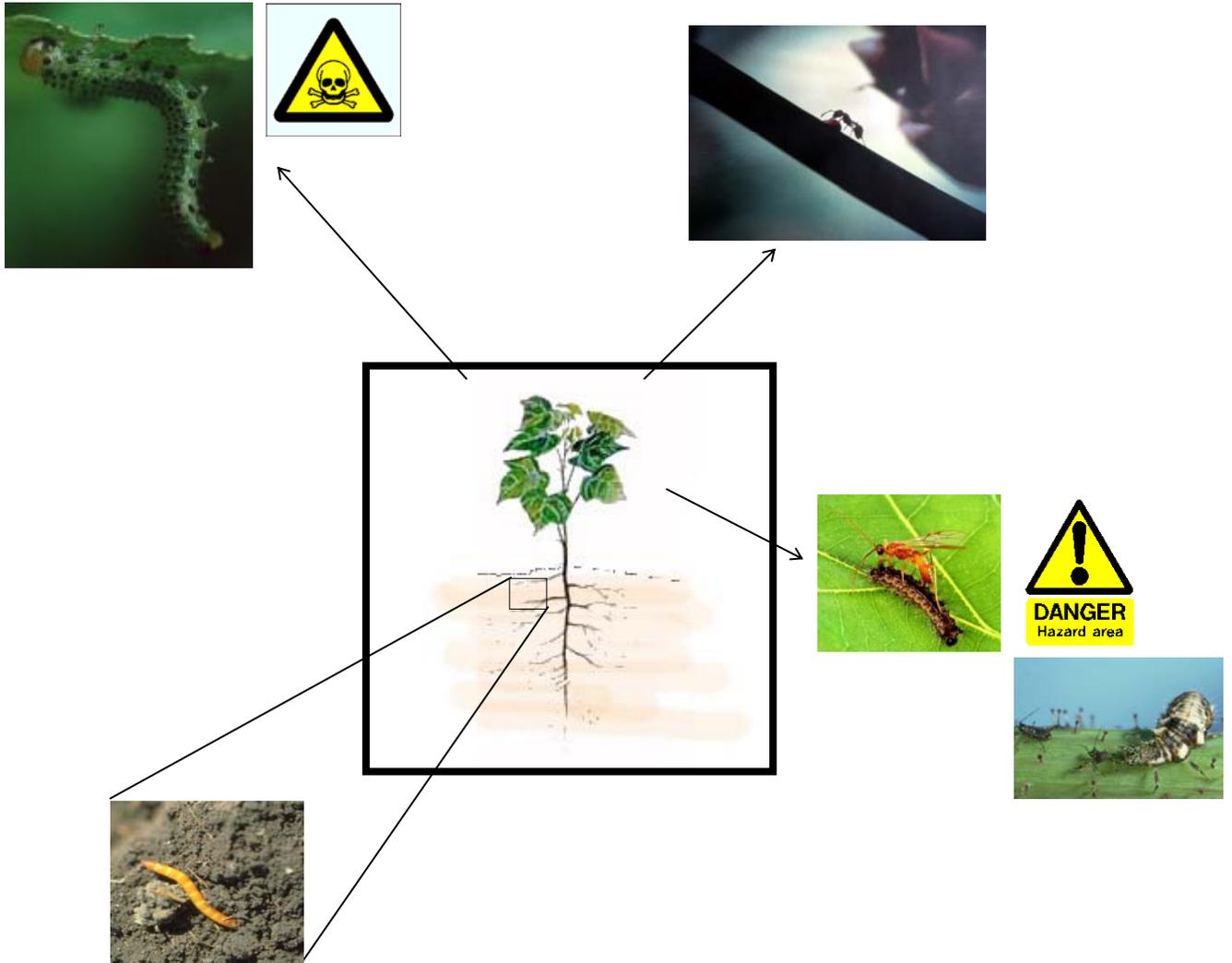


Consequences of belowground induced defence





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Alteration of aboveground plant-insect relations in response to herbivory by root chewing insect larvae (*Agriotes lineatus*) in cotton (*Gossypium herbaceum*)

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Summary

Plants are frequently exposed to both root- and foliar insect herbivores. Nevertheless, the interaction between aboveground (AG) and belowground (BG) systems is rarely studied. Plants are able to defend themselves against herbivorous attackers by means of many different mechanisms; local vs systemic, constitutive vs inducible and direct vs indirect. Belowground herbivores have demonstrated to be able to change direct and indirect plant defense aboveground. Recent studies in cotton (*Gossypium herbaceum*) have shown increased foliar terpenoid aldehyde levels in response to BG-herbivores, which reduces palatability and fitness of herbivores. Furthermore a systemic increase in extrafloral nectar (EFN) production has been found.

We conducted several experiments addressed to the question how BG-herbivory can affect aboveground plant-insect relations. Cotton plants (*G. herbaceum*) were exposed to seven root-feeding insect larvae (*Agriotes lineatus*) inoculated prior to the experiments. Cotton produces EFN as a putative indirect defensive mechanism, as natural enemies, mainly ants, are attracted by this nutritional food source.

In a factorial field experiment insect numbers were recorded over time on cotton plants exposed to root herbivores and control plants. The previously reported increased EFN secretion induced by root herbivory was not reflected in a higher number of insects visiting the plants. The expected increment in number of plant beneficials (ants) and subsequently lower number of herbivores (aphids) was also not observed to be influenced by the plant treatment. We discuss the fact if plants actually displayed higher nectar secretions under conditions suboptimal to the plants. Furthermore is interpretation of our results arguable since the experiment was conducted with a non-natural system.

The effect of BG-induced increment in foliar terpenoids on herbivore behaviour was determined in a bioassay. Under greenhouse conditions the feeding pattern of a leaf feeding larvae was recorded. One individual lepidopteran larva *Helicoverpa armigera* was allowed to feed on a plant exposed to root herbivory in the presence of an undamaged alternative individual. Our data suggests no lower food consumption of the larvae on the BG-damaged plants, nor an effect on larval growth rate. Increased terpenoid levels have shown to deter larvae of generalist lepidoptera and have demonstrated to decrease its fitness. Therefore we question if terpenoid levels were actually increased during the experiment.

In cotton, the composition of volatile emission spectrum changes in response to aboveground herbivory. This is regarded to serve as an indirect defense mechanism as these cues are exploited by parasitoids in search for potential hosts. However, there is increasing evidence that also herbivores might exploit these volatile signals. We studied the possibility of BG herbivory to manipulate the volatile emission spectrum aboveground, which is to our knowledge, a novel aspect in linking the below- and aboveground plant compartments. In a bioassay an attempt was made to test preference of ovipositing females of *Spodoptera exigua* towards a BG-induced and an undamaged control cotton plant. However, these generalist herbivores didn't show any preference. In all cases a higher proportion of eggs was deposited on the cage instead of on either of the two plants. We discuss the use of generalist herbivores in these choice tests.

Furthermore we conducted a volatile odor collection (VOC) from cotton plants subjected to AG, BG and simultaneous above- and belowground herbivory over time. Additionally, plants subjected to the latter two treatments were offered to the specialist parasitoid *Microplitis croceipes* in a dual-choice flight response experiment.

Overall, this experiment remains inconclusive, as our VOC's, analysed by GC-MS, showed a level of background air contamination too high to draw conclusions. Furthermore showed our wasps a level of flight response considered to be too low of to address any preference.

Despite our results, it is likely that effects of changed plant defense, direct as well as indirect, induced by root herbivory, has the capacity to influence multitrophic interactions aboveground. Nonetheless, we discuss the ecological relevance of previously discovered mechanisms in BG-induced AG plant defense. So far, most of the effects of BG herbivores were demonstrated under, for the plant, optimal conditions. In contrast to the greenhouse, plants in nature have to cope with competitors and endure far from optimal conditions, which can alter its strategy in resistance against herbivore attackers. Therefore we plead for more large-scale manipulative field experiments that are required to fully understand the importance of root herbivores. Awareness of the existence and knowledge about mechanisms linking above- and belowground plant compartments has the potential to provide new areas for research on the ecology and evolution of plants.

Key words: *Gossypium herbaceum*; *Agriotes lineatus*; *Helicoverpa armigera*; *Microplitis croceipes*; *Spodoptera exigua*; multitrophic interactions; inducible defense; protective mutualism; extrafloral nectar; terpenoids; belowground herbivory; volatiles; oviposition

Introduction

Much of surface of our planet is covered with vegetation. This implies that either herbivores are too few in number to reduce plant cover significantly, or, more logical, plants seem to be able to defend themselves sufficiently against potential herbivores.

The diversity in organisms having a direct plant-based diet is impressive. Plant consuming organisms range from large mammalian browsers consuming whole plants in a single bite, to small hemipterans parasitising on the vascular transport system. Because insects make up a very large fraction of biomass of life on earth and, additionally, possess enormous diversity, these smaller herbivores pose the biggest threat for plant survival (Karban and Baldwin, 1997).

Although it seems obvious that herbivores can have a considerable negative effect on plant fitness, the consequences of herbivore feeding are usually not fatal. In reaction to the almost inevitable contact with plant-eating species, plants have evolved traits that defend them against, or make them tolerate, damaging effects of herbivores (Karban and Baldwin, 1997). In response, also herbivore species have evolved offensive and adaptive mechanisms that allow them to circumvent plant defences and enhance exploitation of a host plant (Karban and Agrawal, 2002). This systematic arms-race between phytophagous organisms and host plants has led to the enormous diversity in plant defence traits as well as herbivore offensive tactics as we know it today and which has fascinated scientists for generations.

Induced plant response

The diversity of plant parasites and predators has several consequences for the methods in which plants protect themselves. A resistance mechanism effective against one herbivore may be ineffectual or even beneficial for another herbivore species. This suggests that plant defence mechanisms may be directed to universal efficacy instead of specificity against certain herbivorous groups. Generally taken, this is the case concerning constitutive and preformed plant defence expression. The latter indicates defence that is only controlled on the level of preformed precursors, but also functions nearly independent of damage. A high level of these defences should be beneficial in circumstances where likelihood of attack is high or rapid response is vital (Zangerl and Rutledge, 1996; Karban and Baldwin, 1997).

The problem with this uniform, fixed approach in defence tactics is that, as already mentioned, a plant becomes really vulnerable to herbivores that overcome the static defence barrier. Moreover, a constant level of high resistance is accompanied by a constant resource allocation to defence even in periods when no herbivores are present (Agrawal and Karban, 1999). Finally, possible autotoxic effects during storage and release of these defence compounds can be accompanied by negatively effects on plant fitness (Agrawal and Karban, 1999).

Only recently it is recognized that many of the traits and processes that defend plants against herbivores actively change after herbivore attack. Instead of having no control or control on preformed precursor level only, plants are able to respond dynamically and specifically to damage inflicted by certain herbivores and pathogens (Karban and Baldwin, 1997).

Induced synthesis of defence compounds, regulation on transcriptional level, in response to phytophage attack has been observed in many different plant species (Dicke, 1999). It involves specific signal transduction pathways which are activated in response to specific herbivore cues (see box 1).

According to Karban and Baldwin (1997), these so-called induced responses can be grouped in two categories which are not mutually exclusive. Responses that reduce primarily herbivore survival, reproductive output or plant preference are summarised in the term 'induced resistance'. Responses that primarily decrease plants fitness losses without by definition negatively affecting the herbivores are called 'induced defence'. The latter also includes induced plant tolerance and compensatory responses as (re-)allocation of resources (Karban and Baldwin, 1997).

As one herbivore species feeding on a host plant induces specific detrimental resistance compounds which may be beneficial for another species, it has to be mentioned that the terminology described above becomes unpractical. In a multitrophic perspective, plant fitness depends on the total balance of costs and benefits of inducing a particular compound (Agrawal and Karban, 1999). Although the putative term 'induced response' summarises all phenotypic changes in the plant after induction (Van Dam *et al.*, 2003), I will use the term 'induced defence' indicating any defence trait in response to plant damage.

Box 1: Signal transduction

Both abiotic stress as well as biotic factors, such as pathogens, arthropods, or higher organisms, may trigger different plant defence responses. Karban and Baldwin (1997) recognised five partially overlapping mechanistic models to explain how secondary metabolites change after damage. Three of these don't require any specificity in the plant's signalling system, but state that secondary metabolites accumulate in response to imbalances between growth-processes and metabolite production. The generalized stress-response theory posits a non-specific hormonally mediated centralized system of physiological responses. As many induced responses can be affected by stress-related plant hormones, these may play a role in a generalized stress response. Only the active defence response theory, which is now well recognised, posits far more specificity in response to exogenously inflicted damage. Specific cues of attacking herbivores activate plant signal transduction which control biosynthetic pathways. Experiments revealed specific chemical changes following herbivory while no such effects were found in response to mechanical damage. Plants are able to perceive its attacker by specific herbivore elicitors, such as components in caterpillar saliva, which have been identified as inducers triggering "the novo" synthesis of metabolic compounds resulting in a specific plant defence response (Korth and Dicon, 1997).

The ability of plants to produce defensive compounds in response to infestation with herbivores requires signal processing in the plant. Many different signalling compounds have been identified; most of them are centred around different plant hormones as salicylic acid (SA), jasmonic acid (JA) and ethylene (Van Poecke & Dicke, 2003). While there is still a lack of knowledge in the underlying mechanism, the major pathways involved in defence responses include the octadecanoid and the isoprenoid pathway (Dicke and Van Poecke, 2002). The latter pathway is involved in the induction of terpenoids; one of the largest and most diverse group in plants secondary compounds, involved in both direct and indirect defence (Karbon and Baldwin, 1997).

There are generally taken two ways in which a plant reacts to wounding on a specific plant part regarding the spatial scale of response involved. **Systemic induction** involves a plant response in which the damaged plant tissue may produce a signal that is transmitted uniformly throughout the plant or vary between plant parts, causing a response in plant tissue not under attack. **Local induction** is regarded as induced responses restricted to the same plant parts in which the damage has been inflicted (Karbon and Baldwin, 1997).

Recently it has been suggested that defence pathways, involved in defence against herbivores or plant pathogens, can interact. In this so-called 'crosstalk' defence-inducing signals might be transferable between roots and shoots through defence pathways. Soil pathogens could for instance, indirectly activate, or impair, the induction of defence against aboveground herbivores (Van der Putten, et al. 2001, and references cited herein). To obtain better understanding and forecast ecological consequences of these pathway interactions, which could pose a mechanism by which two spatially separated herbivores feeding from the same host plant might interact, more studies in this area are essential.

General plant defensive traits

Direct defence

Direct defensive responses can be mediated by plant morphology, structures such as trichomes, spines and thorns, as well as plant chemicals such as toxins and repellents (Dicke and Van Poecke, 2002). Plants have been especially successful in deploying toxic and digestibility reducing chemicals as a means of discomforting herbivore attackers. This success is limited by the ability of plants cope with broadly effective chemicals as defence, which are likely to be toxic to its producer. Their exceptional anabolic capabilities, that allow them to synthesize an amazing diversity of compounds, partly overcome problems associated with storage and deployment. Second, plants exploit the fundamental physiological differences in their expression of chemicals used for defence (Karbon and Baldwin, 1997). However, plants still face problems of autotoxicity.

Indirect defence

In contrast to a typical bottom up regulation of herbivores by direct defence, indirect defensive traits are based on a top down control of plant detrimental organisms (Townsend et al., 2003). Predaceous and parasitic arthropods can play an important role in the regulation of herbivore populations. The term indirect response refers to those adaptations that result in the recruitment and sustenance of these carnivorous organisms that protect plants against their herbivorous attackers (Turlings and Wäckers, 2004) The two main strategies to arrest the natural enemies are by means of the production of nutritious compounds and herbivore-induced production of specific plant odours

Inducible nutrition

At least during part of their life cycle, most arthropod predators and parasitoids require plant-provided foods. These nutritional supplements include various plant-derived substrates like extra floral nectar (EFN), food bodies, pollen and certain honeydews (Wäckers, 2005).

Obligatory consumers of plant-provided foods are (usually in the adult stage) entirely dependent on non-prey food. This category includes many important herbivore antagonists like syrphid flies and many parasitoid species. A number of studies in biological pest management are aimed at increasing biodiversity and hereby conservation and stimulation of existing natural enemies in agroecosystems. The strategy of sustaining natural existing enemy populations by selectively introducing plants producing accessible (extrafloral) nectar can be a promising method to enhance efficacy of natural control (Wäckers, 2005).

Facultative consumers use plant-derived food as a supplement to the consumption of prey and include many predaceous species as spiders, beetles and ants (Wäckers, 2005). In fact, ants are considered to have played a key role in the coevolution of a range of food-mediated plant-insect mutualisms and are regarded as the most common plant defenders (Koptur, 1992). Early published examples of indirect responses involved intimate ant-plant relations, in which myrmecophilous plants have evolved a range of adaptations providing ants with both protective structures (domatia) as well as various food sources (Belt, 1874; Janzen, 1966). These food providing structures also seem to be partly inducible as the amount of nectar producing glands was shown to increase after herbivore damage in broad bean, *Vicia faba* (Mondor and Addicott, 2003). Wound-induced changes in the secretion as well as in chemical composition of EFN have also been observed on other systems (Wäckers et al., 2001). Both in cotton and in castor damaged leaf secreted higher levels of EFN, hereby guiding ants to the leaf under attack. Furthermore, also changes in amino acid contents, particularly towards those attractive to many ant species, have been observed in response to leaf feeder activity (Smith et al., 1990).

In return, ants provide protection against herbivores, by defending their sugar source and removing herbivores as food for their offspring. While extrafloral nectar usually falls short from providing essential amino acids, it forces ants to seek out supplementary protein sources, thereby stimulating predation (Smith et al., 1990). It is now recognised that the dominant ant species are largely consumers of plant-derived carbohydrates (Tobin, 1994). Only the larvae depend on protein-rich food, as mature stages survive mostly on sugar-rich plant-provided nutrition (Boevé and Wäckers, 2003) (Tobin, 1994).

Inducible volatile signals

Entomophagous arthropod foraging consists of a series of behaviors that are affected by information from the surroundings. Besides visual information (Wäckers and Lewis, 1999) parasitoids utilize, mainly (volatile) chemicals as sources of information. In all stages of actively prey or host searching and selection described as: 1) habitat or host community location, 2) microhabitat location and acceptance, 3) host location and acceptance, chemical information plays an essential role (Dicke et al., 1990). These infochemicals emitted by herbivores and their products could pose reliable information for foraging carnivores. However, direct detection of potential prey is minimized as herbivorous arthropods have been under natural selection to avoid emission of volatile cues that enhance their detectability. Furthermore phytophagous arthropods constitute only a relatively small amount of biomass compared to their host plant.

Indirect detection, mediated by volatiles released by plants damaged by herbivores is now a well recognised phenomenon (e.g. (Sabelis et al., 1984; Dicke and Vet, 1999). Plants actively send these so-called synomones (Dicke and Sabelis, 1988) giving information essential for successful location of herbivores by carnivores. By increasing the hunting efficiency of natural herbivore enemies, which learned to associate emitted plant-derived volatiles with prey (Turlings et al., 1993), the plant is able to increase its fitness by lowering potential feeding damage (Dicke and Sabelis, 1990). However, it has been demonstrated that these defence volatiles can also have a negative side effect for the plant. Volatile cues can be exploited by herbivorous insects in both oligophagous and generalist insects (Bernays and Chapman, 1994) (see box 2).

Box 2: Plant stimuli used by herbivores in host location

The recognition of plants followed by the decision of selecting it as a host plant for oviposition or feeding, performed by herbivorous insects can be divided in three different stages: 1) orientation, 2) landing and 3) assessment (Renwick, 1989). Host plant volatiles are involved at least in the first two stages, as they have shown to induce landing by various gravid insect species (Bernays and Chapman, 1994), whereas assessment of a leaf surface and trigger of oviposition is primarily based on non-volatile contact stimuli. Hence, perception of chemical cues affecting oviposition involves receptors on antennae, tarsi, mouthparts or the ovipositor.

In Lepidoptera, offspring performance is regarded to be primarily based on selection of the ovipositing female for a specific host plant. Many studies have focussed on the link between the two stages (larva and adult) in Lepidopteran life cycle (Singer, 1984; Berdegue *et al.*, 1988), but remarkably, in a high number of cases the correlation between adult host preference and offspring development appears not to be positive one (Berdegue *et al.*, 1988; Fox and Eisenbach, 1992; Singer *et al.*, 1994; Mulatu *et al.*, 2004). Part of this phenomenon has been explained because the relationship between host choice and larval performance varies under different ecological conditions and selection pressures (Thompson, 1988). If we consider only the nutritional benefits as a driving factor in host preference, it would be obvious to expect the phytophagous insect, adult and larva, to choose the optimal food source for oviposition and development.

Factors of influence on the oviposition preference of the female moth could be:

Mobility of the larvae

Larvae of the highly polyphagous beet army worm (*Spodoptera exigua*) are very mobile (Smits *et al.*, 1987) and able to disperse from the site of oviposition. The effect of this could be that a higher fitness of the female moth is reached by selection based on factors improving her own survival instead of direct nutritional benefits for her offspring (Bernays and Chapman, 1994).

Presence of conspecifics

Where *S. exigua* shows gregarious behaviour, the presence of conspecific individuals or egg clutches could also be of influence on host plant selection in a way that presence of conspecific eggs stimulates oviposition. Gregarious feeding by caterpillars appears to enhance the ability of herbivores to exploit their host plants dramatically (Denno and Benrey, 1997).

The presence of feeding stages, or conspecific egg clutches may also deter gravid females from laying their eggs. Chemical mechanisms that ensure adequate spacing of progeny on limited resources include the production of oviposition-detering pheromones as well as recognition of plant constituents released as a result of previous damage (Renwick, 1989). Anderson and Alborn (1999) have shown that above-ground herbivore feeding of *Spodoptera* larva on cotton plants (*G. hirsutum*) can deter female adults from ovipositioning. It has to be mentioned that this effect was found only in larger plants (8 to 10 leaves), where small plants (3-4 leaves) showed the opposite effect.

Natural enemies

Additional stimuli that signal the presence of predators and parasitoids could be very important in host plant selection. The theory of enemy-free space (EFS) (Berdegue *et al.*, 1996) provided by a certain host plant can drive adult moths to oviposit on plants that provide a suboptimal food source for their offspring. With high predation pressure, the trade-off between 'best food' for larvae or 'low predation risk' for adult was shown to result in a preference for a plant that provided adult EFS (Mulatu *et al.*, 2004). This minimization of predation/parasitoid risk could also be a factor which could explain the previous mentioned avoidance by adult females of plants infested with conspecific feeding stages. If cues produced by these larvae can be detected by the adult moth, chances are high that parasitoids are present as well.

Herbivore offensive strategy

Host specificity and female host plant choice are well correlated (Price *et al.*, 1990). In the model of Price, he describes a dichotomy in herbivore strategies assigning causality to the quality of the resource eaten and the choosiness of ovipositing females. Specialist herbivore species which have high host specificity and hence, relatively high quality of consumed resources, tend to have choosy ovipositing females. This in contrast with, generalist species, who do not invest much energy in searching for a host plant, but instead, invest resources in reproduction resulting in high numbers of eggs randomly distributed (Price *et al.*, 1990).

Cotton

The fibres produced in the seed bolls of cotton plants (Malvaceae), constitute the world's most important textile component and has led to its cultivation all over the world. Four out of fifty species are used in agriculture, including *Gossypium herbaceum*, a species originating from South America (Munro, 1994). Insects are one of the major limiting factors of cotton production with yield losses of

over 8 percent per annum (Pedigo, 1999). Cotton is attacked by insects from planting until harvest, with nearly 100 species recorded as pests. Repeated insecticide applications caused traditionally key pests of cotton, including boll weevil [Coleoptera: Curculionidae] and pink bollworm [Lepidoptera: Gelichiidae], to be replaced by other species, particularly generalist leaf feeding insects of the genus *Helicoverpa* spp. [Lepidoptera: Noctuidae] (Pedigo, 1999).

Besides providing the world with suitable clothing material, cotton is also widely used as a model plant in both direct- (McAuslane et al., 1997; Bezemer et al., 2003) and indirect (Röse et al., 1996; Wäckers and Wunderlin, 1999; Wäckers and Bezemer, 2003) defence studies. In the following paragraph, the broad arsenal of adaptations to herbivore attackers in cotton will be introduced.

Defensive traits in cotton

All cotton species in the tribe Gossypieae are characterized by their ability to produce lysigenous pigment glands in vegetative and reproductive tissues. In response to herbivory, these glands contain higher levels of gossypol and biosynthetically related terpenoid aldehydes (McAuslane, Alborn and Toth, 1997). These secondary plant metabolites have been shown to possess toxic, antinutritive, and feeding deterrent effects on many generalist-feeding lepidopterans, including *Spodoptera exigua* and *Helicoverpa zea* (Stipanovic et al., 1988). The response of cotton plants, induced by foliar feeding, was demonstrated to be systemic. Terminal, undamaged leaves from plants previously damaged by larvae contained much greater quantities of terpenoid aldehydes than control plants (McAuslane, Alborn and Toth, 1997). In addition, bioassays revealed that *S. exigua* larvae consumed less of these terminal leaves, containing higher levels of especially hemigossypolone, gossypol and heliocides (McAuslane et al., 2003; Bezemer et al., 2003).

Besides herbivore-induced terpenoids, it is known that foliar pigment glands also contain constitutive volatile mono- and sesquiterpenes (Loughrin et al., 1994) that are continuously present in the plant and are released from damaged leaves immediately after either herbivore or artificial damage is inflicted. Pulse-labeling experiments showed that volatile compounds including several cyclic terpenes and green leaf volatiles of the lipoxygenase pathway were released from storage or synthesized from stored intermediates (Paré and Tumlinson, 1997).

However, concerning these volatile compounds, it has also been demonstrated that cotton plants that have been fed on by lepidopteran larvae actively respond by systemically emitting 'de novo' synthesised volatile terpenes in greater quantities and in different blends (Röse, Manukian, Heath and Tumlinson, 1996; Paré and Tumlinson, 1997). This emission was also observed when plants were treated with exogenous methyl jasmonate (Rodriguez-Saona et al., 2001). Methyl jasmonate is a compound with similar biological properties as jasmonic acid, a plant hormone involved in many induced direct and indirect plant responses (Dicke et al., 1999). The biosynthetic routes leading to different classes of volatiles is represented in figure 1.

The systemic release of these volatile semiochemicals in cotton has been shown to act as an attractant to both a generalist parasitoid (*Cotesia marginiventris*) and a specialist parasitoid (*Microplitis croceipes*) (Röse et al., 1998). Contributing to the evidence that the release of inducible terpenes really functions as a prey-indication signal for foraging natural enemies, hereby benefiting the plant by releasing it from its enemies is given by the study of Loughrin et al. (1994). It was shown in this study that the release of specific plant odours by a damaged plant follows a diurnal cycle, with peak emissions coinciding with the time of active host searching of parasitic wasps.

That these induced chemical changes (volatile or non-volatile) in cotton can also affect behaviour of herbivores was shown in an experiment by Landolt (1993). A higher frequency of landings of females of the generalist cabbage looper moth (*Trichoplusia ni*), was observed on damaged plants compared to undamaged. However, oviposition was significantly higher on undamaged cotton plants. Also gravid female moths of *S. littoralis* preferred undamaged cotton plants over plants that had been fed on by conspecific larvae (Anderson and Alborn, 1999). This seems to

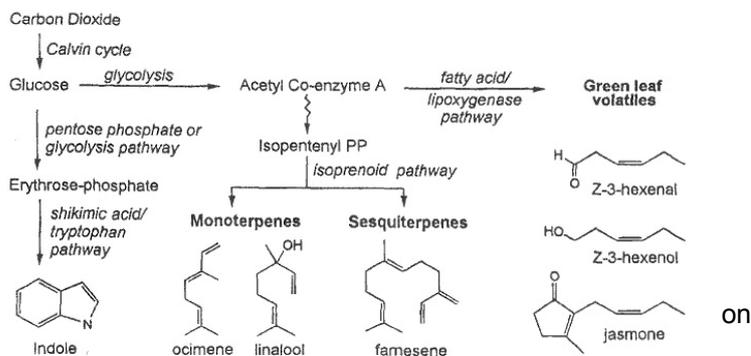


Figure 1 The biosynthetic routes leading to three classes of volatiles (indole, terpenoids, and green leaf volatiles) that are released from cotton plants fed on by *S. exigua*. The squiggly arrow pointed toward isopentenyl pyrophosphate represents the current uncertainty of this biosynthetic route (Eisenbreich et al., 1996).

indicate that it can be important for insects to assess health and infestation status when searching for a suitable host plant for their offspring.

Cotton has the ability to produce extrafloral nectar in order to accomplish a higher degree of protection by attracting and accommodating sugar dependent natural enemies. Besides constitutive extrafloral nectar production, providing a prophylactic protection, cotton plants have demonstrated to actively adjust their production in response to their biotic environment (Wäckers et al., 2001). Induced EFN production in cotton can be triggered by nectar removal (Wäckers, unpublished data) and tissue damage (Wäckers and Wunderlin, 1999; Wäckers and Bezemer, 2003). From a study by Wäckers & Wunderlin (1999) it is concluded that induction of extrafloral nectar secretion constitutes a general response by the plant to tissue damage. As both herbivore feeding and mechanical damage raised nectar production to similar volumes, induction does not seem to be representing a herbivore-specific mechanism. Wäckers et al. (2001) showed that the increase of EFN production in response to herbivory is primarily a local effect. It is suggested that this localized effect of raised EFN secretion may enhance the effectiveness of natural enemy recruitment as it can result in an accumulation of natural enemies on the leaf under attack (Wäckers et al., 2001).

In cotton, ants are the most common plant defenders attracted by EFN (Koptur, 1992). As ants tolerate no insects near their nutritious and well predictable food source, ant attended plants have shown to result in a significant improvement in plant fitness (Cautle and Rico-Gray, 2003). Well documented fitness benefits of ant attendance, combined with the fact that food supplements are difficult to reconcile with other functions, are convincing arguments for the interpretation that this adaptation represents an example of an indirect defensive response (Turlings and Wäckers, 2004).

This aboveground induced recruitment of predators by means of induced nutrition fits well in the optimal defence theory. This theory predicts that defensive traits will be produced and allocated in a pattern that optimises the trade-off between defensive costs and benefits (Zangerl and Rutledge, 1996). Extrafloral nectar allocation patterns in cotton generally fit prediction by the optimal defence theory (Wäckers and Bonifay, 2004). However, not all types of EFN induction may be adaptive to the plant, especially when induction across the root-shoot barrier is concerned (Wäckers and Bezemer, 2003).

Belowground versus aboveground induced plant defence

Belowground insect herbivory has received relatively little attention in the ecological literature, despite the fact that subterranean herbivores are very common and can be quite damaging. Field experiments showed that suppression of root boring moth larvae could increase mean cumulative seed production by 28% and survival by 18% (Maron, 1998). Moreover, there is increasing awareness of the importance of belowground herbivores in the structure and function of ecological communities by for example limiting species in reaching high cover abundances (Masters, 2004). It is believed that in some cases escape from its specific soil dwelling phytophages, mainly causes an introduced exotic species to become established and subsequently invasive (Müller-Schärer et al., 2004).

Despite the fact that, in nature, all plants are almost constantly exposed to simultaneous above- and belowground herbivores (Van der Putten et al., 2001), induced plant responses have mainly been studied in their aboveground parts (Karban and Baldwin, 1997). Recently, there is an increasing interest in studying the interaction of aboveground and belowground compartments as a whole, rather than isolated aboveground studies (Bezemer et al., 2003; Ban Dam et al., 2003; Wäckers and Bezemer, 2003). It is acknowledged that insects can interact when feeding in different compartments on a shared host plant (Masters and Brown, 1992), but the extent to which interactions of this sort has its effects on

above- and belowground plant fitness is still poorly known (Maron, 1998). The indirect interaction between below- and aboveground organisms is mediated by their common host plant. Although I will focus in this report primarily on root feeding organisms as the belowground counterpart, it has to be mentioned that the existing studies included several different functional groups like mycorrhizal fungi (Van der

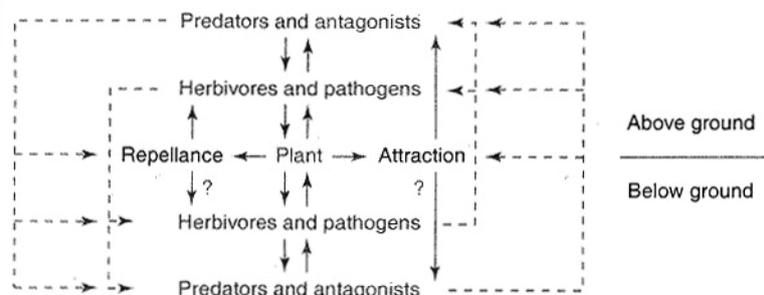


Figure 2. Basic interaction scheme of above- and belowground multitrophic interactions. Straight lines are direct effects; dashed lines are indirect effects. Question marks refer to effects that are largely unknown. In plants, direct defence aboveground has been studied much more than direct defence belowground. Examples of root-carnivore interactions are extremely rare (Van der Putten et al., 2001).

Putten et al., 2001) and soil detritivores and decomposers (Wurst and Jones, 2003) with which aboveground herbivores can indirectly interact. Current knowledge about basic interactions below – and aboveground is given in a schematic diagram (figure 2) according to Van der Putten (2001). Within the systems studied in linking above- and belowground insect herbivores, only one studied the impact on parasitoid behaviour (percentage of parasitism or number of parasitoids per plant). In a field study, population abundances of both parasitoids and its host were greater on plants subjected to root herbivory (Masters et al., 2001). Root herbivory may cause physiological changes within the host plant leading to a change in the nutrient (C:N) balance (Masters et al., 1993). As roots are essential for taking up water and nutrients, the activities of root herbivores may induce a water stress response, which is known to lead to the accumulation of soluble amino acids and carbohydrates in foliage (Gange and Brown, 1989) (Masters et al., 1993). It is suggested that this is the mechanism causing root feeders to have a positive effect on the foliar feeder and its parasitoids. However, recent studies on cotton have shown that below- and aboveground interactions are not only the consequence of a physiological change in primary chemistry (C:N balance), but can also be mediated by the plant defence system. Several compounds involved in aboveground defence are actually produced (primarily) in the roots including terpenoids as gossypol in cotton. Root herbivory by *Agriotes lineatus* resulted in an increase in terpenoid levels in the foliage (Bezemer et al., 2003; Bezemer et al., 2004). This resulted in lower performance of the foliage feeding insect *Spodoptera exigua* feeding on cotton plants previously exposed to the root herbivore (Bezemer et al., 2003). Also the distribution of plant defences aboveground were influenced. Root herbivory resulted in an overall increase in secondary plant compounds, in contrast to foliar damage which showed only locally increased level of terpenoids, mainly in the costly immature foliage. However, the level of induction after root herbivory was much lower compared to foliar herbivory. Furthermore, plants exposed to root herbivory also had significantly higher levels of terpenoid aldehydes in root tissue, while no such effect was found following foliar herbivory. Additionally, plants simultaneously exposed to root and foliar herbivory appeared to induce primarily above-ground at the cost of belowground defence (Bezemer et al., 2004).

Concerning belowground induced indirect defence in cotton, recent studies showed that extrafloral nectar secretion can also be stimulated by root herbivore feeding. Cotton plants (*Gossypium herbaceum*) showed a nectar production 18 times higher in plants fed on by the root chewing herbivore (*A. lineatus*) compared to undamaged control plants (Wäckers and Bezemer, 2003). In contrast to aboveground induction, resulting in a local increase in EFN production of the damaged leaf, belowground induction caused a systemic increased level of nectar production in all leaves (unpublished data Lommen & Wäckers, 2002). In the same experiment, exposure to both above and belowground herbivores resulted in an intermediate response, showing a systemic raise in nectar production with a peak at the damaged leaf.

As it is shown in several studies that cotton leaves are also protected indirectly by systemic release of herbivore-induced volatiles that attract natural enemies of herbivores (Röse et al., 1996; Röse et al., 1998; Rodriguez-Saona et al., 2001), no study has been carried out on the effect of belowground herbivory on volatile emission spectrum.

In this study we intend to determine the ecological consequences of belowground induction on aboveground plant-insect interactions. We conducted several experiments investigating the effect of both direct and indirect induced defences by root herbivores on aboveground insects in cotton. These consisted of a comparative field study, three preference tests including a larvae (feeding) and adult (oviposition) and parasitoid (flight response). The latter was tested by simultaneously collection with volatile emissions.

1. Field study

In a factorial field experiment, we investigated the possible effects of root herbivory on insect visitation of extrafloral nectaries in cotton plants. This study primarily focussed on the change in EFN production and distribution pattern over the plants following belowground attack. As Wäckers & Bezemer (2003) showed a dramatical increase in EFN production in response to root herbivory, we hypothesised this could also be reflected in the number of insect visitors depending on sugar as a carbohydrate source. As it is assumed that EFN secretion is part of an indirect defence strategy, it might result in higher numbers of plant beneficials (predators and parasitoid), and consequential lower numbers of herbivorous species.

Furthermore, it was shown that this belowground induction is systemic throughout the plant (unpublished data Lommen & Wäckers, 2002). We hypothesise that this might also be reflected in the distribution of insects on the plant. To investigate this, cotton plants (*Gossypium herbaceum*) grown in a greenhouse were exposed to the root chewing parasite (*Agriotes lineatus*) and transported

immediately to a field site in Heteren (Holland). Recordings of insect activity and distribution over the leaves were carried during 16 days. Special attention was given to plant beneficial organisms, including ants and parasitic wasps, visiting the EFN glands. As ants tend to attack food competitors near their nutritious food source (Cuautle and Rico-Gray, 2003), the experiment was also carried out with plants on which ants were excluded.

Ants are, besides EFN also attracted to sugar-rich honeydew producing sternorrhyncha species including homopterans like aphids. In this ant-homopteran mutualism, the ants protect the sap-sucking aphids in exchange for a nutritional reward, which is essentially a waste product of their sugar-rich diet of phloem sap (Gaume et al., 1998). However, when both EFN and honeydew are available as a carbohydrate source, foraging theory predicts that consumers will sometimes ignore low-quality food items when higher-quality items are readily available (Charnov, 1976; Engel et al., 2001). As in belowground induced cotton plants, EFN might be superior in quantity to honeydew, aphids will suffer more predation which will ultimately result in lower numbers of aphids. Conclusively we hypothesise that:

Aphid infestation will be higher in plants where ants are excluded

On belowground attacked cotton plants with ant access, aphid infestation will be lower compared to control plants.

2. Herbivore feeding preference

It was shown that herbivory, belowground, aboveground and in combination, resulted in an increase in terpenoid levels in roots and foliage in cotton. This resulted in reduced herbivore performance and even changed feeding distribution pattern (Croxford et al., 1989; Bezemer, Wagenaar, Dam and Wäckers, 2003). We hypothesise that induced plants may stimulate feeding stages to leave the plant. In a bioassay, one belowground induced and one undamaged control plants will be offered to a single generalist herbivore *Helicoverpa armigera* (Lepidoptera: Noctuidae). Spatial feeding pattern over the two plants will be recorded.

3. Herbivore oviposition preference

In nature, the consequences of local or systemically increased terpenoid production (volatile or non-volatile) in response to herbivory may actually act also in another way.

In Lepidoptera, a female in search of a suitable host plant, the herbivore-induced volatiles emitted from potential host plants could be important for avoiding oviposition sites with high risk of competition, parasitism or predation. Furthermore, it has been shown that food quality can be poor in a previously (above and belowground) attacked plant, due to induced defence (Bezemer, et al., 2003).

Behavioural studies show that ovipositing females of *S. littoralis* distinguish between undamaged cotton plants and plants damaged by feeding of conspecific larvae (Anderson and Alborn, 1999). We hypothesise that undamaged cotton plants will be preferred over belowground induced individuals by ovipositing female moths. In a two-choice bioassay individual gravid moths (*S. exigua*) were given the opportunity to oviposit on a belowground attacked or an undamaged cotton plant.

4. Belowground induced emission of volatile semiochemicals

a. Volatile collection

As already mentioned, several studies have demonstrated that cotton plants that have been fed on by lepidopteran larvae actively respond by systemically emitting 'de novo' synthesised volatile terpenes (Röse, Manukian, Heath and Tumlinson, 1996; Paré and Tumlinson, 1997). Compounds systemically released and specifically induced by caterpillar feeding were (*Z*)-3-hexenyl acetate, (*E*)- β -ocimene, linalool, (*E*)- β -farnesene, (*E,E*)- α -farnesene, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene and (*E*)-4,8-dimethyl-1,3,7-nonatriene. The latter two homoterpenes are regarded as a strong attractant for several carnivorous and parasitic arthropods as they are observed to be induced by herbivory in several other plant species in addition to cotton (Dicke and Sabelis, 1990).

As belowground herbivory alone and in concert with foliar feeding has demonstrated to induce terpenoid levels aboveground, it may well be that also the emission of volatiles is changed. Could simultaneous belowground and aboveground feeding change aboveground volatile emission? It may well be that these plants exert a shorter lag-time of induction or produce higher levels or just a different blend of volatile signals. This could also involve a change in the behaviour of foraging parasitic wasps. To our knowledge, only one study has been conducted to systemic release of root herbivore-induced plant volatiles. This study, involving a system of root-feeding larva (*Delia radicum*) on cabbage plants, demonstrated that belowground induced plants were systemically producing parasitoid-attracting volatiles (Neveu et al., 2002).

b. Flight response of parasitoid

Furthermore, it is imaginable that the aboveground SOS signals are increased when a plant is confronted with enemies at two spatially separated fronts. In accordance with the optimal defence theory (Zangerl and Rutledge, 1996), indirect defence aboveground might be intensified in order to minimise damage on valuable photosynthetic tissue.

To investigate the effect of belowground herbivores, cotton produced volatiles were collected over time from individual plants attacked by either root herbivores, foliar feeders or both. Specific attention will be given to compounds already shown to be induced systemically in response to aboveground feeding. In addition, plants attacked in both root and shoot are tested for parasitoid attraction against plants only fed on by an aboveground herbivore only.

Materials and Methods

Plants

Cotton plants (*Gossypium herbaceum* (L.)) [Malvaceae] were grown in a climate controlled greenhouse at 60% RH, 16L:8D; daytime temperature 23°C ($\pm 1^\circ\text{C}$); night time 18°C ($\pm 1^\circ\text{C}$). Daylight was supplemented by 400 W metal halide bulbs (1 per 1,5 m²). Each cotton seedling was grown individually in square (13x13cm) containers, filled with 1,5 dm³ of a 50:40:10 peat/sand/clay mixture. Plants were watered three times a week.

Belowground plant induction treatment (BG)

Belowground induction of cotton plants was obtained by allowing wireworms, larvae of click beetles [Coleoptera, Elateridea], to feed on the roots. Wireworms are generalist root-feeding larvae with a larval developmental time of 3 to 5 years. They are considered as pest species in cultivation of various crops including wheat (Blot *et al.*, 1999), potatoes (Jansson and Lecrone, 1991), sugarcane (Samson and Calder, 2003) and cotton (Anonymous, 1996).

Wireworms of the species *Agriotes lineatus* were collected at a pasture near Wageningen, the Netherlands, at a density of approximately 50 individuals per m². After each experiment, larvae were recovered from the containers by hand sorting roots and soil. Subsequently, they were placed in moist soil at 15°C and fed with potato, so that they could be reused for following experiments.

In order to allow the plant to respond, root herbivores were always introduced 7 days before the start of the experiments. This introduction was carried out by placing 7 late instar individuals on the soil surface, allowing them to burrow into the soil. Prior to introduction, larvae, were starved for four days. During greenhouse experiments, the activity of root feeding insects was checked by analysing the EFN production of individual plants fed on by wireworms, compared to undamaged control plants. Wäckers and Bezemer (2003) showed that in response to wireworm feeding activity, EFN production increased significantly compared to the control plant, within the first week. To confirm belowground induction, EFN was removed 4 days after wireworm were introduced. After 48 hours EFN was again collected and weighted using methodology described in Wäckers and Bezemer (2003).

1. Field experiment

On the 9th of August 2004, 40 cotton plants with 6 true leaves were transferred in their containers to a research field in Heteren (Holland), after having been grown in the greenhouse for 4 weeks. In order to investigate the effects of belowground feeding and ant attendance on aboveground plant-insect interactions, plants were divided in four groups with each group given a different treatment (table 1). Plants were positioned in a single row, grouped in blocks and ordered per treatment respective to the order in table 1. Ants were excluded with a water barrier, by placing the container on one disc placed in a second filled with water.

Field code	Ant access	Presence of root herbivores
+A+W	+	+
-A-W	-	-
+A-W	+	-
-A+W	-	+

Table 1: Block design of plant treatments in field experiment

Monitoring of insect activity, abundance per plant per leaf, was carried out by inspecting all leaves of each plant 12 times during the 19 days following wireworm inoculation. Insects were collected using an exhaustor and put in vials containing 70% alcohol to be identified in the lab.

The recordings of numbers of insects visiting the extrafloral nectaries were square root transformed before analyses using repeated measures analyses of variance (RMANOVA). The distribution of the insects over the leaves was characterized by its centre of gravity and its mean deviation from the centre, which were defined respectively as:

$$\sum \left(\frac{\#insect / leaf}{total\#insect / plant} \right) * leafnumber$$

$$\sqrt{\sum \left(\frac{\#insects / leaf}{total\#insects / plant} * (centreofgravity - leafnumber)^2 \right)}$$

Again effects of the different treatments on the distribution of total insect numbers and ant attendance were analysed using RMANOVA's.

2. Herbivore feeding preference

In order to determine feeding preference of the generalist larva *Helicoverpa armigera* [Lepidoptera: Noctuidae], a bioassay was conducted in which aboveground herbivores were offered both a belowground induced plant and an alternative plant.

Two cotton plants (*G. herbaceum*) of same developmental stage (7 true leaves) were placed in large meshed cylindrical cages (height 1 meter, diameter 35 cm). We discriminated two different sets of plant pairs: control pairs (C) in which none of the plants were belowground induced and BG-pairs, in which one of the plants was fed on by root herbivores. The experiment was conducted in a greenhouse with the same climate regime as the chamber in which cotton plants had been grown. Larvae were obtained from an established culture maintained at 25°C (+/- 1°C) with RH of 70% and reared on a cornflower-based diet (Elzinga *et al.*, 2002). Prior to the experiment, the culture was reared one generation on excised cotton leaves. In both pairs (C and BG) a pre-weighed 4th instar larva was introduced on the third true leaf of one of the two plants. Within BG-pairs, larvae were placed on the belowground induced plant, in the C-pairs on a marked, random plant. Larvae were allowed to move freely over the two plants during the following 5 days, after which larvae were removed and weighing was repeated.

Spatial feeding pattern of *H. armigera* was analysed by recording the amount of feeding holes and analysing the total area consumed per leaf per plant. All leaves were photocopied and numbered per plant according to increasing age. A leaf-area scanner was used to determine number of feeding holes and consumed leaf area per plant. The average number of feeding hole per plant was calculated based on these parameters. The distribution of leaf consumption over the plant was characterized by its centre of gravity and its mean deviation from the centre, which were defined respectively in equation 1 and 2.

Furthermore, a distinction was made between mature and young (last 3) leaves. The experiment was repeated 6 times. As our data was not normally distributed, statistical analyses was carried out using non-parametric tests (Mann Whitney-U).

3. Herbivore oviposition preference

In order to determine the effect of root herbivory on oviposition preference, adult female moths were offered two host plants with different treatments. Two-day-old gravid females of the generalist herbivore *Spodoptera exigua* (Hübner) [Lepidoptera: Noctuidae] were individually given the choice to deposit their eggs on a cotton control (C) or a belowground induced plant (BG) in a meshed cage (1.3- x 1.3- x 1.3-m).

Cotton plants (*G. herbaceum*) were of same developmental stage (7 true leaves) and placed in the cage in their containers at approximately 70 cm distance. The experiment was conducted in a greenhouse with the same climate regime as the chamber in which cotton plants had been grown. Insects were taken from an established culture maintained at 25°C (+/- 1°C) with RH of 70% and reared on a cornflower-based diet (Elzinga *et al.*, 2002). Prior to the experiment, the culture was reared one generation on excised cotton leaves. After emergence, male and female moths were allowed to mate for two days, before introduction in the cage and allowed to oviposit the following 5 days. During the experiment, the gravid females were fed with a 1:1 honey-water solution, provided in a Petri-dish with a drenched cotton plug positioned halfway between the two cotton plants. Every day during the following 5 days, distribution and numbers of eggs as well as clusters were recorded. The experiment was repeated 10 times.

4. Belowground induced emission of volatile semiochemicals

a. Volatile collection

This experiment was carried out to study the effect of separate and simultaneous below- and aboveground herbivore feeding on volatile emission spectrum. Plant odors were collected from cotton plants (*G. herbaceum*) exposed to four different herbivore feeding treatments:

- plants experiencing foliar herbivory only (AG)
- plants exposed to both root and foliar herbivores (AG+BG)
- undamaged cotton plants (CC)
- background air (conducted with a container with soil and roots only, cotton stem was cut of just below soil surface) (BC)

Volatile collection took place in a climate cabinet with a capacity of four one-month-old plants to be analyzed simultaneously. During the collection period, plants were provided with artificial light. Plants were individually placed in their containers under a cylindrical glass chamber (H:D; 50:24.5 cm), positioned on a stainless steel ring (H:D; 30:24,5 cm). Soil containers were covered with aluminum foil in order to minimize spread of soil-borne odors. The incoming airflow originated from a pressurized cylinder (L050H[®], Hoekloos BV, Holland) and was cleaned by heated catalyst purification (Parker ChromGas, Model Gas generator 3500[®]). Purified incoming air was injected at the top of the cylindrical glass chamber at a rate of about 0,45 l/min. Sampling took place at a point halfway the height of the glass dome, by pulling air and emitted volatiles over a volatile collection trap at a rate of about 0,18 l/min. The remaining excess air escaped through small openings at the base of the glass chamber, causing a positive pressure which insures a barrier against incoming contaminated air. Collector traps (15 cm long x 5 mm o.d.), contained 50 mg of Super-Q (catalog no. p/n 2735; Altech Associates, Deerfield, IL) as an adsorbent. Sampling of volatiles was carried out at three times: just before foliar herbivory (t=0), as well as 48hrs and 96 hrs following onset of foliar herbivory. Glass cylinders were cleaned between daily collections by putting them overnight in a 80°C stove. Aboveground feeding damage was inflicted by exposing plants to four larvae of *H. armigera* (2nd instar), introduced in two separate clip cages (2,5 cm²) placed on the two lower leaves of the plant. Sides of the clip cages that touch the leaves were cushioned to prevent abrasion of the leaf and were supported by bamboo sticks.

H. armigera caterpillars, used for assessing aboveground feeding damage, were reared on excised cotton leaves. Larvae were allowed to feed 48 hrs following the first volatile collection.

Just before the second odor sampling, clip cages and caterpillars and any remains were gently removed by a brush. In order to exclude any effect caused by attaching the clip cage, empty clip cages were applied at the same time on undamaged control plants (CC).

Analysis of volatiles

Volatiles were extracted from the collector traps by washing the adsorbent with 150µl dichloromethane (VWR International, Leuven) and put in glass vials together with internal standards (n-octane and n-nonyl acetate) and subsequently stored at minus 80°C. Finally samples were sent to Neuchatel on dry ice to be analyzed by a GC-MS.

Volatile collections were analysed with a Hewlett Packard HP 6890 (Agilent 6890 Series GC system G1530A), coupled to a mass spectrometer operated in electron impact mode (Agilent 5973 Network Mass Selective Detector; transfer line 230°C, source 230°C, ionization potential 70 eV, scan range 33-280 amu). A 3 µl aliquot of each sample was injected in the pulsed splitless mode onto an apolar capillary column (HP-1, 30 m, 0.25 mm ID, 0.25 µm film thickness, Alltech Associates, Inc, USA). Helium at constant pressure (18.55 psi) was used as carrier gas flow. Following injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min and subsequently to 200°C at 5°C/min followed by a post-run of 5 min at 250°C. The detected compounds were quantified based on comparison of their peak area with those of the internal standards and identified by comparison of retention times with those in previous analyses (Róse et al., 1996). Compounds were identified by comparison of their mass spectra with those of the NIST 98 library and by comparison of their spectra and retention times with those of authentic standards. Collector traps were rinsed with 1,5 ml dichloromethane (VWR International, Leuven) before and after use. Concerning AG, AG+BG and CC plants, the experiment was repeated 4 times. The BC treatment was repeated 3 times with and 3 times without wireworms feeding on the root system.

b. Flight response of parasitoid

To study if belowground herbivory has an effect on the attractiveness of the plant to an insect of third trophic level I conducted a dual choice flight experiment. The flight response of a female parasitic wasp *Microplitis croceipes* [Hymenoptera: Braconidae] towards two cotton plants (AG and AG+BG) was observed within a nylon gauze covered tent (H:W:L; 2,5-:2,0-:2,7m.) located in a climate-controlled room with the same climate regime as where cotton plants had been grown. To ensure a constant airflow from plants to the wasp release platform, an electric fan (30 cm diameter, 40W) was placed behind the nylon gauze, 30cm from the plants. Plants were placed in their containers on a table located in the tent at a mutual distance of 30cm . During the experiment, plants were switched after every 6 releases and replaced after every 12 releases. Wasps were released from a 22cm high platform, at 1m distance downwind from the cotton plants. The first landing by the wasp on either of the two plants was recorded as choice. If the wasp did not land on either plant within 3 flights or within 6 minutes, this individual was scored as 'no choice'.

Cocoons of *M. croceipes* were obtained from the Lab of Dr Joe Lewis, USDA-ARS, Tifton GA which has an established rearing as described by Lewis & Burton (1970). Wasps were allowed to emerge in a climate-controlled room (60% RH, 16L:8D; (23 C± 1°C : 18°C (± 1°C)) in Plexiglas cage (40 x 40 x 40 cm) with one window covered with nylon gauze. Adults were fed a 1:3 sugar-water solution. In order to increase responsiveness of the parasitoids, females were allowed to antennate and parasitize a third instar *H. armigera* larva reared on artificial diet, within 6 minutes before release.

A first flight experiment was carried out with 80 wasps using plants just after second volatile collection (after 48 hrs of herbivore feeding). Wasps which initially had been scored as 'no choice' (n=50), were tested in a second experiment under the same experimental conditions two days after their first release. To test for significant preferences in first and second dual-choice experiments I separately analyzed the data using binomial probability function.

Results

1. Field experiment

Total insect visitation

The majority of insect visitors were belonging to the order of the Diptera and are considered neither harmful or beneficial for the plant (figure 3).

Especially species belonging to the family of *Sepsidae* and *Chloropidae* were very dominant in numbers. In addition to ants, several other species considered to be beneficial for the plant including ladybirds (Coccinellidae),

lacewings (Chrysopidae: both adult and larval stages) and a variety of parasitic wasps (Apocrita) (appendix 1) were recorded on the extrafloral nectaries. As these nectaries are considered a being rather inconspicuous (Wäckers, 2005), it was remarkable to observe Ichneumonid species actively scanning each leaf for nectar within one hour after plants were located in the field.

As we were interested in the effect of belowground herbivory on total insect visitation, only results of plants on which ants were excluded are shown. However, ants were by far not significantly affecting insect visitation. The total number of individuals recorded feeding on EFN on plants treated with root herbivores did not significantly differ from control plants (RMANOVA: $F=0,59, df=1,18, p=0,45$). The effect of time was significant where numbers of both treatments decrease over time. (appendix 1). Despite the fact that much of the variance is caused by within plant differences (see figure 4), our data suggest a negative effect of wireworm feeding in the long run on number of insects visitors.

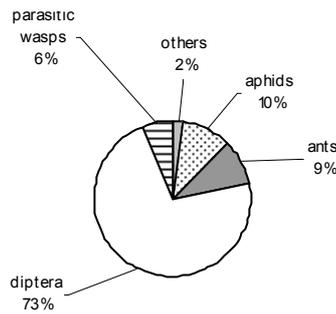


Figure 3 Total number of individual insects recorded over the total experimental period divided per category. In total, all 40 plants were sampled 12 times over 19 days. This makes 480 samples recording a total of 1556 insects.

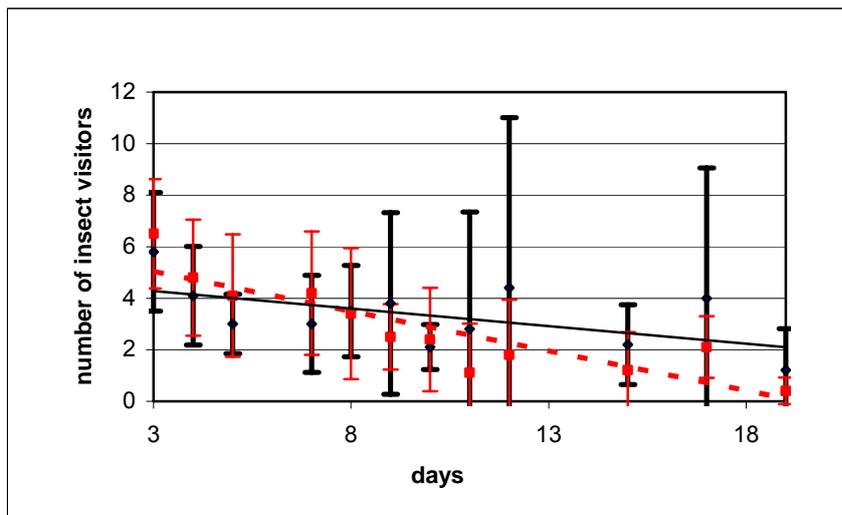


Figure 4 Effect of wireworm treatment (ants excluded) on insect abundance. The number of insects recorded is set against time and given as means (\pm SD) per plant per day. Recordings were carried out 12 times over 16 days. First recording took place at day 3; 3 days after wireworms were introduced followed by immediate transportation to the field. Wireworms present (red square, $n=10$) and without wireworms (black diamonds, $n=10$)

The value of the center of gravity of insect visitors was significantly affected by the presence of wireworms (RMANOVA: $F=6,26, df=1, 18, p=0,02$). Our data suggests that insects tend to be more concentrated on higher leaves when the plants root system is fed on by wireworms (graph in appendix 2). Most insects were recorded around leaf position 5 (forth developed leaf) increasing in time (see top two graphs figure 5).

Deviation of insect visitors around the center was not significantly affected by the presence of wireworms (appendix 2). Insignificant difference seemed to be mainly caused by high within plant variation (lower graphs figure 5). However, p-value attains significance level, suggesting more variation in insect leaf distribution when the plant is under belowground attack.

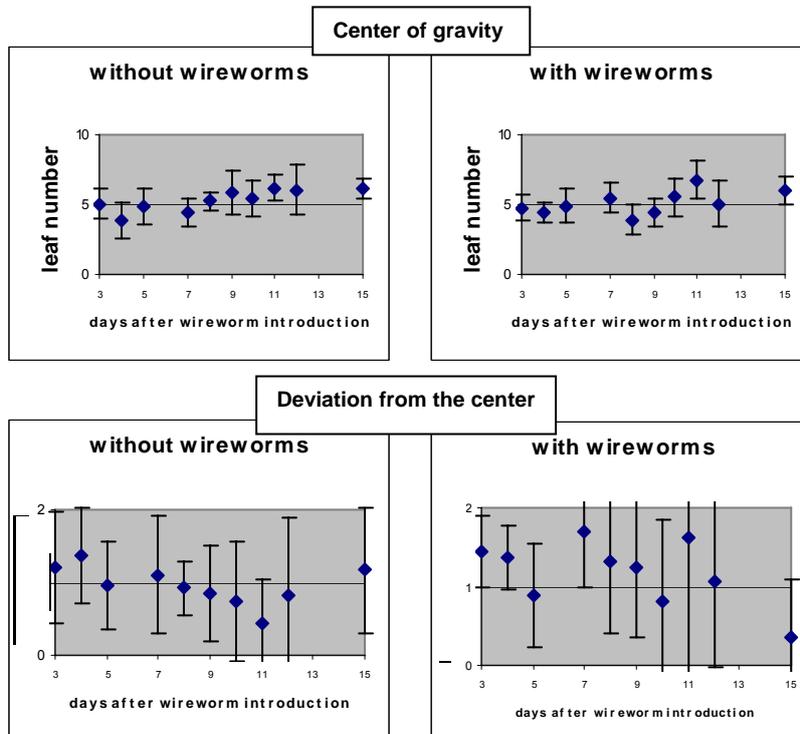


Figure 5 Effect of wireworm introduction on spatial distribution of insect visitors within a plant. Center of gravity (top two graphs) and deviation from the center (bottom two graphs) is set against time and given as the mean (\pm SD) per plant. Recordings were carried out 12 times over 16 days. First recording took place at day 3; 3 days after wireworms were introduced followed by immediate transportation to the field. Left two graphs show recordings on plants with root herbivory (n=10), right ones show recordings of the controls (n=10).

Ant attendance

Ants visiting the extrafloral nectaries were randomly collected and identified. All attending ants belonged to the species *Lasius niger*. A negligible number of ants were observed to chase away other nectar feeders from the leaves, which was also seen in the fact that number of insect visitors on plants with ant access didn't differ from plants not accessible to ants. Furthermore ant numbers were decreasing gradually and varied greatly within plant treatments (figure 6). Total number of individuals and intraplant distribution did not differ between plant treatments (appendix 3). Although numbers of observations were very low, there seemed to be a trend of ants being concentrated in the higher leaves in plants without belowground root herbivory (figure 6). Low deviation from the center in both treatments, especially after day eleven, was mainly the result of low number of recordings.

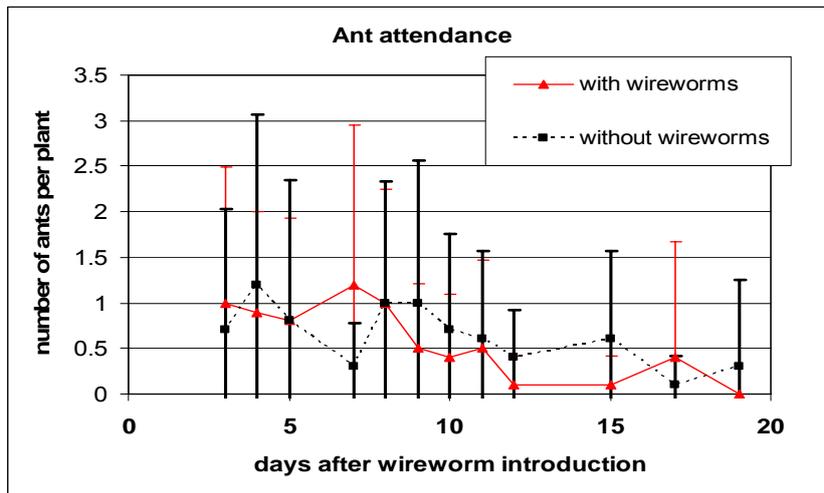
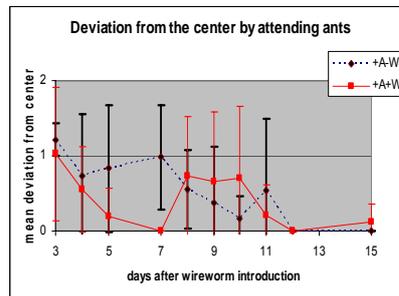
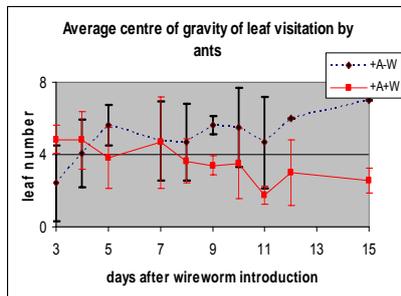


Figure 6: Temporal and spatial distribution of ants on plants with root herbivores (red triangles, n=10) and controls (black squares, n=10).

Observations were carried out 12 times over 16 days. First recording took place at day 3; 3 days after wireworms were introduced followed by immediate transportation to the field. Top graph shows number of individuals attending plants set against time and given as means (\pm SD) per plant.

Center of gravity (bottom left graph) and deviation from the center (bottom right graph) is set against time until day 15 and given as means (\pm SD) per plant.



Herbivores

The number of herbivorous insects observed feeding on cotton plants during the experimental time period was very low. Herbivores consisted primarily of sap sucking aphids of the species *Aphis gossypii* and *Aphis fabae*. Both were able to reproduce on the plants and were observed being attended by ants, which could be the cause of higher aphid infestation level on plants with ant access which is indicated in our data (figure 7). Over time, numbers of individuals increased, but stayed insignificant for statistical analysis.

During the experimental time period, three lepidopteran egg clutches were recorded, all on plant without root herbivory. Two of these, both recorded on the last day, were identified as members of the family *Noctuidae*. The other egg deposit couldn't be identified because caterpillars were all parasitized by an *Ichneumonid* wasp. Also some individuals of thrips (*Thysanoptera*) were observed feeding on EFN, sometimes destroying the nectaries, but total numbers stayed very low.

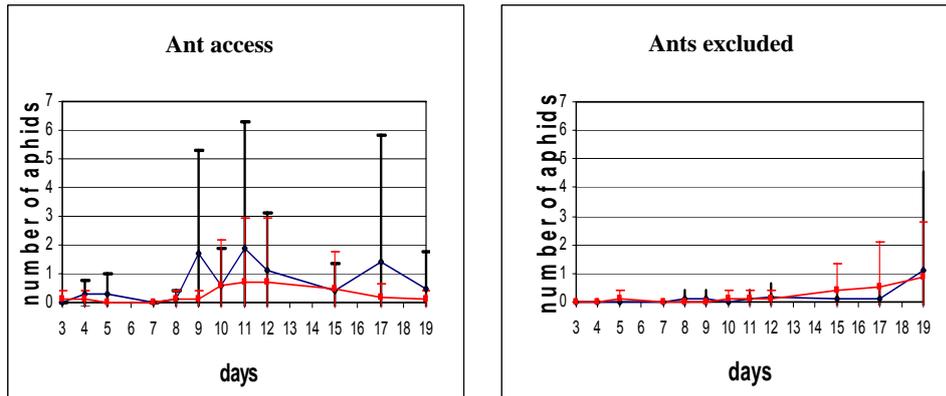


Figure 7 Effect of wireworm introduction and access of ants on aphid infestation. Number of aphids on plants with ant access (left graph) and plants without ants (right graph) is set against time and given as the mean (\pm SD) per plant. Recordings were carried out 12 times over 16 days. First recording took place at day 3; 3 days after wireworms were introduced followed by immediate transportation to the field. Red triangles show plants with root herbivory (n=10), black squares show recordings of plants without belowground herbivores (n=10).

2. Herbivore feeding preference

Because of a possible viral infection of our cotton plants, a scheduled repetition of the initial experiment did not take place. This resulted in the experiment having only six replicates. The total EFN production in plants with root herbivory was significantly higher (more than 5 times) compared to control plants (ANOVA: $F=57,89, df=1, 10, p=0,00$), indicating active feeding of wireworms. *H. armigera* food consumption, measured as the area of foliage consumed showed no significant treatment effects (fig 8a). This concerned foliar consumption on the alternative plant as well as on the plant where caterpillar was introduced. Including feeding damage from immature leaves only shows no difference. Also aspects of spatial feeding pattern of the fourth instar larvae did not result in any clear differences between treatments (fig. 8bc)

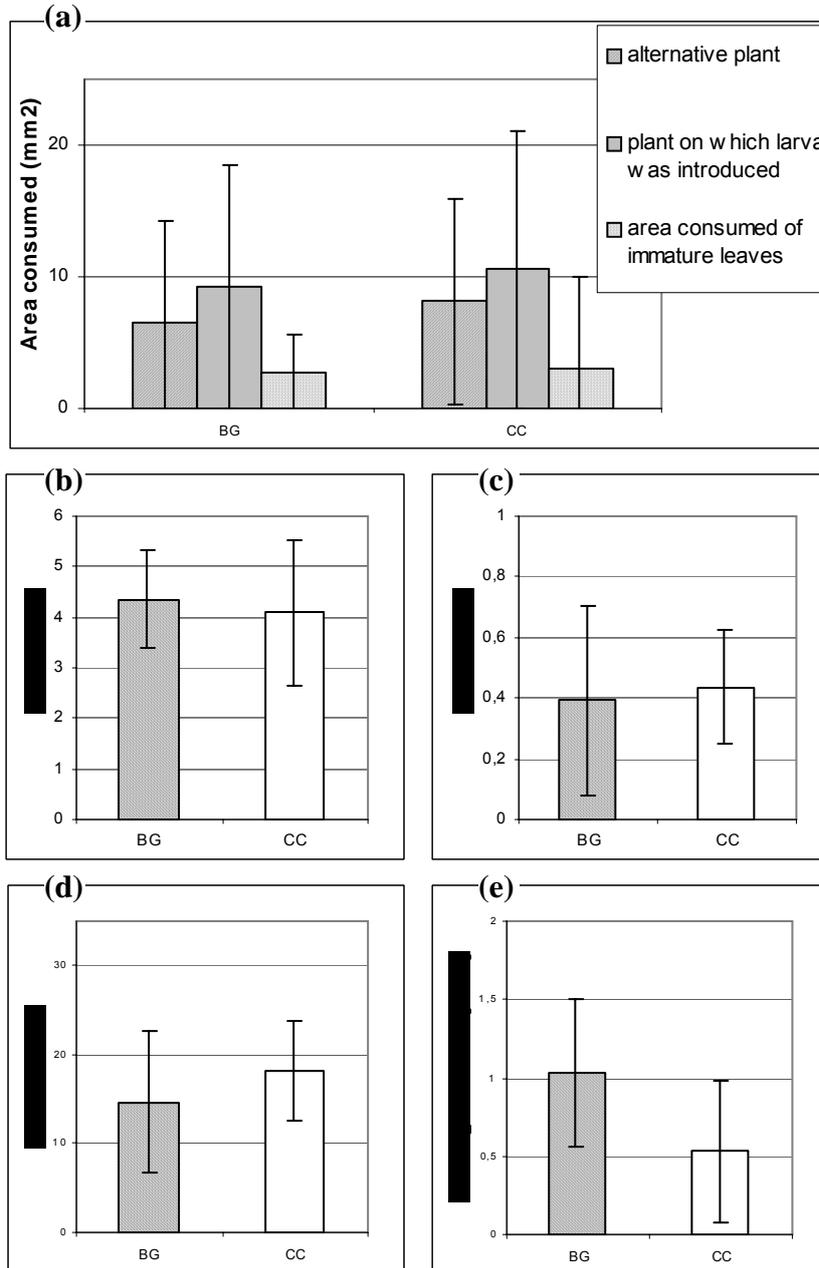


Figure 8 Individual leaf consumption of *H. armigera* larvae placed on cotton plants either exposed to root herbivory by *A. lineatus* (BG) or undamaged controls (CC). In both cases larvae were also offered an alternative control plant. Over five days larvae were able to move freely between the two plants. In the lower four graphs, comparisons were made based on data of plants on which the larva was initially placed. Mean values (\pm SD) are shown for total leaf area consumed (a), center of gravity (b), deviation from the center (c), total number of feeding holes per plant (d) and mean feeding hole area (e).

Although not significant, feeding holes of plants under belowground attack have a tendency of being a bigger and subsequently lower in number compared to control plants. This suggests that that larvae were feeding longer on the same spot on plants that were fed on simultaneously by root herbivores.

Larval performance

The only parameter measured indicating larval performance was the weight increase during the experiment. Mean growth rate over five days of *H. armigera* larvae did not differ when allowed to feed on two control plants (CC) or on a plant pair which included one plant with root herbivores (fig 9). This data suggest that there was no significant nutritional benefit for the larvae in either of the plant treatments.

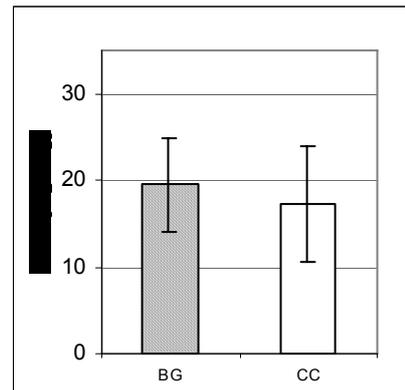


Figure 9 Relative growth rate (RGR) of *H. armigera* larvae placed for five days on cotton plants either exposed to root herbivory by *A. lineatus* (BG) or on undamaged controls (CC). In both cases larvae were also offered an alternative undamaged plant and were able to move freely over five days between the two plants.

3. Herbivore oviposition preference

During the experiment no eggs were deposited on either of the two plants. All *S. exigua* females laid their eggs on the cage, instead of preferring to lay on plant tissue. In following trial experiments, several pupae of both female individuals were allowed to emerge inside the oviposition arena. This method was shown to result in higher proportion of the total numbers of eggs deposited on the cotton plants (Anderson and Alborn, 1999). Nevertheless numbers of eggs deposited on the plants were insignificant. Trial experiments conducted with the other generalist lepidopteran species *Mamestra brassicae* and *Helicoverpa armigera* also showed a higher total proportion of eggs laid on the cage.

4. Belowground induced emission of volatile semiochemicals

a. Volatile collection

The samples extracted from the collection traps did not contain considerable amounts of cotton specific volatiles. Relative to the large amount of unrelated compounds to cotton were measured, indicating a high level of impurity of the sampled air. This completely overshadowed compounds related to cotton, making analyses of this data inappropriate. Relative amounts of emitted volatiles of a trial experiment, focusing only on inducible compounds and using a different extraction method, are shown in appendix 4.

Although also these collection revealed a lot of impurities, there were measurable amounts of compounds shown to be emitted following aboveground damage in cotton. Within these compounds, no specific trend can be observed. Since specific inducible compounds as (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene and (*E*)-4,8-dimethyl-1,3,7-nonatriene were also measured in relative high concentrations in our cotton control plant, makes this data unreliable for interpretation. Furthermore, there were no replicates in this trial experiment.

b. Parasitoid flight response

Wasps of both experiments showed rather marginal attraction to the damaged plants, resulting in insignificant numbers of completed flights for both groups tested. In the first experiment, of the 80 wasps released, only 18 wasps completed their flight to one of the two plants. Although the proportion of wasps choosing one of the two plants in the second experiment was higher, the number of 'no choice' recordings were still very high (figure 10). It is not very elegant, let alone of ecological relevance to draw conclusions from recordings with this proportion of completed flights. However, *Microplitis croceipes* females displayed no preference for plants exposed to both below- and aboveground (BG+AG) in both first ($p>0,1$) and second experiment ($p>0,1$).

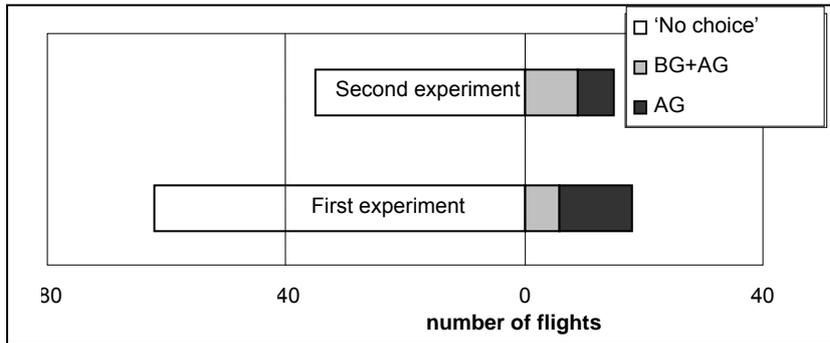


Figure 10 Flight response of the specialist parasitoid *M. croceipes* tested a two-choice experiments to plants attacked by the polyphore host *H. armigera* (AG) alone and to plants damaged simultaneously with the root herbivore *A. lineatus* (BG+AG). Wasps released in the first experiment had no preliminary experience with cotton in contrast to wasps released in the second experiment. This group consisted of re-tested wasps that were initially recorded as having 'no choice' in the first experiment. Shaded bars indicate the numbers of wasps that landed on each source. Open bars show those that did not land on either plant and were recorded as 'no choice'.

Discussion

1. Field experiment

We hypothesized that, under the experimental circumstances EFN secretion of nectaries located on foliage of cotton plants would be raised in response to BG-feeding and that this would be reflected in a higher number of insect visitors. However, data resulting from our field experiment do not indicate an effect of root herbivory on quantity of insects visiting extrafloral nectaries. Our hypothesis was based on data showing that under laboratory conditions cotton plants inoculated with wireworms produced significantly more EFN during the first week as to compared control plants. (Wäckers and Bezemer, 2003). The fact that our experiment was not conducted under controlled conditions might have influenced our results. First of all, this experimental design did not allow us to measure the amount of EFN that was actually secreted by plants during the experiment. It is possible that the assumed difference in EFN secretion between control plants and individuals inoculated with wireworms did in fact not occur. I consider that abiotic stress factors associated with the abrupt transfer of plants from the greenhouse to the field could have overshadowed the effect of BG-herbivores. Although plants were on one side partly protected by a mesh screen, they were nevertheless exposed to relatively low humidity, full and direct sunlight and severe wind. Furthermore, wind caused incidental rupture of leaves in these fragile greenhouse plants. These stress factors, not endured in a greenhouse, could have influenced my data. Cotton has been intensely studied and overall results indicate that specificity of direct defense induction may be limited (Croxford et al., 1989; Alborn et al., 1996). Additionally, concerning induced indirect defence, it is known that *G. herbaceum* raises secretion of EFN in response to mechanical tissue damaged without requiring any herbivore-specific elicitor (Wäckers and Wunderlin, 1999). This lack of specificity in defense response can lead to a reaction of the plant in the absence of herbivory (Wäckers and Wunderlin, 1999). These 'false alarms' could have played a major role in our experiment. As nectar secretion could have been primarily induced by adverse environmental conditions like mechanical damage due to wind, which might have overshadowed the effect of wireworms.

However, there appears to be an effect of belowground herbivory on insect distribution. It was shown that insects recorded on plants inoculated with wireworms were significantly more concentrated higher in the plant as compared to control plants. Contradictory, this effect was accompanied by an almost significant higher spread around the mean, suggesting a more even distribution over the plant, instead of a stronger preference for the higher leaves. Thus insects on plants under belowground attack were found primarily on the younger leaves, but displayed a wider distribution. This last observation fits the findings of Lommen and Wäckers (unpublished data, 2002) that belowground EFN induction is systemic throughout the plant, which can be expected to be reflected in a more even distribution of nectarivorous insects.

An explanation for the fact that insects were concentrated higher in BG-induced plants could be provided by the optimal defense theory. This theory predicts that defensive traits will be produced and allocated in a pattern that optimises the trade-off between defensive costs and benefits (Zangerl and Ruthledge 1996). While plants were observed to be exposed to a relatively high level of mechanical damage, the plant has to set priorities in resources allocation for compensatory growth. Under stressful conditions, the plant allocates resources from reserves stored in the roots for compensatory growth to the most valuable parts, in this case the upper leaves that represent the highest remaining photosynthetic potential (Van Dam et al., 2001). This phenomenon was also shown in allocation of secondary metabolites in cotton when fed on simultaneously by foliar- and root feeders (Wäckers and Bezemer, 2003). It appeared that allocation of defense compounds (terpenoid aldehydes) was mainly focused on the aboveground compartment, at the expense of belowground defense. Moreover, increased amount of resources allocated to the higher leaves at the expense of lower leaves, could imply a higher state of defense. This could be subsequently revealed in higher secretions of EFN. Furthermore, the optimal defense theory might provide us with an explanation for another observed phenomenon. During the course of the experiment insects tended to be concentrated higher in plants. Parallel to this, plants were getting more and more damaged over time, which was accompanied by an presumable increased resource allocation towards the top leaves. Subsequently higher levels of EFN could have been secreted, resulting in higher numbers of insect visitors on these terminal leaves. On the other hand, this increment in center of gravity of insect visitation over time might also be (at least partly) explained by the fact that plants developed their top leaves during the experiment.

In support of future experiments that try to elucidate the ecological consequences of belowground inducibility of EFN, I suggest to check the effect of BG-herbivores first under circumstances comparable to conditions plants are exposed to in the field. Under, from the plants perspective, 'less favorable' conditions, plants might react in a different way as observed under optimal greenhouse

conditions. However, I agree that laboratory and greenhouse experiments are still very important to be able to recognize the existence of individual mechanisms involved in plant defense. But to determine the ecological importance of such mechanisms in plant response, field experiments, in which plants endure simultaneous abiotic stress factors in concert with exposure to herbivores, are essential.

It is assumed that EFN secretion is part of an indirect defense strategy, a 'protective mutualism' in which predators and parasitoids protect plants from herbivory in exchange for nutritional rewards, (Wäckers et al., 2005). A putative elevation in EFN secretion might therefore result in higher numbers of plant beneficials and consequential lower numbers of herbivorous species. On the other hand, it is found that aphid performance can be greatly increased by feeding of root herbivores by increased levels of foliar nitrogen (Gange and Brown, 1989). Our data revealed no effect of inoculation with wireworms on numbers of ants nor on aphid recordings. This may once more be explained by exposure to a high degree of mechanical damage that overshadowed the effect of wireworm infestation (see above). Moreover, comparison of treatments was hampered by a low number of recordings of both carnivores and herbivores and a high variation within the treatments.

Although only marginal numbers of ants were observed attending the extrafloral nectaries, our data suggests that on plants accessible to ants, aphid infestation seemed to be higher. Besides being attracted to EFN, ants are also attracted to sugar-rich honeydew producing sternorrhynchae species including homopterans like aphids. Both recorded aphid species (*A. fabae* and *A. Gossypiae*) have shown to be tended by *L. niger*, the only ant species observed. In this ant-homopteran mutualism, the ants protect the sap-sucking aphids in exchange for nutritious honeydew (Gaume et al, 1998). When both EFN and honeydew are available as a carbohydrate source, foraging theory predicts that consumers will sometimes ignore readily available low-quality food items when higher-quality items are also present (Engel et al., 2001; Charnov, 1976). However, there was no significant competitive effect between the two sugar sources. Aphid numbers were not affected by the possible effect of wireworm infestation, although EFN was assumed to be superior in quantity to honeydew especially in BG-induced plants. So in this experiment, plants didn't receive a higher level of protective mutualism provided by foraging ants when infested with wireworms. Again we can question whether the expected increase of EFN secretion actually took place. Secondly, as sugar concentrations of both EFN and honeydew were not measured, it is hard to say if a higher quality (and quantity) of extrafloral nectar may have outweighed the quality of aphid honeydew. Moreover, we have to take into account that we're not dealing with a natural system. A relatively high proportion of nectar was obviously consumed by the high numbers Dipteran visitors, neither beneficial nor directly detrimental for the plant. This may have influenced our data resulting in lower numbers of ants attending the plants, whilst *L. niger* tolerated these insects. In contrast, natural systems showed that ants associated with cotton attacked any competitors feeding from the same source. Due to the fact that neither ants nor aphids were acquainted with cotton, drawing general conclusions from this experiment is rather disputable.

Overall, it is still unknown what to consider as the ultimate cause for the phenomenon that foliar nectar is induced in response to root herbivory. The induced secretion of EFN following shoot damage fits well in the optimal defence theory, as recruitment of aboveground predators has proven to increase plant fitness. I'm not aware of any studies showing an increase of belowground predation by ants in relation to raised levels of EFN production. Wäckers and Bezemer (2003) proposed three explanations from different viewpoints for the reported induction. First it was stated that induction represents just a physiological constraint, rather than being adaptive in terms of plant defense. Changes in source/sink balances in response to root damage could cause accumulation of carbohydrates in the foliage resulting in additional nectar production. Secondly, it could represent an adaptive trait for the root feeder that has been shown to be reduced in performance by a concurrent attack by aboveground counterparts (Maron, 1993). In this way root herbivores could benefit from a higher state of aboveground defense, which brings us to the final explanation. A higher state of aboveground defense, revealed in elevated prophylactic secretion of foliar EFN in response to belowground attack, could help the plant to avoid an expensive war on two fronts.

Whatever the ultimate cause of this phenomenon, if we want to examine the ecological consequences this might potentially have on aboveground population dynamics on a three-trophic level, we have to show first the relevance of BG-induced EFN secretion in natural systems in which a plant has to endure harsh conditions.

2. Herbivore feeding preference

In our experiment, it was observed that the foliar herbivore *H. armigera* was very mobile and fed on both offered plants, belowground induced as well as control plant. Our results show that under these experimental conditions, root herbivory did not persuade the foliar feeder to feed less from the BG-damaged plant, when offered undamaged alternative host plant. The *H. armigera* larva seemed not affected by a possible effect imposed by belowground herbivores, in contrast to previous studies carried out by Bezemer et al. (2003). In that experiment it was shown that root-feeding insect larvae had a negative effect on a foliar feeder presumably mediated by the plants defence system, revealed in increased allocation secondary plant metabolites to the leaves. Increased levels of foliar terpenoids in cotton resulted in a lower performance and avoidance behaviour in *S. exigua* larvae.

I argue that the effect of belowground herbivory on larvae feeding preference was due to the fact that induced terpenoids may not have affected our foliar feeders. Terpenoid aldehydes have unquestionably demonstrated its negative effects on many generalist-feeding lepidopterans, including *Helicoverpa spp* (Stipanovic et al., 1988). In truth, a possible change in foliar defense compounds was not verified as no analyses of terpenoid levels was carried out in our experiment. Since in our experiment inoculation of belowground herbivores took place only one week before larvae were introduced, it is uncertain if this was a sufficient period for any significant expression of direct defense to occur in aboveground plant parts. Previous studies using the same system showed significant increases of foliar terpenoid levels after four (Bezemer et al., 2003) and five weeks (Bezemer et al., 2004) following inoculation of root herbivores. Therefore, the observed behaviour of larvae not using the alternative plant as a putative low-terpenoid refuge could be explained by the fact that there was no significant difference in foliar terpenoid concentrations between the two plants. Although wireworm feeding activity was verified, reflected in a fivefold increase in EFN secretion of plants inoculated with wireworms, we are unsure of the actual period between exposure to root herbivores and increased allocation of defense compounds to the leaves. Therefore I question if the behavior of larvae was influenced by the presumed presence of these compounds in leaves during our experiment. For further experiments, I suggest to incorporate a period of at least four weeks between inoculation of wireworms and introduction of the larvae, to be sure of sufficient time for the plant to respond. It is also advisable to measure concentrations of defense compounds in foliage in order to be more certain about the source that causes a possible change in feeding behavior of your foliar feeder.

In addition, it has been shown that preference for a certain host plant could be induced in larvae after only one day of feeding experience. In polyphagous *S. littoralis* larvae, feeding preference at a later stage was influenced by previous feeding experience (Anderson et al., 1995). Furthermore, it was also demonstrated that an orientational response could be induced by host plant associated volatile cues in *S. littoralis* larvae previously feeding of the plant (Carlsson et al., 1999). Larvae reared on a synthetic diet did not show any attraction to the odor of cotton leaves, whereas cotton-reared larvae were highly attracted. As larvae in our experiment were reared on excised cotton leaves, harvested from undamaged plants, larvae could display an initial preference for undamaged plants. However, no feeding preference was observed during the five days the larvae were allowed to feed both BG-induced and control plants, suggesting no effect of previous feeding experience. Moreover, an innate behavioral response of this polyphagous herbivore to numerous odors of the wide range of host plants the larvae might encounter during its development is not very likely. The larva is probably better served by an ability to modify its behavior after having experienced a novel food source. This type of phenotypic plasticity without much (innate) preference most likely optimizes foraging in these generalist herbivores (Carlsson et al, 1999).

The possible effect could furthermore be overshadowed by short term changes in leaf palatability associated with damage. Experiments in cotton demonstrated that low levels of AG-damage may induce local changes in palatability which causes *S. littoralis* larvae to avoid the site of damage (Croxford et al., 1989). Furthermore, this could be a direct effect of increased terpenoid levels in response to AG-herbivory. However, Bezemer et al. (2003) showed that terpenoid concentrations were mainly increased in the valuable immature leaves in response to leaf feeding, while BG-damage showed an overall- and BG+AG feeding an intermediate level of increment. If the plants were induced belowground, this might involve a higher proportion of leaf consumption on the terminal leaves of the BG-damaged plant relative to larvae on the control plants, since levels of foliar terpenoids in these plants were high throughout the plant. However, in our experiment, larvae on BG-induced plants did not consume a higher proportion of terminal leaves. This observation further adds to the conclusion that BG-induction might have been neglectable, or at least at a very low level.

Although wireworm activity was reflected in a five-fold increase in overall EFN secretion, another mechanism proposed by Masters et al. (1993) could be responsible for the observed feeding pattern. Our data indicate a tendency of larvae to feed longer on the same spot on leaves of BG-induced plants. Although they performed equally, feeding on BG induced plants could represent a nutritional advantage. In agreement with Masters' theory, root feeding causes a stress response in the plant, reducing water and nutrient uptake, resulting in higher concentrations of nitrogen in the foliage. However, in our experiment, we ensured that plants were not water stressed. Besides, has it been shown that the induction of water stress in plant tissue by a root-feeding insect can be offset to a certain extent by supplying plants with ample water (Gange and Brown, 1989). In addition, it was demonstrated by Bezemer et al. (2004) that when supplied with ample water, root herbivory by *A. lineates* had no effect on foliar nitrogen levels in cotton. Nevertheless, phytophages may change nutrient levels in plant tissue by manipulating source-sink relations. An altered flow in the plants vascular transport system in response to root damage, has been demonstrated to cause a physiological change aboveground (Rhodes, 1987).

Finally, on the short term, according to Masters et al. (1993) belowground herbivory might pose an additional disadvantage for the plant by improving nutritional value of the plant tissue aboveground. However this effect might be reversed after four weeks of root herbivore feeding, when aboveground expression of direct defence could be more apparent. In that case, it would be obvious that larvae would try to circumvent high terpenoid levels by switching to low terpenoid tissue, as has already been reported (McAuslane et al., 1997). Nevertheless, to draw ecological conclusions concerning consequences of root feeding by these long-lived, persistent insect larvae, effects should be measured over even longer periods of time. There is still a lack of experiments investigating more chronic exposure to root herbivores, with perennial plant roots becoming progressively more damaged in following years. As Maron (1998) showed that root herbivores only affected plant fecundity negatively in the third year of the experiment, where roots were extensively engraved and damage. As cotton is a perennial species, the exposure to sessile and persistent wireworms over prolonged time periods of several years could also represent a cumulative impact. Subsequently it might result in higher resource allocation to reproduction and dispersal, in agreement with the enemy escape theory, instead of plant growth (Thompson, 1988).

Overall, above and belowground interactions are only of relevance if they occur in the field. In contrast to optimal laboratory conditions, in nature, plants have adapted to survive under conditions far from optimal. Plants have to compete continuously for resources, whereas for instance, it is accepted that the majority of plants in the field are water stressed (Hsiao, 1973). Clearly, if root herbivory can affect levels of water, nitrogen and defensive compounds in plant tissues, this is likely to have important consequences for foliar-feeding insects associated with such plants. However, especially the possible impact of induced secondary compounds in nature can be overestimated. Until now this phenomenon was only demonstrated under optimal conditions, while this induction can be costly under low nutrient conditions and subsequently force a plant to tolerate higher levels of infestation (Blossey and Hunt-Joshi, 2003). Furthermore, it can't be overlooked that putative mechanisms on how BG- and AG herbivores might interact (nutrients and plant defence) are typically interlinked. Production of both direct and indirect plant defence compounds depend on nutrient uptake by the roots. N-based defensive compounds as well as amounts of volatiles produced when attacked aboveground (Van der Putten et al., 2001). Serious effects of stress factors have to be taken into account when extrapolating results of laboratory studies into the field. This is also demonstrated by the fact that cotton plants in laboratory experiments showed no major herbivore-induced fitness reductions (Bezemer et al., 2003), documenting their ability to effectively compensate for leaf and root herbivory under optimal conditions (Maron, 1998). To fully understand the importance of root herbivores, more large-scale manipulative field experiments are required. A problem is designing these experiments that demonstrate conclusively impacts of root herbivores considering that the test organism is not readily observable – hence assumptions have to be made regarding its activities. Additionally do these field experiments that involve root feeders comprise techniques as local elimination by use of soil insecticides, that may have indirect effects on the parameters of interest (Masters, 2004). However, to get an idea of the importance of root herbivores in terrestrial ecosystems, more field data is essential.

3. Herbivore oviposition preference

In our experiment, the gravid female moths were only marginally ovipositing on either of the host plants offered. This makes me doubt about the use of generalist lepidopteran species in preference experiments. The parasite/grazer hypothesis predicts that females of grazer species (i.e., herbivores

that can complete development on more than one host) are not likely to show strong host plant oviposition preferences (Thompson, 1988). This in contrast to parasitic herbivores (i.e., herbivores that complete development on one host plant), that invest much energy in searching a specific host plant. The strategy of highly polyphagous insects, such as *S. exigua*, implies that eggs are deposited rather randomly over plants (Smits *et al.*, 1987), thus leaving more energy available for reproduction. Ultimately the increase in fitness resulting from a higher reproductive potential must compensate for any fitness reduction resulting from development on a possible nutritionally inferior host. In addition, grazers including beet army worm (*S. exigua*) tend to have highly mobile larvae, which are able to disperse easily from the site of oviposition. As polyphagous larvae have, at least to a certain extent, the ability to determine their own host plant (Carlsson *et al.*, 1999), this might partly explain the phenomenon that lepidopteran females don't show particularly choosy behavior.

Furthermore, we hypothesised that the adult moths would prefer undamaged plants over plants infested with root herbivores for oviposition. As it was shown that BG-induced allocation of secondary metabolites can hamper larval development (Bezemer *et al.*, 2003), inhibition of oviposition could clearly increase survival of its offspring. However, in a remarkable high number of studies in lepidopteran behaviour the correlation between adult host preference and offspring development appears not to be a positive one (Berdegue *et al.*, 1988; Fox and Eisenbach, 1992; Singer *et al.*, 1994; Mulatu *et al.*, 2004). This means that in the presence of a preferable alternative, adult female herbivores deposited their eggs on a nutritional inferior plant. Part of this phenomenon can be explained by the general hypothesis of selection pressures that could shape host plant use by insect herbivore species (Thompson, 1988). In addition, to indicate the complexity of the system, also larval diet has demonstrated to influence adult oviposition behavior (Anderson *et al.*, 1995). Adults preferred to oviposit on plants on which they fed as larvae.

Finally, I believe that an effect of continuously rearing of insects on artificial diet, by putting no selection pressure on host choice by the female moths, can not be ruled out. Larvae were taken from a culture reared for several generations on synthetic diet, while no wild collected moths had been introduced into the culture. If genetic covariance does occur between preference and performance, this could have certainly be of significance in our experiment. However, it has to be mentioned that this genetic relationship is not distinguished yet. Furthermore, some tests showed that prolonged time periods of selection, including several generations, did not change oviposition preference (Thompson, 1988 and references cited herein).

Nonetheless, the effects of changed plant secondary chemistry, induced by root herbivory, obviously can change relations aboveground in second and higher trophic levels, including repellence or avoidance of aboveground herbivores (Van der Putten *et al.*, 2001). However, it is still not clear what plant-related cues (volatile or non-volatile) are exploited in the host plant selection process. The role of olfaction in host plant choice seems apparent among non-conspecific plants. Results from cage and olfactometer assays, suggest that volatiles emitted from cotton (*G. hirsutum*) and pigweed (*Amaranthus hybridus*) play a role in the tendency for female *Spodoptera* to oviposit primarily on pigweed, which lacks the volatile compound gossypol (Showler, 2001). Among conspecific host plants, behavioural studies showed that ovipositing females of *S. littoralis* preferred undamaged cotton plants over plants damaged by feeding of conspecific larvae (Anderson and Alborn, 1999). In addition, in this experiment it was shown by electrophysiological techniques that females were able to detect these herbivore-induced volatiles on sensilla located on the antennae. A direct effect of induced volatiles on herbivores was found in Lepidoptera in cotton (Jönsson, 2005). A cage experiment demonstrating female moths (*S. littoralis*) being deterred by odours of cotton plants infested by conspecifics, gives evidence of the role of olfaction in host plant selection. This phenomenon was also observed in an olfactometer experiment, where the odour from intact plants (*Zea mays*) was attractive to moths (*Ostrinia nubilalis*), but odours emitted by injured plants (infested or mechanically damaged) acted as a repellent (Schurr and Holdaway, 1970). Jönsson (2005) stated that avoidance of plants releasing induced odours can be adaptive to decrease the risk of predation, parasitism, competition and malnutrition for offspring. This seems an obvious theory, but it does not include the fact that in many preference/performance studies, no correlation was found. What non-volatile contact chemicals are concerned, it was demonstrated by electrophysiological response on the sensilla of the proboscis that detection of sugars and amino acids by of *S. littoralis* is possible (Blaney and Simmonds, 1988). Assuming that *Spodoptera* can also detect secondary plant metabolites like foliar terpenoids, it is evident that sensitivity to these compounds could play a role in the selection of host plants for oviposition. When a female moth searches for an optimal diet for her offspring, she should certainly

take presence of terpenoids into account, while these can seriously hamper larvae performance (Stipanovic et al., 1977).

Ultimately, I think that the choice of oviposition site is mediated mainly by a combination of olfactory cues and contact chemoreception, but strong preference is mainly an adaptive trait in specialist phytophagous insects. As studies have shown a changed behavioural response of ovipositing herbivore insects towards aboveground damaged plants, it seems reasonable to assume that also belowground damage has the potential to affect host preference. I suggest that among the most important subjects for further research in this area, are studies investigating the consequences of BG-damage in natural systems on ovipositing behaviour focussing preferentially on more oligophagous herbivores.

4. Belowground induced emission of volatile semiochemicals

a. Volatile collection

No conclusive results were obtained from our experiment addressing the effect of belowground herbivory on foliar emission of volatiles. Since also the trial experiment showed ambiguous results, a possible effect of root feeding remains unidentified. I don't think it is very interesting to speculate about the causes why our volatile collection samples revealed such relative low proportion of cotton-related plant odors. However, the experiment will be repeated in the lab of T. Turlings later this year to reveal the possible impact of BG-herbivory on aboveground volatile emission in cotton.

b. Parasitoid flight response

Since inducible compounds have shown to be released in significant amounts in response to AG herbivore feeding (Röse et al., 1996), they present a reliable indication of the presence of a potential host. Especially inducible compounds that are systemically released in response to herbivore damage may be easily detected by parasitoids from a distance since they are released in large amounts throughout the entire plant (Röse et al., 1996). However, in our experiment the specialist parasitoid *M. croceipes* completed only a small number of flights to both plants offered (AG and BG+AG). Despite the fact that wasps were allowed a preflight experience consisting of antennation and oviposition of a diet-fed host larva. This has shown to activate host-searching behavior of this wasp (Röse et al., 1997) and consequently proved to increase the number of completed flights compared to naïve wasps. Also a second experiment, in which the wasps were released a second time after having been recorded as having no choice in the first experiment, resulted in a low number of wasps landing on either of the plants offered. Therefore, the experience with plant-related odors, color or shape in the first experiment, did not improve the parasitoid's host searching behavior. I question if this could have made a difference, since it is considered that attraction of specialist *M. croceipes* parasitoids to cotton plants is innate (Röse et al., 1998), although they are considered as excellent learners and experience can modify innate responses. As *M. croceipes* uses visual as well as olfactory cues to locate a host site (Wäckers and Lewis, 1994) and plants were clearly visible for the wasps in our experiment it is still unclear why wasps were not responding to the presence of herbivore damaged plants. I discuss two reasons for the observed overall low level of response. First, the flight response test took place during conditions of low atmospheric pressure. Although the experiment was situated under climate controlled circumstances, it has been reported that factors as low atmospheric pressure could seriously influence behavior. Second, the internal state of the insect can have affected the behaviour of the parasitoid. Nutritional state has for instance been shown to affect whether a parasitoid engages in search for hosts or food (Van Lenteren and Bakker, 1979). However, this effect of energy deprivation was likely limited in our experiments, as we ensured that our wasps were never restricted in their food and water supply.

Despite the fact that one cannot draw conclusions from the low number of completed flights, I like to comment on the results that were obtained. The completed flights did not indicate a clear difference in plant attraction. Since it is still unclear if, and in what way the volatile blend was changed when the plant was simultaneously attacked in root and shoot, I hypothesize that this could include a higher release of constitutive mono- and sesquiterpenes. For a specialist endoparasite like *M. croceipes* these non-specific compounds released following BG-damage may not provide useful cues for locating a leaf-feeding host. In contrast to a generalist with a broad range of host species it can parasitize, these compounds were shown to increase plants attractivity (Röse et al., 1998). It may explain our findings that BG+AG plants were not preferred over AG damaged plants. Due to low information specificity of what inflicted the plant damage, the chances of the wasps encountering a host in which it can develop are not increased.

Secondly, I hypothesized that the composition of the volatile blend could be changed in such a way that volatiles specific to the AG-herbivore are released in higher amounts by BG-herbivory as it was also shown to increase non-volatile terpenoids in the foliage (Bezemer et al., 2003). In that case, BG herbivory could increase host searching efficiency of aboveground parasitoids, and may under some circumstances represent a potential competitive advantage. However, it is still uncertain how plant emitted odors determine plant attractivity for a host searching wasp; quality, quantity or both? In a recent study it was demonstrated that parasitoids have preferences for specific herbivore-attacked plant species. Hoballah et al. (2002) showed that cowpea odors were preferred over maize odors by naïve *Cotesia marginiventris*. This was despite the fact that cowpea released much less odors compared to maize. So there is some evidence showing that quantity of odors is not by definition positively correlated with attractiveness. Consequently, when investigating the effect of simultaneous herbivory in root and shoot on volatile emission, the whole spectrum of compounds should be taken into account, regardless of their quantities. However, it has to be mentioned that overall, parasitoids may use non-specific plant volatiles in the first stages of habitat or host community location. Once such an area has been located, wasps may rely on more specific cues such as host frass (Röse et al., 1997), which could be learned associatively during host encounters.

Several studies have linked below and aboveground compartments in a multitrophic context. In a field study, Masters et al. (2001) found greater population abundances of parasitoids on thistle plants subjected to root herbivory. It is questionable whether the increased abundance of parasitoids had anything to do with a changed volatile spectrum. It is more likely that the root herbivores, which showed to increase performance and abundance of aboveground hosts, had mainly density-dependent knock-on effects for numbers of recorded parasitoids. This seems also the case in a study involving the effect of root infection by arbuscular mycorrhizal fungi on presence of insect parasitoids. Parasitoid searching efficiency was reduced on plants inoculated with certain mycorrhiza species combinations (Grange et al., 2003). Although the most likely cause of these findings was an observed differences in plant size, caused by these mycorrhizal combinations, an effect of herbivore-produced plant volatiles couldn't be rule out. AM fungi can have significant effects on chemistry of host plant leaves, and may therefore alter plant chemistry in such an extent as to affect the attractiveness of damaged leaves to a foraging parasitoid (Gange and West, 1994). Overall parasitoid searching efficiency is known to be influenced by host-plant characters like nutrient status and distribution of secondary metabolites. As several studies have revealed these characters can change as a consequence of belowground herbivory (Masters et al., 1993; Bezemer et al., 2004), this can have both direct effects on the parasitoid and indirect host plant mediated effects.

5. General discussion & prospects

Plants are embedded in a complex network of interactions with other plants, above- and belowground resource availability, and above- and belowground herbivores (Blossey and Hunt-Joshi, 2003). As all these components interact and in concert (not in isolation) determine the plants fitness and their role within plant communities, underlying mechanisms are very difficult to elucidate. Interpretation of their ecological relevance will be even more difficult.

Models predicting root feeders would be negatively affected by competitively superior aboveground herbivores may be limited to early successional habitats (Masters et al., 1993). This is regarded a result of the fact that later successional communities include higher numbers of perennial plants. This importance of life-history strategy for root herbivores seems evident as these perennial plants store more energy in belowground plant parts (Masters et al., 1993). Maron (1998) suggests that aboveground feeding in these plants causes translocation of stored assimilates (primarily C-based compounds) from roots for compensatory growth. This results in a decrease in C:N ratio which in turn results in a higher food quality for belowground herbivores. In contrast annual plants do not possess this storage strategy, so primary productivity products will be directly diverted to compensatory growth aboveground, reducing root biomass, which in agreement with Masters model (1993) negatively affects belowground herbivores by limiting food quantity (Maron, 1998). In later successional habitats, root feeders have shown to become more abundant (Blossey and Hunt-Joshi, 2003) and appear to pose a powerful force in driving plant performance and plant community composition (Masters, 2004). Subsequently interaction strengths between plants and above- and belowground herbivores are dynamic and change over time (Van der Putten et al., 2001). This may lead to alternation in selecting forces, in which root herbivores change seats from adapting passengers in early successional stage to driving the selection and shaping plant communities during later stages in succession.

Consequently, the evolution of plant communities should be viewed in the perspective of an aboveground- belowground multitrophic selection arena. An example of belowground herbivores

changing plant traits is shown in a study carried out by Maron (1998). Results from experiments investigating joint effects of above and belowground insect indicated that feeding by persistent and sessile root herbivores can potentially impose strong selection effects on the host plant. Perennial lupine plants (*Lupine arboreus*) showed increase growth and subsequent fast seed set in response to belowground herbivory. They seemed to alter their strategy before they die from belowground herbivory (Maron, 1998). In comparison with the spring annual, *Arabidopsis thaliana*, this species probably defends itself also by escape in time, rather than chemical defence (Van der Putten et al., 2001). In addition, exposure of plants over longer periods to sessile and persistent herbivores such as root feeding insect larvae and nematodes drives plants to require efficient dispersal mechanisms in order to escape their belowground attackers (Masters, 2004). Finally, awareness of the existence of links between above- and belowground multitrophic interactions provides new areas for research on the ecology and evolution of plants. In this research plant defence should be included in examining links between above and belowground interactions (Van der Putten et al., 2001). It will increase our knowledge about the functional aspects of plant life-histories and the evolution of (induced) direct and indirect plant defence.

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Appendix 1

Total numbers of parasitic wasp species visiting plants with different species recorded on cotton during the field experiment. Numbers were too low to be able to suggest an effect of plant treatment on numbers of parasitoids visiting the plants.

Plant treatments: +/- A: ant access and ants excluded respectively

+/- W: plants with or without wireworm infestation respectively

	Plant treatment				total
	-A+W	+A-W	+A+W	-A-W	
Hymenoptera					
Apocrita					
<i>Ichneumonidea</i>					
<i>Ichneumonidae</i>	2	1	3	1	7
<i>Braconidae</i>	2	1	1	3	7
<i>Cynipoidea</i>					
<i>Eucoilidae</i>	1	1	3	4	9
<i>Charipidae</i>	1	-	-	-	1
<i>Chalcidoidea</i>					
<i>Mymaridae</i>	-	-	1	-	1
<i>Trichogrammatidae</i>	2	-	-	1	3
<i>Euritomidae</i>	-	-	-	1	1
<i>Eulophidae</i>	10	3	6	5	24
<i>Encyrtidae</i>	1	1	2	1	5
<i>Pteromalidae</i>	3	3	4	6	16
Proctotrupoidea					
<i>Scelionidae</i>	1	3	-	-	4
<i>Platygastridae</i>	-	-	1	1	2
<i>Diapriidae</i>	-	1	-	-	1
<i>Ceraphronoidea</i>					
<i>Ceraphronidae</i>	5	4	4	7	20
Total	28	18	25	30	101

Appendix 2

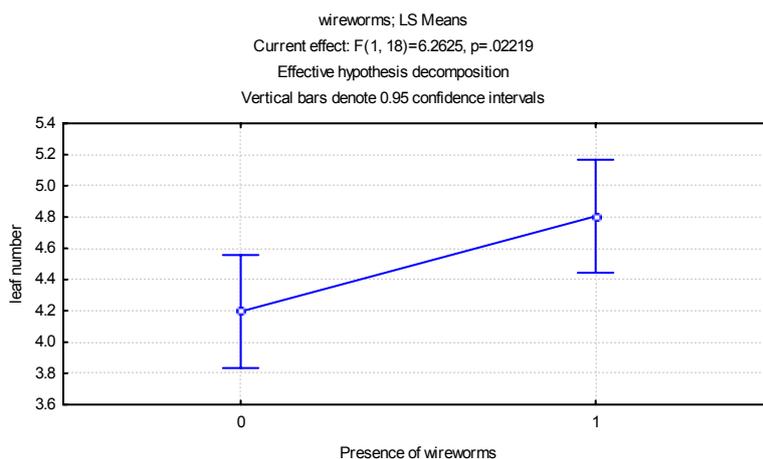
Repeated Measures Analysis of Variance (rmanova) of total insect numbers visiting the cotton plants.

Total numbers					
	SS	df	MS	F	p
Intercept	2747.27	1.00	2747.27	576.69	0.00
wireworms	2.82	1.00	2.82	0.59	0.45
Error	85.75	18.00	4.76		
TIME	673.13	11.00	61.19	18.94	0.00
TIME*wireworms	35.38	11.00	3.22	1.00	0.45
Error	639.65	198.00	3.23		

centre of gravity					
	SS	df	MS	F	p
Intercept	4860.84	1.00	4860.84	1365.36	0.00
wireworms	22.30	1.00	22.30	6.26	0.02
Error	64.08	18.00	3.56		
TIME	110.08	11.00	10.01	2.80	0.00
TIME*wireworms	33.37	11.00	3.03	0.85	0.59
Error	708.93	198.00	3.58		

deviation from center					
	SS	df	MS	F	p
Intercept	233.55	1.00	233.55	525.05	0.00
wireworms	0.99	1.00	0.99	2.23	0.15
Error	8.01	18.00	0.44		
TIME	41.91	11.00	3.81	7.72	0.00
TIME*wireworms	5.55	11.00	0.50	1.02	0.43
Error	97.68	198.00	0.49		

Graph showing the effect of wireworm treatment with respect to the location of the center of gravity of total insect visitation. Presence of wireworms (1) suggests a higher center of gravity compared to control plants (0).



Appendix 3

Repeated Measures Analysis of Variance (rmanova) of total number of ants recorded on the cotton plants during the field experiment

Total ant attendance	(analysis untill day 15)				
	SS	Degr. of Freedom	MS	F	p
Intercept	95.22	1.00	95.22	15.57	0.00
wireworm	0.32	1.00	0.32	0.05	0.82
Error	110.06	18.00	6.11		
TIME	12.38	9.00	1.38	1.69	0.10
TIME*wireworm	8.08	9.00	0.90	1.10	0.36
Error	131.94	162.00	0.81		

center of gravity	(analysis untill day 15)				
	SS	Degr. of Freedom	MS	F	p
Intercept	472.01	1.00	472.01	19.00	0.00
wireworm	1.08	1.00	1.08	0.04	0.84
Error	447.11	18.00	24.84		
TIME	45.82	9.00	5.09	1.56	0.13
TIME*wireworm	12.67	9.00	1.41	0.43	0.92
Error	527.17	162.00	3.25		

deviation around center	(analysis untill day 11)				
	SS	Degr. of Freedom	MS	F	p
Intercept	9.63	1.00	9.63	13.95	0.00
wireworm	0.16	1.00	0.16	0.23	0.64
Error	12.42	18.00	0.69		
TIME	1.20	7.00	0.17	0.75	0.63
TIME*wireworm	1.63	7.00	0.23	1.03	0.42
Error	28.63	126.00	0.23		

Appendix 4

Collection of volatiles of plants exposed to different treatments in a trial experiment without replicates. Results only show volatiles known to be associated with an induced response in cotton. Numbers represent amounts collected relative to other amounts in the sample. Quantification was not possible because no internal standards were taken in the analyses. Overall, result remains inconclusive because the measured concentrations seem quite ambiguous.

compound/treatment	(CC)	(AG)	(BG+AG)	(BG)	[BC]
(Z)-3-Hexenol	0	0	0	0	0
(Z)-3-Hexenyl acetate	0	0	0	0	0
linalool	87	249	454	95	0
(E)-4,8-dimethyl-1,3,7-nonatriene	5800	7490	3900	3060	0
(E)- β -farnesene	236	123	376	137	0
(E,E)-4,8,12-trimethyl-1,3,7,11-tridecatraene	243	138	8	391	0
indole	0	0	0	0	0
(E)- β -caryophyllene	122	65	15	338	0
α -pinene	32	61	53	27	10
β -myrcene	57	0	0	0	0
(E)- β -ocimene	36	38	294	12	0

Appendix 5

Individual leaf consumption of *H. armigera* larvae placed on cotton plants either previously exposed to root herbivory by *A. lineatus* or undamaged controls. In both cases larvae were also offered an alternative control plant. Over five days larvae were able to move freely between the two plants. Except from the total leaf consumption, comparisons were made based on data of plants on which the larva was initially placed. Transformation did not lead to a normal distribution of our data. Results are analyzed by a nonparametric Man-Whitney U test. No significant differences were to be observed.

	Rank Sum	Rank Sum	U	Z	p-level	Z	p-level
	Group 1	Group 2				adjusted	
Total consumed per plantpair	39	39	18	0	1	0	1
leaf consumption per plant	45,5	32,5	11,5	1,040833	0,297954	1,042657	0,297108
Center of gravity of leaf consumption	36	42	15	-0,48038	0,630954	-0,48038	0,630954
deviation from the center of leaf consumption	33	45	12	-0,96077	0,336669	-0,96077	0,336669
RGR	46	32	11	1,120897	0,262333	1,120897	0,262333
# feeding holes per plant	34	44	13	-0,80064	0,42334	-0,80487	0,420893
average area of feeding hole	50	28	7	1,76141	0,07817	1,76141	0,07817
consumption of young leaves	40	38	17	0,160128	0,87278	0,161546	0,871663

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Before I started my thesis six months ago at the NIOO-CTE in Heteren, I had great expectations of my research. However, as one thing became apparent to me, it is that an ecologist has to deal with the fact that nature doesn't reveal its mysteries easily. Perhaps this also fascinates so many researchers, driven by their curiosity to try to at least partly understand the elementary mechanisms of interspecies interactions we can observe all around us. I enjoyed working with those enthusiastic people sharing the same fascination in the multitrophic interactions workgroup. First of all, I would like to thank Felix Wäckers, who introduced me into the scientific world. I liked our informal discussions about all topics related to your passion, interactions between plants and insects. Furthermore I like to thank Roel Wagenaar, best wireworm collector in Holland and Gregor Disveld for their assistance and great chats in the Fytotron about old philosophers and the Dutch soccer competition. Yde Jongema, thank you for introducing me into the complex world insect taxonomy and showing me the spectacular collection in the laboratory of entomology. It was always great fun identifying my insects with you, while talking about our adventures in Fryslân. Although not all volatile collections went as planned, I want to thank Nicole van Dam and Cristina Tamò for their great help during these experiments. I want to show my gratitude to Paul van Rijn for his statistical assistance and our dialogues about our outdoor hiking expeditions while sharing the same office. Finally to all my friends, corridor mates, brothers and parents, thanks for your support and inspiration you gave me by sharing your opinions with me, allowing me to reflect on my thesis work from totally different perspectives.

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