been suggested under laboratory and field conditions. Volatile compounds produced by plants can have both odour masking and repellent effects on the herbivores. This means that plants can reduce herbivore attack when they grow in the neighbourhood of the resistant plants. (Jactel *et al.*, 2011; Karban, 2007; Hambäck *et al.*, 2000). Another mechanism suggested is that volatile emissions from resistant or herbivore damaged neighbours can induce resistance in the undamaged host plants and reduce the attractiveness and suitability of the host plants against herbivore attack (Arimura *et al.*, 2009; Frost *et al.*, 2008; Heil & Silva Bueno, 2007). For example, alder trees, *Alnus glutinosa*, proximate to defoliated conspecifics were found to be more resistant to the alder leaf beetle than trees having healthy conspecifics neighbours (Dolch & Tschamtkke, 2000). Similarly, experimentally clipped branches of the sagebrush, *Artemisia tridentata*, reduced herbivore abundance and leaf damage on adjacent undamaged conspecific and heterospecific plants (Karbon *et al.*, 2006).

In a recent study, volatile emissions from neighbouring non-host plants were shown to be adsorbed on the host plant leave surface and increase the resistance of the receiving plants against herbivore attack (Himanen *et al.*, 2010; Karban, 2010). The chemical compounds emitted by the resistant neighbours were adsorbed and then re-released by the receiving undamaged plants that affect their attractiveness to herbivores. For example, when mixed with *Rhododendron tomentosum* plants, a non-host plant of green leaf beetle, the leaves of birch plants, *Betula spp*, have been shown to respond to the non-host plant volatiles and start producing these compounds and found repellent against the beetles. However, plant neighbours producing secondary metabolites, e. g., in response to herbivore feeding, affects the undamaged

plants directly; through the avoidance of the herbivores, or indirectly through increasing visitations of the natural enemies of the herbivores (Barbosa *et al.*, 2009; Agrawal *et al.*, 2006).

## 2 Herbivore-induced responses in plants

Since the initial report on proteinase inhibitor (PIs) by Green and Ryan (1972), numerous defensive responses have been identified in many plant species that are induced by herbivore-feeding or mechanical wounding. In their fascinating work, Green and Ryan (1972) showed that potato and tomato plants accumulate PIs throughout the plant tissues after damage on the leaves by adult *Colorado potato beetles*. This was the starting point for broad field of studies involving herbivore-induced changes in plants (e. g. Agrawal & Karban, 1999; Dicke & Baldwin, 2010; Heil, 2010; Karban & Baldwin, 1997). Studies have been made to understand the mechanisms of biochemical responses in plants in response to herbivore feeding and their ecological effect on growth and development of the herbivores directly and also indirectly through the multitrophic setting.

After hatching, insect larvae start feeding on the green foliage of the host plant selected by the female and can cause a significant loss of plant biomass. In response to the feeding damage by herbivores, the plant starts to produce secondary metabolites. In many cases both volatiles and non-volatile chemicals are produced. These compounds have been shown to affect the host plant preference of the herbivores, to reduce the growth and development of their offspring and/or to attract the natural enemies of the feeding herbivores (Erb *et al.*, 2012; Dicke & Baldwin, 2010; Heil & Karban, 2010; Kost & Heil, 2008; Rasmann & Turlings, 2007; Farmer, 2001). The production of such secondary metabolites after herbivore damage has been shown to affect the defence against herbivores in both at aboveground and belowground systems. (Kost & Heil, 2008; Rasmann & Turlings, 2007; Kost & Heil, 2006; Heil, 2004; Farmer, 2001; Agrawal, 1998; Paré & Tumlinson, 1996).

Two systems have been suggested to explain the mechanisms involved in herbivore-induced defences in plants. The induced responses are classified into “induced resistance” and “induced defence” and both traits help plants to limit

effects of herbivore attack by reducing the preference and performance of herbivores or by attracting their natural enemies. The induced responses that reduce herbivore survival, reproductive output, or preference for a plant are termed “induced resistance”. Induced resistance has been discussed from the herbivore’s point of view, and it does not necessarily benefit the plant. For example, the investment in induced resistance may exceed the benefit from reduced herbivore damage, or induced resistance may render the plants more vulnerable to other potential danger (Karban, 2011; Agrawal, 2005; Karban & Baldwin, 1997).

The induced responses that minimize the negative fitness consequences of the subsequent herbivore attacks on plants either by repelling them or by attracting the natural enemies (predators and parasitoids) of the herbivores are termed as “induced defences”. Induced defences are viewed from plant’s point of view, and the plant gains benefit from these responses in all circumstances. Hence, induced responses plants help them to develop resistance against herbivores and allow them to confront herbivores directly; by affecting either herbivore preference or their reproductive success on host plant, or indirectly; by attracting the natural enemies of the herbivores (Karban, 2011; Dicke & Baldwin, 2010; Heil & Karban, 2010).

## 2.1 Extra-floral nectar production

Herbivore feeding induces plant defence by producing the extra-floral nectar (EFN) that is exploited as alternative food source by carnivorous arthropods. EFN-production has been described in *ca.* 1000 plant species belonging to at least 93 different families (Kessler & Heil, 2011; Karban & Baldwin, 1997). Using an acquisitive approach towards indirect defence, many plants attract ants as well as other predators and parasitoids to their above ground parts by secreting nectar from extrafloral nectaries in order to increase their own reproductive fitness. In the context of EFN-production, vegetative plant parts such as stem and leaves as well as the flowers are actively involved (Heil *et al.*, 2010; Wäckers *et al.*, 2007; Röse *et al.*, 2006; Wäckers *et al.*, 2001). In response to feeding damage by *S. littoralis* larvae, for example, foliar EFN-production in castor *Ricinus communis* and cotton *Gossypium herbaceum* plants has been increased 2.5 and 12 folds respectively, as compared to the control plants or mechanically damaged plants (Wäckers *et al.*, 2001). These plants efficiently adjust their nectar production in order to recruit the predators and parasitoids to the site of attack when exactly needed (Kessler & Heil, 2011; Kost & Heil, 2008; Röse *et al.*, 2006). Kost and Heil (2006) have shown that herbivore-induced EFN production in Lima bean *Phaseolus lunatus* plants results in

increased numbers and duration of visits by carnivorous arthropods e.g. ants and wasps. Moreover, it has also been found previously that EFN production increases under the external application of jasmonic acid (a plant-hormone) on lima bean *P. lunatus* plants that help in reducing the amount of leaf damage indirectly via ants and wasps (Heil, 2004).

## 2.2 Herbivore-induced plant volatile production

A second strategy of induced indirect defence is the production of volatile organic compounds in response to various stimuli e.g., herbivore infestation, pathogen infection, mechanical damage etc. Volatile compounds produced specifically in response to herbivore infestation are known as herbivore-induced plant volatiles (HIPVs). Damaged plants release HIPVs from their exposed herbaceous parts (from leaves and flowers) and from roots as well. HIPVs provide airborne signals either to attract natural or deter ovipositing females from their host plants (Hare, 2011; Dicke & Baldwin, 2010; Heil & Karban, 2010; Arimura *et al.*, 2009; Turlings & Ton, 2006; Rasmann *et al.*, 2005).

Furthermore, studies have shown that some plant species emit HIPVs both locally at the site of damage and systemically distal to damaged parts (Röse & Tumlinson, 2005; Loughrin *et al.*, 1994; McCall *et al.*, 1994). The compounds emitted locally are stored in the tissues and released rapidly in response to the general wounding or mechanical damage to the tissues, while other compounds are *de novo* synthesized by the plant and are released systemically in response to herbivore feeding (Paré & Tumlinson, 1997a). Production of the latter compounds is induced by elicitors present in larval saliva and regurgitate (Alborn *et al.*, 1997). The mechanism of *de novo* compound production is known, but their importance in plant resistance e.g., by affecting the herbivore host selection behaviour has not been studied (Paré & Tumlinson, 1999; Paré & Tumlinson, 1997b).

## 2.3 Ecological significance of HIPVs

HIPV emission from the plant and its perception in the surrounding organisms is quite fascinating because different insect species use these chemicals as information for survival in a complex environment. At tritrophic level, HIPVs attract the natural enemies (predators and parasitoids) of the herbivores in order to reduce the further level of herbivore infestation (Dicke & Baldwin, 2010). For example, HIPVs from maize *Z. mays* plants infested by *S. littoralis* attract the parasitoids *C. marginiventris* and *Microplitis rufiventris* towards infested

parts (D' Alessandro *et al.*, 2006). Similarly, a lima bean plant *P. lunatus* releases HIPVs after the infestation of the two-spotted spider mites *Tetranychus urticae* from the vegetative parts and attract predatory mites *Phytoseiulus persimilis* for protection against an increasing population of *T. urticae* (Dicke & van Loon, 2000). A recent investigation has shown that tobacco *N. attenuata* releases (*E*)-isomers of green leaf volatiles (GLVs) instead of (*Z*)-isomers (produced on mechanical damage) after the infestation of *Munduca sexta*. This isomeric change in GLVs [(*E*)-isomers] ultimately influence the foraging efficiency of the generalist hemipteran predator *Geocoris spp.* towards the exact location of the neonates and eggs on the plants (Allmann & Baldwin, 2010). In addition, HIPVs can provide protection to the below-ground plant parts against the attacking herbivores e.g., the roots of *Z. mays* plant infested by root-feeding beetles *Diabrotica virgifera virgifera*, release (*E*)- $\beta$ -caryophyllene into the soil that can help in the foraging of entomopathogenic nematode *Heterorhabditis megidis* towards the infested roots (Rasmann *et al.*, 2005) (Fig. 1). As HIPVs provide benefits to both the natural enemies and the infested plants, thus they can be categorized as synomones (Table 1).

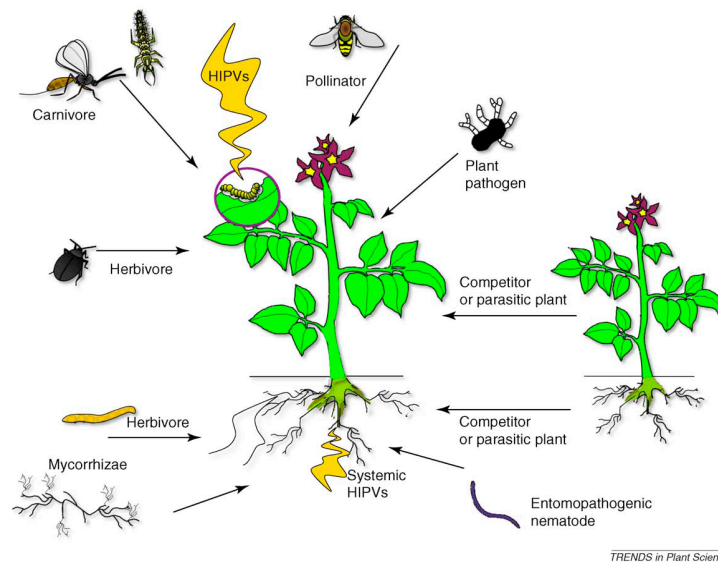


Figure 1. Herbivore-induced plant volatile associated interactions among different organisms (signal receivers) around a damaged plant (signal emitter) (Dicke & Baldwin, 2010).

Table 1. Types and functions of the allelochemicals (Arimura et al., 2009).

Allelochemical	An infochemical <sup>a</sup> that mediates an interaction between two individuals that belong to different species.
Allomone	An allelochemical that is pertinent to the biology of an organism (organism 1) and that, when it contacts an individual of another organism (organism 2), evokes in the receiver a behavioral or physiological response that is adaptively favorable to organism 1, but not to organism 2.
Kairomone	An allelochemical that is pertinent to the biology of an organism (organism 1) and that, when it contacts an individual of another organism (organism 2), evokes in the receiver a behavioral or physiological response that is adaptively favorable to organism 2, but not to organism 1.
Synomone	An allelochemical that is pertinent to the biology of an organism (organism 1) and that, when it contacts an individual of another organism (organism 2), evokes in the receiver a behavioral or physiological response that is adaptively favorable to both organism 1 and organism 2.
Antimone	An allelochemical that is pertinent to the biology of an organism (organism 1) and that, when it contacts an individual of another organism (organism 2), evokes in the receiver a behavioral or physiological response that is adaptively favorable to neither organism 1 nor organism 2.

<sup>a</sup> An infochemical is a chemical that, in natural context, conveys information in an interaction between two individuals, evoking in the receiver a behavioral or physiological response. Pheromone and allelochemical are subcategories of infochemical.



Plants can also resist better against attacking pathogens by using HIPVs. The production of GLVs [C6–aldehydes, –alcohols and –acetates], a prominent and specific part of HIPVs, can reduce pathogen attack. For instance, C6-aldehydes isolated from transgenic *Arabidopsis thaliana* overexpressed with 13HPL have been found active agents against a fungal pathogen *Botrytis cinerea*. Similarly (*E*)-2-hexen-1-ol enhance the resistance of citrus *Citrus jambhiri* against *Alternaria alternata*, while *in vitro* studies on cis-3-hexanol and (*E*)-2-hexenal has shown their anti-growth properties against *Pseudomonas* (Felton & Tumlinson, 2008; Kishimoto *et al.*, 2008; Prost *et al.*, 2005; Gomi *et al.*, 2003).

The HIPVs can also affect the herbivore host selection indirectly (Barbosa *et al.*, 2009). It has been suggested that HIPVs can trigger a signalling mechanism in the neighbouring healthy plants and reduce their attractiveness to herbivores (Arimura *et al.*, 2009; Heil *et al.*, 2008). However, the function of HIPVs in plant-plant communication through eavesdropping is still under debate. Some plant species such as lima bean, cotton, poplar, black alder, sagebrush, sitka willow, tobacco, maize have shown intraspecific signalling while tobacco and tomato has been shown to induce defence responses when exposed to damaged sagebrush volatiles as interspecific signalling (Heil & Karban, 2010; Heil, 2008).

### 3 Objectives of the study

The main objectives of the study were to evaluate:

- ⇒ Whether HIPV emissions from damaged cotton plants provide associational resistance against ovipositing *S. littoralis* moths on undamaged plants in conspecific and heterospecific plant patches. Furthermore, what underlying mechanism confers such resistance, i.e. whether HIPVs affect oviposition behaviour directly or indirectly through plant-plant communication.
- ⇒ The effect of systemically produced volatile compounds on oviposition behaviour in *S. littoralis* females i.e., whether *de novo* synthesized volatile compounds among HIPVs, that are detected by mated female *S. littoralis* moths, are sufficient for oviposition repellency.
- ⇒ The effect of herbivore-induced changes in cotton on pre-mating behaviours i.e., activation, and attraction, and mating behaviours i.e., duration of mating, spermatophore size and mating success, in male *S. littoralis* moths.
- ⇒ Whether herbivore-induced changes in cotton plants affect pre-oviposition behaviours i.e., onset time of calling, time spent in calling, overnight calling rhythms and calling durations in virgin *S. littoralis* females.

## 4 Study system

The Egyptian cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae) is a generalist herbivore that feeds on a wide range of wild and cultivated plants including cotton (*Gossypium hirsutum*) (Fig. 2), alfalfa (*Medicago sativa*) and clover (*Trifolium alexandrinum*) in the agroecosystem in North Africa and the Middle East (Brown & Dewhurst, 1975).



Figure 2. Female *Spodoptera littoralis* on cotton leaf surface.

Under laboratory and field condition, female *S. littoralis* moths have shown to select plants that are suitable for oviposition and larval feeding (Sadek *et al.*, 2010). A hierarchy in the selection among host plants has also been observed under laboratory conditions (Thöming *et al.*, unpublished). Female *S. littoralis* moths have shown preference among different plant species as well as between the host plants depending on their quality. *S. littoralis* moths have shown to reduce oviposition on cotton plants that have previously been damaged by their own larvae. In addition, ovipositing moths were able to assess the quality of the plants that has been damaged by a root feeding heterospecific herbivore (Anderson *et al.*, 2011; Anderson & Alborn, 1999). The positive and negative effects of a host and non-host plants on reproductive behaviour has also been reported in *S. littoralis* moths (Sadek & Anderson, 2007). In response to larval feeding, cotton plants undergo both qualitative and quantitative changes in

volatile emission and also that the emission of volatiles are affected by the diurnal and nocturnal circadian rhythms (Loughrin *et al.*, 1994; McCall *et al.*, 1994). In addition, systemic induction of volatiles has been reported in damaged cotton plants, since these plants release volatile compounds from undamaged leaves apart from the leaves that have been damaged by herbivores (Röse & Tumlinson, 2005; Paré & Tumlinson, 1998).

Similarly, alfalfa and clover also have shown damage-induced changes in the emission of volatiles from the vegetative parts. Chemical analysis revealed an overlap in the profile of volatiles between herbivore-damaged alfalfa and cotton plants (Kigathi *et al.*, 2009; Blackmer *et al.*, 2004). Resistance against attacking herbivores by volatiles from damaged clover volatiles has been reported (Jiang *et al.*, 1997). Electrophysiological studies have shown that female *S. littoralis* moths smell these changes in their host plants. The HIPVs are detected by olfactory receptor neurons housed in sensilla on the antennae (Jonsson & Anderson, 1999) and processed in the central nervous system (Sadek *et al.*, 2002). Female moths have also exhibited modulation behaviour in the detection and perception of plant volatiles before and after mating and that the modulation has been seen at both peripheral and central levels (Saveer *et al.*, 2012; Martel *et al.*, 2009).

## 5 Experiments

### 5.1 Associational resistance via HIPVs against oviposition in *S. littoralis* (Manuscript-I)

In this study we investigated if HIPV-emissions from herbivore-damaged host plant neighbours provide resistance to the undamaged host plants by reducing the probability of herbivore attack on them. Bioassays were performed in the laboratory and under field conditions to test the behavioural effects and possible mechanism of HIPV on ovipositing *S. littoralis* moths with different combinations of host plants.

In the laboratory two pairs of undamaged plants, serving as oviposition plants, were placed on opposite sides inside a cage. Another two pairs of plants were placed just outside the far ends of this cage, serving as neighbouring plants (Fig. 3). Two "plant patches" were thus created, one where the undamaged plants inside the cage were adjacent to undamaged plants outside the cage (undamaged emitters), and one where the other pair of undamaged plants inside the cage were adjacent to plants with ongoing herbivory by *S. littoralis* larvae (damaged emitters) outside the cage.

Three experiments were conducted to investigate the effects of associational resistance via HIPVs within heterospecific plant patches. In each experiment, plants from two species were tested at a time by placing them either at the oviposition (receiver) or the neighbouring (emitter) plant positions. In the first and second oviposition experiment, undamaged cotton plants were used as receivers and damaged and undamaged alfalfa or clover plants were used as emitters. In the third oviposition experiment, undamaged alfalfa plants were used as receivers while damaged and undamaged cotton plants were used as emitters. To investigate the active range of HIPVs on oviposition behaviour, the undamaged cotton plants were distributed inside the oviposition cage at



Figure 3. Laboratory setup to test the influence of HIPVs on the oviposition behaviour in *S. littoralis* (Zakir et al., 2012).

three different distances i.e., 30, 60 and 90 cm away from the damaged plants that were placed outside the cage.

In the field, oviposition experiments were performed using greenhouse-potted cotton plants as a source of HIPVs. Oviposition cages were placed in the field to surround groups of the field-cultivated cotton plants (Fig. 4). Potted plants were taken to the field at the time expected for the beginning of oviposition. For oviposition, *S. littoralis* pupae were placed in the centre of the oviposition cage, buried under a few mm of moist soil, and were allowed to emerge and to then mate inside the cage. After placing the cages in the field, four potted plants that had been damaged by *S. littoralis* larvae for 7 days were placed between the two cages of each pair in the first trial and four undamaged plants were placed outside the far side of each cage in the pair. In both the laboratory and field experiments, the number of eggs was recorded.

In case of intraspecific effects of HIPVs, female moths of *S. littoralis* laid more eggs on cotton plants (receivers) that were adjacent to undamaged cotton plants (emitters) than on plants that were adjacent to cotton plants damaged by *S. littoralis* larvae (emitters) (Fig. 5A). The same result was observed under field conditions, where females laid a significantly higher proportion of egg batches on plants with undamaged neighbouring cotton plants compared to

plants with damaged cotton neighbours (Manuscript-I). The study provides evidence that HIPVs from damaged neighbours are involved in the resistance of the undamaged conspecifics by inhibiting the attraction of the ovipositing *S. littoralis* moths (Agrawal *et al.*, 2006; Atsatt & O'Dowd, 1976).

We also demonstrate that the associational resistance via HIPVs is not a general phenomenon among the host plants of *S. littoralis* tested in this study. Volatiles emitted from herbivore-damaged alfalfa (Fig. 5B) and clover plants did not reduce oviposition on neighbouring undamaged cotton plants, while HIPV-emissions from damaged cotton neighbours reduced oviposition on both undamaged alfalfa and cotton plants (Fig. 5C). Species-specific limitations have also been observed in other plant species. For example, wild tobacco *Nicotiana attenuata* plants growing nearby experimentally clipped sagebrush suffered significantly less leaf loss compared to tobacco plants growing near unclipped sagebrush plants, whereas reciprocal effects were not observed (Karban & Maron, 2002). We found that HIPVs are involved in providing resistance to the undamaged plants against female *S. littoralis* oviposition, as the females were having no physical access to the damaged plants. However, a few examples are available from the herbivore-plant interactions where induced volatiles have been shown oviposition repellent effects. For instance, tobacco plants exhibit herbivore-induced quantitative and qualitative changes in volatile production and were found oviposition repellent for the *Heliothis virescens* and *Manduca sexta* moths under laboratory and field conditions, respectively (De Moraes *et al.*, 2001; Kessler & Baldwin, 2001). For ovipositing females, HIPVs can indicate lowered food quality, increased pressure from natural enemies and risk of competition on the damaged plant (Rasman *et al.* 2005; Dicke & Baldwin 2010; Heil & Karban 2010).

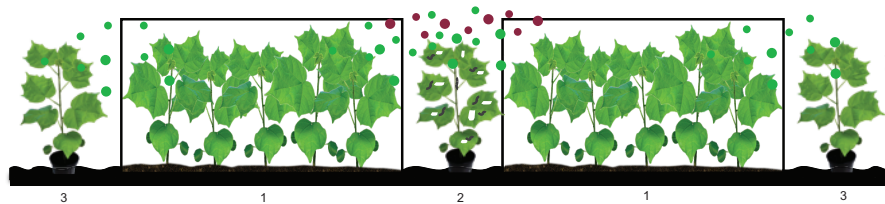


Figure 4. Experimental setup to test the influence of HIPVs on oviposition behaviour in *S. littoralis* under field conditions (Zakir *et al.*, 2012).

We found that associational resistance extended to at least 60 cm from the nearest damaged cotton plant (Fig. 6). This corresponds well with the distance of HIPV induced resistance observed in sagebrush (Karban *et al.*, 2006) and lima bean (Heil & Adame-Alvarez, 2010). However, HIPVs from cotton may act over even longer distances as female choice in our experiments was limited

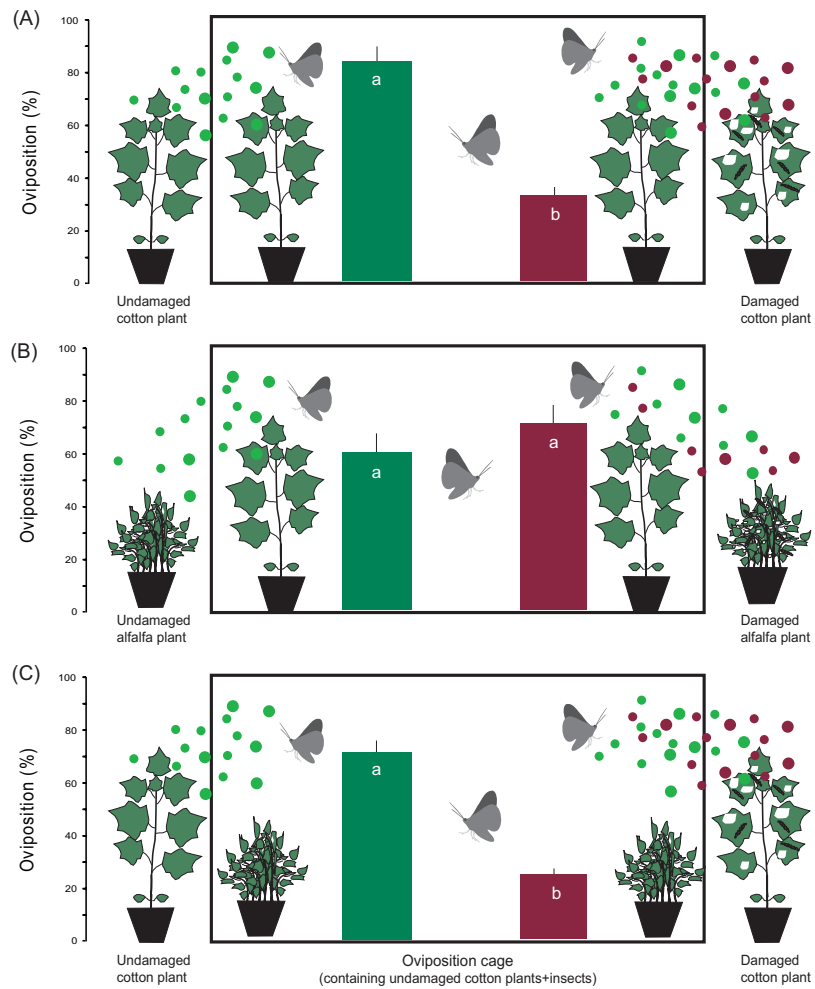


Figure 5. Associational resistance via HIPVs among conspecific and heterospecific plant patches against oviposition in *S. littoralis*. (A) represents intraspecific interactions among conspecific plant patches where damaged cotton neighbours provide resistance to the undamaged cotton plants, (B) represents interspecific interactions among heterospecific plant patches where damaged alfalfa plants neighbours were tested for oviposition preference on undamaged cotton and (C) represents the interspecific interactions among heterospecific plant patches where damaged cotton neighbours provide resistance to undamaged alfalfa plants against oviposition in *S. littoralis*. Paired-sample t-test was used for statistical analysis. Different letters represent significant effect in plant selection and the level of significance was selected at  $\alpha = 0.05$ .



by the experimental cage size. Volatiles from damaged alder trees reduced resistance at least 1 meter from the damaged tree (Dolch & Tschardtke, 2000). No difference in larval feeding was found between leaves from pre-exposed to HIPVs and unexposed cotton plants. The overall leaf area consumption was 49% for leaves on cotton plants pre-exposed to HIPVs and 51% for unexposed cotton plants (Table 2).

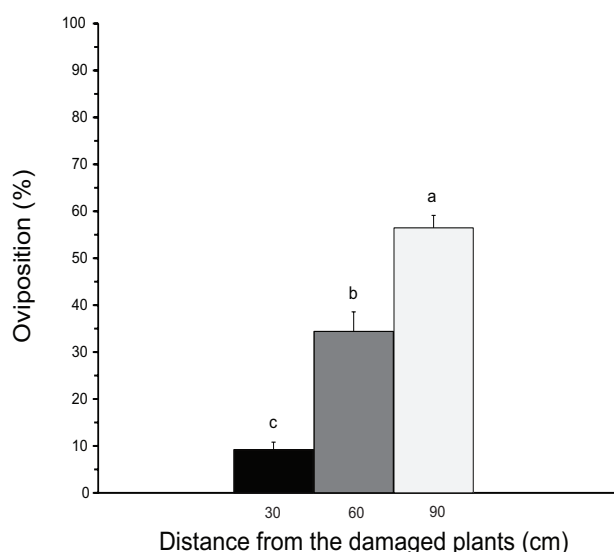


Figure 6. Oviposition preference of *S. littoralis* among three undamaged cotton plants placed at different distances from damaged cotton plants ( $n = 12$ ). GLM-ANOVA with cages as blocks and Tukey's post hoc test was used for statistical analysis. Bars represent mean ( $\pm$ SEs) percentage of eggs on all plants within the cages and different letters on bars show significant difference in selection among the undamaged plant over distance for oviposition at a level of  $\alpha = 0.05$ .

## 5.2 Signal specificity and reliability in *S. littoralis* (Manuscript-II)

In this study we investigated which HIPV-compounds emitted from damaged cotton are behaviourally active. We hypothesized that *de novo* synthesized volatile compounds among HIPVs would be sufficient for oviposition repellency. Systemic production of these volatile compounds would be reliable indicators for the female to avoid plants already under attack by other herbivores. The laboratory experiments were performed in cages where an undamaged cotton plant was placed at one side and another undamaged cotton plant was placed on the other side of the cage and the distance between the plants was around 80cm. The odours to be tested were added through a

Table 2. Leaf area consumption from youngest leaf (YL1), top side-shoot leaf (TSSL), 5th oldest true leaf (OTL5) and 2nd oldest true leaf (OTL2) from HIPV pre-exposed vs. unexposed cotton *G. hirsutum* plants by third instar larvae of *S. littoralis* during a period of 12 hours.

Treatments	Larval feeding duration (hrs)	Leaf area consumption (mm <sup>2</sup> )			
		YL1	TSSL	OTL5	OTL2
Pre-exposed	12	120 ± 15	100 ± 20	200 ± 25	140 ± 20
Unexposed		130 ± 5	90 ± 15	240 ± 30	150 ± 12
N		17	18	15	17
P *		0.863	0.700	0.628	0.831

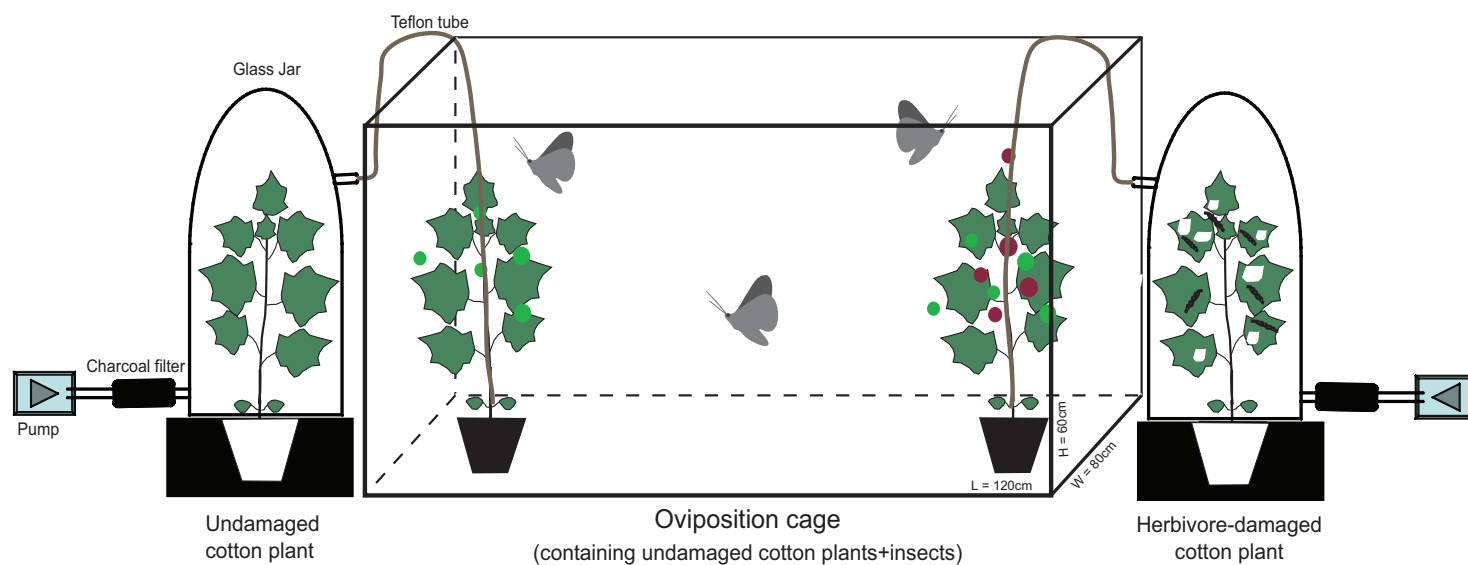
\* Paired-sample *t*-test was used to compare the leaf area consumption between cotton plants pre-exposed and unexposed to HIPVs in all replications (N = 15-18)

delivery system releasing the odour along the stem of the experimental plants (Fig. 7).

Five different oviposition experiments were performed in the laboratory. In the first experiment, female *S. littoralis* moths were allowed to choose between undamaged cotton plants with volatiles added from either a plant with on-going damage by *S. littoralis* larvae or volatiles from an undamaged plant. In the second experiment, headspace from cotton plants with ongoing larval feeding was tested. In the next series of experiments the moths were offered a choice between undamaged control plants and undamaged plants with added synthetic compound mixtures. Field experiments were performed in cages placed in pairs with in a 1400 m<sup>2</sup> cotton field.

We found that the addition of HIPVs from herbivore-damaged cotton plants to an undamaged cotton plant reduce their preference during oviposition in *S. littoralis* females. The females laid fewer eggs on undamaged cotton plants when odours from plant with ongoing damage by conspecific larvae were added (Fig. 8a, A). In our previous study, HIPV emissions from damaged cotton plant neighbours were found to provide resistance to the undamaged plants against ovipositing *S. littoralis* females (Zakir *et al.*, 2012). However, it was unknown which volatile cues females use during selection of plants for oviposition.

This study further demonstrates that among the electrophysiologically active compounds, a blend of seven *de novo* synthesized volatile compounds from herbivore damaged cotton plants reduced oviposition in *S. littoralis* (Fig. 8b, D). The results showed that *de novo* synthesized volatile compounds that are systemically produced by the plant reliably signal damaged plants (Table 1: Manuscript-II). In addition, these compounds are produced in high amounts and in specific ratios after herbivore damage to the plant (McCormick *et al.*, 2012; Dicke, 2009). Whereas, a blend of the remaining 11 electrophysiologically active compounds (Fig. 9), that are not *de novo* produced by the damaged plants, did not affect the oviposition preference in *S. littoralis*. Antagonistic effect of a blend of *de novo* volatiles compounds within the whole blend of HIPVs shows that these volatiles may signal to the ovipositing females about the presence of the conspecific larvae on the damaged plants and are sufficient to affect female oviposition behaviour and help them to evaluate the available food resources (Dicke & Baldwin, 2010; Heil & Karban, 2010; Arimura *et al.*, 2009).



*Figure 7.* Laboratory oviposition set-up: The undamaged plants were placed inside the cage with one plant on each side of the cage, 80 cm apart. To the undamaged plant of one side undamaged plant volatiles or hexane (control) was added, while the other plant was either HIPVs or mixture of synthetic compounds was added.

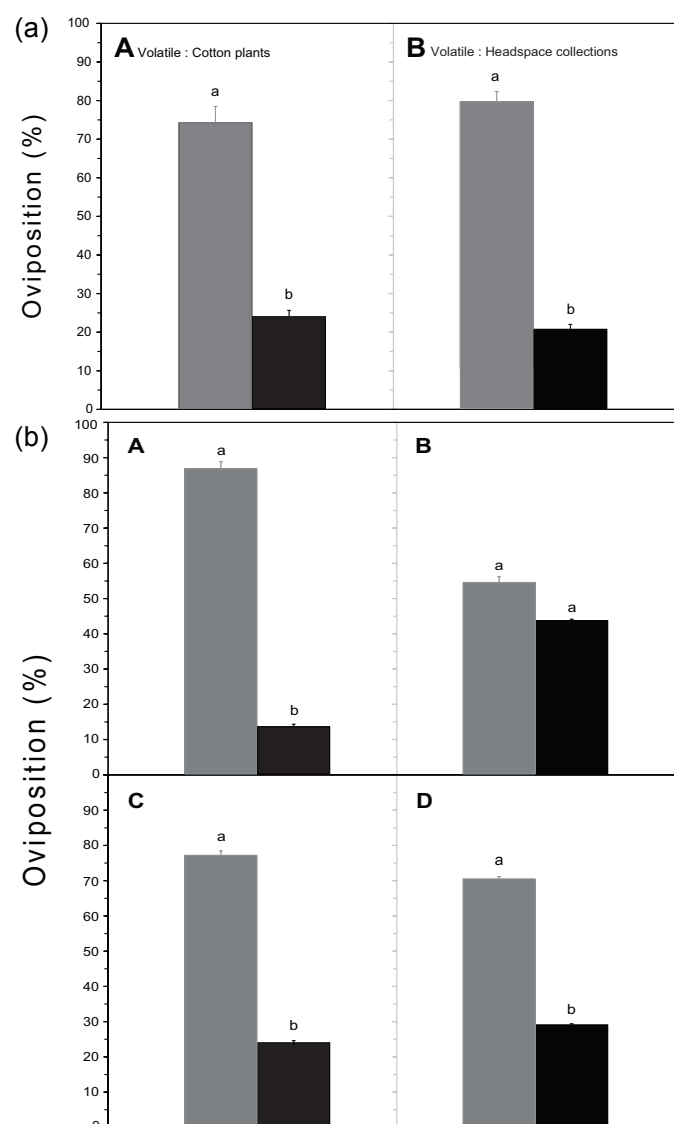


Figure 8. (a) Female *S. littoralis* moths were allowed to oviposit on undamaged cotton plants with added volatiles. Bars represent the means (+SEs) and the percentage of egg deposition was compared after adding the volatile emissions directly from (A) damaged (■) vs. undamaged (■) cotton plants, and (B) headspace collections from damaged cotton plants (■) vs. hexane control (■). (b) Undamaged cotton plants were added (A) with a synthetic mixture of all eighteen GC-EAD active compounds, (B) *non-de novo* (=11) compounds among GC-EAD active compounds, (C) *de novo* (=7) compounds under laboratory conditions and (D) the seven *de novo* compounds under field conditions. Paired-sample t-test was used for statistical analysis. Different letters represent significant effect in plant selection and the level of significance was selected at  $\alpha = 0.05$ .

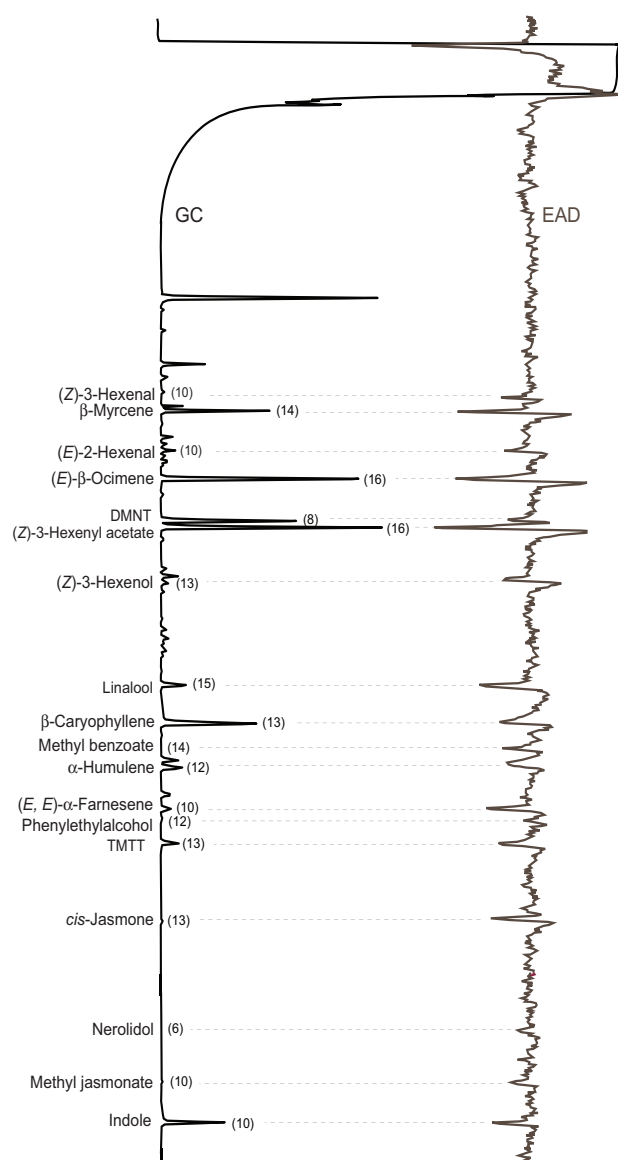


Figure 9. Averaged GC-EAD trace of responses from antennae of mated female *S. littoralis* moths (right trace) towards headspace collections of cotton plants damaged by the larvae of *S. littoralis* (left trace). The number of significant antennal responses for each compound is shown in brackets.

### 5.3 Host plant quality and reproductive behaviours in *S. littoralis* (Manuscripts III, IV)

So far, the effect of herbivore-induced chemical changes in host plant quality has been studied in insect herbivores, particularly in Lepidoptera, either through oviposition behaviour or through the performance of the offspring (Howe & Jander, 2008; Agrawal, 2001; Karban & Baldwin, 1997). However, it has not been investigated how herbivore-induced chemical changes influence pre-oviposition i.e., mating and calling behaviours, in herbivores.

To evaluate the effect of herbivore-induced changes on mating behaviour, individual plants of either undamaged or damaged cotton plants were transferred to the Plexiglas cages. Two larvae of *S. littoralis* were placed in a rectangular pocket of fine mesh, opened at one side, and the second true leaf of the cotton plant covered with the mesh around 48 hours before the start of the mating experiments. At the onset of the scotophase, five females were released from the top-side of the cage that were allowed to come in contact with the plants and three males were released from the glass tubes near the base of the plants. Duration was calculated from male activation till onset of the successful mating.

We found that herbivore-damaged larval host plants affect male attraction to female pheromone and mating behaviour in *S. littoralis*. Activation of males for females was reduced when damaged cotton plants were present compared to undamaged plants. We also observed a reduction in mating frequency and onset of mating in *S. littoralis* moths in the presence of damaged cotton compared to the undamaged plants. The reduction and delay in mating in the presence of damaged plants shows that male *S. littoralis* are affected by herbivore-induced changes in plant cues (Fig. 10). The impact of herbivore damage on precopulatory and mating behaviours in male moths, in this study parallels the effect of damaged plants on oviposition behaviour of mated female *S. littoralis* (Zakir *et al.*, 2012; Anderson & Alborn, 1999).

Furthermore, *S. littoralis* male moths were more attracted to pheromone in the background of undamaged plants than in the background of herbivore-damaged plants (Manuscript-III). Concurrent effects of host plant odours on male moth attraction towards female sex pheromones have been seen in several studies. These studies have shown that host plant volatiles synergizes the male response to female sex pheromone in moths (Schmiera & Guerin, 2012; Varela *et al.*, 2011; Witzgall *et al.*, 2008; Tasin *et al.*, 2007; Landolt & Phillips, 1997). In addition, we observed that male *S. littoralis* moths found females faster in the presence of undamaged cotton plants compared to the damaged cotton plants (Paper III). In a wind tunnel study on male codling moth *Cydia pomonella*, a similar effect has been shown. Males were attracted more rapidly towards

female sex pheromone in the presence of host plant background odour, compared to when only sex pheromone was present (Schmera & Guerin, 2012).

Similarly, we observed a significant reduction in the frequency of virgin *S. littoralis* females calling in the presence of damaged cotton plants compared to when undamaged cotton plants were present (Fig. 11). In this context, our study provides an initial insight into the negative effects of herbivore-induced host plant compounds on calling behaviour in virgin females. Delayed onset time of calling as well as shift in calling peaks during early scotophases were also observed (Manuscript-IV). The modulated calling behaviour in virgin female moths may provide a new direction to understand effects of host plant quality on mating success (Manuscript-III) and oviposition decisions in *S. littoralis* moths (Zakir *et al.*, 2012).

A reduced number of calling females combined with a reduced calling time on damaged plants indicate that pheromone release by the female moths is influenced by plant volatiles that indicate the quality of the plants as food for the progeny. It is known that cotton plant undergoes chemical changes after damage by the larvae of lepidopteran herbivores (Bezemer *et al.*, 2004; McCall *et al.*, 1994). These changes are known to influence growth and development in young conspecifics, host plant selection adult conspecifics during oviposition (Bezemer *et al.*, 2003; Anderson & Alborn, 1999; Alborn *et al.*, 1996) and can also attract natural enemies of the young (Paré & Tumlinson, 1999). Reduced calling behaviour in *S. littoralis* females has earlier only been reported in the presence of non-host plants (Sadek & Anderson, 2007).



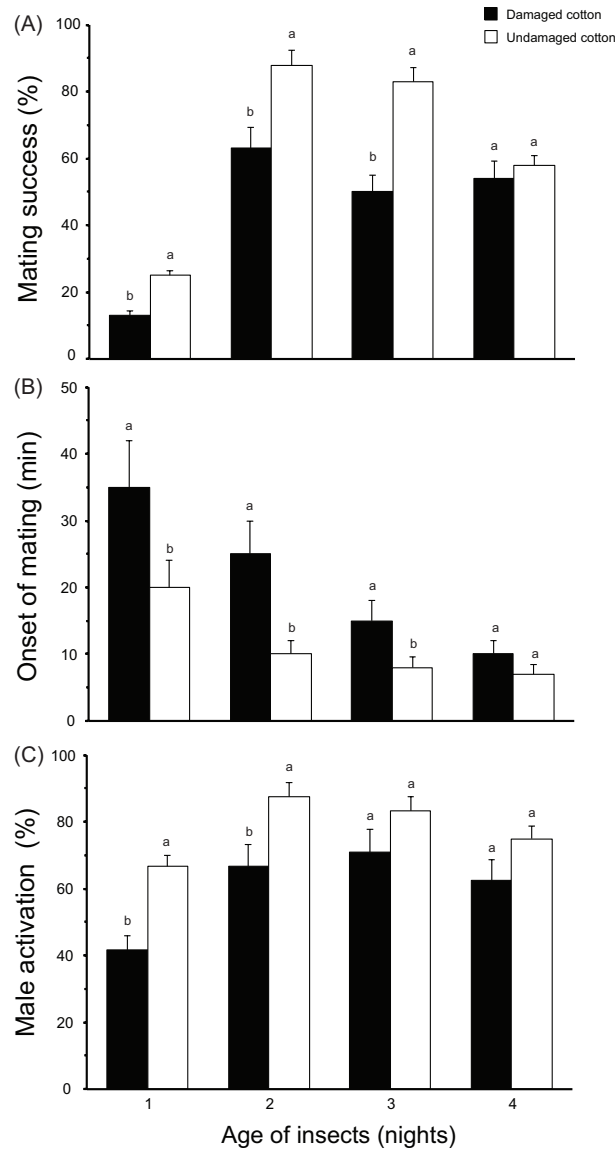


Figure 10. Percentage of mating success (A), mean onset time of mating (B) and percentage of male activation (C) in *S. littoralis* under damaged and undamaged cotton plants. Male and female moths were allowed to mate in the presence of either damaged cotton plants (black bars) or undamaged cotton plants (white bars), over a period of consecutive four days. Bars represent means and standard errors (+SEs). Chi square test was used for statistical analysis ( $n = 32$ ). Different letters within the bars of each age is to show significant effect ( $P < 0.05$ ).

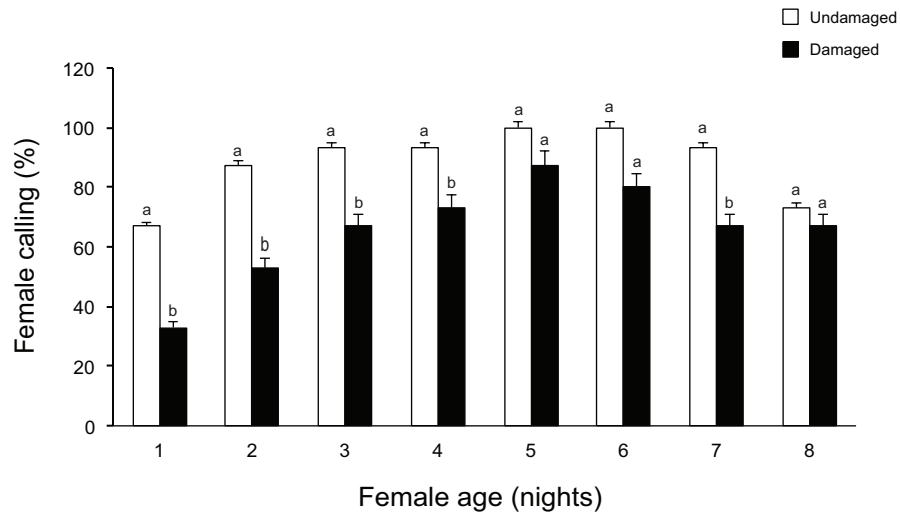


Figure 11. The proportion of *S. littoralis* females exhibited calling behaviour in the presence either damaged (black bars) or undamaged (white bars) cotton plant over eight consecutive nights. Bars represent means and standard errors (+SEs). Chi square test was used for statistical analysis. Different letters within the bars of each age is to show significant effect and the level of significance was selected as  $P < 0.05$ .

## 6 Summary, conclusion and future directions

This study provides evidence that herbivore-induced changes in cotton influence reproductive behaviours including pre-oviposition, male attraction to females, female calling and mating behaviours as well as oviposition, in the generalist moth *S. littoralis*. We found that herbivore-induced changes in cotton plants reduce male *S. littoralis* attraction and activation towards conspecific females and reduced the mating success (Manuscript-III). The wind tunnel experiments showed that males were attracted towards the female sex pheromone when undamaged cotton plants were present in the background and/or that males avoided sex pheromone released from damaged plants in the background. A possible mechanism could be that the HIPV emissions from damaged host plants have a masking effect and that the male moths are unable to find a female in the presence of the damaged plants (Agrawal *et al.*, 2006). Another possible mechanism could be that odours from the damaged cotton plants may have antagonistic effect on male attraction towards female sex pheromones and that male moths avoided the sex pheromone when offered with damaged cotton plant odours.

It would be interesting to find the volatile chemical compounds responsible for male avoidance and the mechanism behind it at a neuronal level. Though there is no evidence about the presence of olfactory neurons co-localized with pheromone and plant volatiles detecting cells in single sensilla on female *S. littoralis* moth antenna (Binyameen *et al.*, 2012), but studies are needed to know in male moths as been found in some coleopterans (Andersson *et al.*, 2010). It seems that the information is processed at central or higher brain levels in male *S. littoralis* moths. Neurophysiological studies using functional imaging (Saveer *et al.*, 2012), can help in understanding the effects of induced plant volatile antagonistic with female sex pheromone. In addition, to confirm the effect of HIPVs on male mating disruption, reflected in no-choice mating

experiments, further behavioural assays are required under laboratory and field conditions. Possibly, HIPVs could synergize pheromone-mediated mating disruption, since it could affect female mating on plants or plant patches and oviposition.

An effect of herbivore-induced changes in cotton plants on female *S. littoralis* calling behaviour was observed. We found a significant reduction in the frequency of calling in virgin *S. littoralis* females in the presence of damaged cotton plants. Furthermore, the onset of calling was also prolonged and females were found calling mostly in the late hours of the scotophase. In this context, our study provides an initial insight into the effects of herbivore-induced host plant compounds on calling behaviour in virgin females (Manuscript-IV). These results indicate that virgin female moths are able to assess the quality of the plant and adjust calling behaviour accordingly. In addition, the increased activity of females found in the presence of damaged plants indicates increased dispersal activity elicited by HIPVs. The results suggest that herbivore-induced changes may be repellent also for females or that they affect host choice behaviour and resting time when in contact with the damaged plants. It will be ecologically interesting to identify the chemical cues responsible for both male and female behavioural changes and to elucidate the mechanism affecting pre-oviposition reproductive behaviour in *S. littoralis*.

Emissions of HIPVs from herbivore-damaged cotton plants provide associational resistance to undamaged plants against ovipositing *S. littoralis* females. In the presence of damaged cotton plant neighbours, we found a reduction in oviposition by *S. littoralis* on undamaged plants and also that the associational resistance effect via HIPVs was seen in both conspecific and heterospecific plant patches. However, no effect was found when damaged alfalfa and clover plants were used as neighbouring plants in the plant patches (Manuscript-I). The unidirectional effect we found shows that there is a differential influence among plants in a habitat. The fitness of a specific plant is directly affected by its neighbours and this may favour specific constellations of plants.

The active range of the HIPVs i.e., how far the effect can be observed, is an important parameter to determine the strength of a defence strategy adopted by the plant would work against a generalist herbivore searching plants for oviposition. We found that the active range of the HIPVs repellent for oviposition in *S. littoralis* is at least 60 cm. Reduced oviposition not only on the nearest neighbouring plant indicate that the volatiles travel horizontally and could repel the females at a distance. The effects of the HIPVs is thus not localised to the damaged plant and its immediate neighbours, but has the potential to affect a larger patch of plants. This could affect competition the

risk of parasitization and predation by natural enemies not only on the species that causes the initial damage, but also have ecological consequences for other plant and herbivore species in the near environment. Further studies are needed to investigate the effects and mechanisms of associational resistance via HIPVs in other specialists and generalist herbivores as well as other host and non-host.

To understand the mechanism behind, it is important to investigate the specific volatile compound(s) that are behaviourally active (de Bruyne & Baker, 2008). Our experiments showed that a blend of seven *de novo* synthesized volatile compounds were sufficient to repel oviposition on undamaged plants (Manuscript-II). In this study, we have used a combination of classical and modern approach to identify the behavioural relevant compounds. Laboratory approaches such as GC-EAD and neurophysiological studies are used by insect physiologists for identification and understanding the ecological relevance of identified volatile compounds during insect host location behaviours. For instance, out of six EAG active compounds from the infested broad bean *Vicia faba* plant, 6-methyl-5-heptene-2-one was found as highly attractive for the aphid parasitoid *Aphidius ervi* (Du *et al.*, 1998). The use of state-of-art techniques is a need of the time to understand the mechanisms and the chemical cues at sensory levels of the herbivores. Perception and detection of systemically produced *de novo* volatile compounds in mated *S. littoralis* females may indicate the level of reliability of signalling cues in generalist herbivores. *De novo* synthesis of volatile compounds can reliably indicate the presence of another herbivore feeding on the plants and avoid them to oviposit on plants surrounded with these volatiles. In addition, future studies are needed to fully understand the ecological significance of specific signalling cues induced by herbivore feeding and providing reliable information to ovipositing insect herbivores.

In the light of the above discussion on herbivore-induced plant defence against herbivores, we conclude that the risk of herbivory in a plant patch can largely be reduced if the host plants are efficient in producing HIPVs in response to the herbivore attack. The influence of HIPVs was found in both unmated male and female as well as on mated ovipositing *S. littoralis* females. In an environment with damaged plants, male moths were found not responding to the females as efficient as when only undamaged plants were present, which indicates HIPV-emitting can modulate male mating behaviour. An effect was also seen on female calling and oviposition behaviour. Thus, the presence of HIPVs has been shown to effect reproductive behaviour at different levels, calling, mating and oviposition. This makes it possible to use HIPVs as a part of push-pull strategies and can increase plant resistance by (i)

pushing the herbivores away as well as (ii) by pulling the natural enemies of the herbivores towards the host plants (Cook *et al.*, 2007). In order to cope with the herbivore attack, under field conditions, screening of push-pull components from HIPVs blend could become important. Functional and mechanistic knowledge of HIPV interactions with insect herbivores could help agricultural systems in monitoring and controlling insect pests in a sustainable and environmentally safe manner. Thus, the best combination of different approaches can contribute to Integrated Pest Management programs.

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