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PhD Thesis

Interactions between cotton plants mediated by volatile organic compounds: prospects for pest control?

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Summary

Cotton (*Gossypium* spp) is one of the most studied plants in the world due to its great economic importance. This highly valuable crop is cultivated worldwide primarily to produce textile fibers and, to a lesser extent, for animal forage and cooking oil production.

As for all plants, cotton is involved in several beneficial and antagonistic interactions and in nature it has evolved several direct and indirect defenses to ward off antagonists and to facilitate beneficial interactions. To protect and enhance the performance of cultivated cotton, various pest-management strategies are currently applied. For instance, pesticides, herbicides and fertilizers are intensively used in cotton cultivation. In addition, genetically modified varieties have been created in order to tolerate herbicides or enhance resistance to pest insects. Although these techniques result in short term economic returns, development of resistance in pests, weakening of natural plant defenses, environment pollution with agrochemicals, and unpredictable effects of introducing genetic modified crops, have increased the need for alternative, more environmentally friendly pest control strategies. This need is further enhanced by human activities that facilitate the introduction and dispersal of non-native invasive organisms. To fight these pests, bio-control and integrated pest management (IPM) strategies have been proposed, but much knowledge is lacking to achieve the full potential of these strategies. For instance, a comprehensive understanding of the plant's direct and indirect inducible defenses against insects and pathogens under natural conditions is essential. Studies on indirect plants defenses have shown that volatile organic compounds (VOCs) emitted by herbivore-attacked plants, are able to attract the natural enemies of pests and may repel pests. Moreover, these herbivore-induced plant volatiles (HIPVs) may also serve as signals that alert undamaged leaves and neighboring plants to incoming attack and may prime them for enhanced defense induction. Recent studies suggest that this priming of neighbors has potential for application, by enhancing pest resistance in cotton, but the underlying mechanisms are still poorly understood.

The aim of my thesis was to contribute to a better understanding of VOC-mediated signaling among cotton plants by elucidating the biochemical and molecular mechanisms that are involved in priming and/or induction. With a number of controlled plant exposure experiments I could show that cotton HIPVs change the physiology of receiver plants by means of defense-related gene activation, changes in plant hormones levels, as well as increases in levels of secondary metabolites. These changes resulted in increased VOC emissions by receiver plants and reduced palatability of the plants to caterpillar pests. These changes were

found to be plant genotype-specific and seem to be mainly driven by VOCs that are emitted in the later stage of the caterpillar infestations (inducible volatiles). This notion was based on an experiment where plants were exposed to the volatiles of relatively freshly damaged plants or of plants with older damage. We further tested the importance of the different classes of volatiles by exposing plants to different sets of authentic, pure versions of VOCs. Unfortunately, due to high variation and difficulties in controlling dispenser emission rates, we could not determine single or group of compounds responsible for defense induction in neighboring plants. We hypothesized that intact plants may require a full natural blend, a specific ratio or other non-tested compounds in order to activate defense responses.

Furthermore, we also analyzed field-collected leaves from plots with and without mechanically damaged (i.e. topped) plants and we could confirm that damage-induced volatiles can enhance the production of defense compounds in the plant itself and in neighboring plants.

This thesis highlights the potential using volatile-mediated interactions among cotton plants for the development of a novel IPM strategy that enhances cotton resistance to pests. Still, further studies are required in order to identify potent volatiles compounds that may eventually be applied for cotton protection.

Keywords: *Gossypium ssp.*, *Spodoptera spp.*, plant-plant signaling, volatile organic compounds, plant defenses

Résumé

Le coton (*Gossypium* spp.) est la plante modèle la plus étudiée à cause de son énorme importance économique. En effet, ce genre est cultivé partout dans le monde, principalement pour ses fibres, mais aussi comme fourrage et huile de cuisson. Comme toutes les plantes, le coton interagit de manière bénéfique ou antagoniste avec plusieurs organismes grâce à différents traits développés au cours de l'évolution. Pour protéger et augmenter la performance des cultures de coton, plusieurs stratégies de lutte contre les ravageurs sont aujourd'hui appliquées. Par exemples, des pesticides, herbicides et fertilisants sont utilisés intensivement sur le coton. De plus, des variétés génétiquement modifiées ont été créées afin de les rendre résistantes contre les ravageurs ou tolérantes aux herbicides. Bien que ces techniques donnent un retour rapide en termes économiques, le développement des traits de résistances des ravageurs, l'affaiblissement des défenses naturelles des plantes, la pollution environnementale due aux produits agrochimiques et les effets imprévisibles liés à l'utilisation de cultures génétiquement modifiées, ont mis en exergue l'urgent besoin de stratégies alternatives plus respectueuses de la nature. Cette nécessité prend tout son sens à la lumière de l'activité humaine, qui aujourd'hui facilite extrêmement l'introduction et la dispersion d'organismes invasifs. Des plans de contrôle biologique et des stratégies de lutte intégrée contre les ravageurs (IPM) ont été lancés dans cette voie, mais il y a encore une grande marge de progression à réaliser avant de connaître et rejoindre le potentiel total de ces stratégies. Par exemple, une compréhension plus complète des défenses naturelles, directes, indirectes et inductibles des plantes contre les insectes et les pathogènes est essentielle. Des études sur les défenses indirectes des plantes ont montré que les composés organiques volatiles (COVs) émis par une plante attaquée par des herbivores sont capables d'attirer les ennemis naturels des ravageurs et de repousser ces derniers. De plus, les COVs peuvent être utilisés comme signaux d'alerte par des parties intactes de la plante elle-même ou d'une plante voisine, résultant en un effet de « priming » pour des défenses améliorées en cas d'attaque future.Des études récentes suggèrent que ce « priming » des individus voisins a un grand potentiel d'application dans le renforcement de la résistance aux ravageurs du coton. Cependant, les mécanismes à la base de ce phénomène sont encore mal connus. Le but de cette thèse est de contribuer à une meilleure compréhension des rôles des COVs dans l'interaction entre les plantes de coton, en mettant en lumière les mécanismes biochimiques et moléculaires sous-jacent des phénomènes de "priming" et d'induction des défenses.

Via différentes expériences, en exposant des plantes aux COVs, il a pu être prouvé que ces derniers peuvent changer la physiologie de la plante, en activant l'expression des gènes de défense et en altérant les niveaux des phytohormones et des métabolites secondaires de défense. Ces changements ont mené à une augmentation des émissions des COVs par les plantes exposées et ont réduit l'attraction des chenilles envers ces mêmes plantes. Ces changements sont spécifiques au génotype de la plante et semblent être dus aux COVs émis par le coton après une infestation prolongée des chenilles (COVs inductibles). Cette dernière hypothèse est basée sur les résultats d'une expérience ou nous avons exposé des plantes à des COVs émis par des plantes avec des dégâts frais ou avec des dégâts plus vieux. L'importance des différentes classes d'odeurs a également été testés en exposant des plantes à plusieurs combinaisons de versions de synthèse et authentiques de certains COVs. Malheureusement, à cause de la grande variance et les difficultés dans le contrôle du taux d'émission des distributeurs d'odeur, il n'a pas été possible d'identifier le simple ou groupes de COVs responsable(s) pour l'activation des défenses dans les plantes voisines L'hypothèse qu'une plante intacte nécessite soit un bouquet d'odeurs complet et naturel, soit un ratio spécifique entre certains composés ou bien d'autres composés non-testés pour activer une réponse de défense a donc été émises. De plus, lors de l'analyse de feuilles de coton collectées dans des champs de culture avec des plantes endommagées mécaniquement (i.e. écimage) ou intactes, nous avons observé comme les composés organiques volatiles émis par les plantes endommagées peuvent induire la production de certains composés de défense dans la plante elle-même et parfois dans la plante voisine également.

Cette thèse met en lumière le potentiel d'utilisation de certaines composés organiques volatiles pour le développement d'une nouvelle stratégie IPM visée à augmenter la résistance du coton aux ravageurs. Toutefois, de plus amples investigations sont nécessaires pour identifier des composés spécifiques puissants qui pourraient être in fine appliqué et protéger activement le coton.

Mots-clés : *Gossypium* spp., *Spodoptera* spp., signal entre plantes, composes volatiles organiques, défenses des plantes

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Inspiring Citations:

Do what you can, with what you have, where you are.

Theodore Roosevelt

In the long history of humankind (and animal kind, too) those who learned to collaborate and improvise most effectively have prevailed.

Charles Darwin

If you want something you’ve never had, you must be willing to do something you’ve never done.

Thomas Jefferson

Happiness can be found, even in the darkest of times, if one only remembers to turn on the light.

Dumbledore, J.K. Rowling

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General introduction

Plant defenses

Plants are the key players in agricultural and natural ecosystems, occupying a central role in different multi-trophic interactions above- and belowground. Plants are subject to a plethora of abiotic and biotic stresses that include numerous pathogens, parasites and herbivores. To face these threats, plants have evolved several direct and indirect, constitutive and inducible defenses, which are regulated by the expression of genes, signal pathways and phytohormones (Ballaré, 2011).

Direct defenses include morphological traits and secondary metabolites, while indirect defenses include, for instance, volatile organic compounds (VOCs) and extrafloral nectaries that attract and reward the pest's natural enemies (Turlings & Wäckers, 2004; Pearce et. al, 2020 *in press*). Direct defenses are often not lethal to herbivores, but can still reduce damage by making the tissues less accessible and less palatable, reducing herbivore growth, and making the plants less attractive as ovipositional sites (Hagenbucher *et al.*, 2013).

Natural enemies of herbivores are key players in plant indirect defensive strategies. Predators and parasitoids, which represent the third trophic level, may actively contribute to the plant protection by controlling herbivore pressures (Turlings & Wäckers, 2004; Mumm & Dicke, 2010; Kessler & Heil, 2011). Plants can regulate the behavior and development of natural enemies using different strategies and metabolites (Turlings & Benrey, 1998). Natural enemies use VOC emissions as cues to find host or prey and can be further sustained by plant-provided food, shelter and oviposition substrates (Turlings & Wäckers, 2004).

Plant defensive traits have been constantly evolving in natural ecosystems, adapting to local herbivore pressures, but in agricultural systems, domestication processes have frequently weakened these defenses (Chen *et al.*, 2015a; Stenberg *et al.*, 2015). Indeed, domestication has transformed wild ancestral plants into modern crops by selecting for agronomic traits that make plants more useful and increase yields (Meyer *et al.*, 2012). Selection for higher production has shifted the finely tuned natural balance of resource investments at the cost of defense (Gepts, 2004). The neglect of defensive traits in breeding strategies has also been more recently indirectly facilitated by the simultaneous use of pesticides on cultivated field, providing a quasi-pest-free environment, but their effects might not be beneficial in the long term (Pimentel, 1995; van der Werf, 1996; Aktar *et al.*, 2009).

Cotton pest management and defenses

Cotton is cultivated worldwide mainly to produce textile fibers and is considered to be one of the dirtiest crops in the world. Indeed, up to 22.5% of the world's known pesticides are used on this cultivated plant to face the attack of a wide range of arthropod pests (Naranjo *et al.*, 2008; Hagenbucher *et al.*, 2013). These extensive applications of chemicals have helped to increase crop yields, but at the same time have had negative impacts on the environment (Pimentel, 1995; van der Werf, 1996; Aktar *et al.*, 2009). Among these impacts, soil and water pollution and pest resistance are the most explicit. To resolve these problems and to reduce the use and the impact of dangerous chemicals, biocontrol solutions are being considered and need to be evaluated under natural conditions.

The total number of arthropod species recorded on cotton worldwide is now exceeding 1300, but less than 40 are defined as key pests (Naranjo, 2011; Trapero *et al.*, 2016). Among these, lepidopteran and sucking insect pests are strongly represented. Lepidopteran species comprise the pink bollworm (*Pectinophora gossypiella*), old world bollworm (*Helicoverpa armigera*), corn earworm (*Helicoverpa zea*), spiny bollworm (*Earias insulana*), spotted bollworm (*Earias vitella*) and several armyworms (i.e. *Spodoptera frugiperda*, *S. exigua* and *S. littoralis*). On the other hand, sucking insect pests include, aphids (e.g. *Aphis gossypii*), thrips (e.g. *Thrips tabaci*), mirids (e.g. *Lygus hesperus*) and other hemipterans (Jabran & Chauhan, 2019). To face these threats in nature, cotton has evolved a variety of highly characteristic constitutive or inducible defensive strategies, regulated by the expression of different specific genes (Liu *et al.*, 2017; Zebelo *et al.*, 2017). Direct defenses involve toxic gossypol and related terpenoids (e.g. heliocides), which are produced in cotton subepidermal pigment glands and are known to have insecticidal and fungicidal properties (McAuslane *et al.*, 1997; Puckhaber *et al.*, 2002; Stipanovic *et al.*, 2006) (Fig. 1). These constitutively present secondary metabolites are also inducible and accumulate following herbivore attack (Bezemer *et al.*, 2004; Opitz *et al.*, 2008). Cotton also displays several indirect defense traits such as emissions of herbivore-induced plant volatiles (HIPVs) that may repel herbivores or attract their natural enemies (Loughrin *et al.*, 1994; McCall *et al.*, 1994). In addition, cotton is known to produce extra-floral nectar that could serve as a food reward for natural enemies (Eleftheriou & Hall, 1982; Röse *et al.*, 2006).

Figure 1. Cotton leaf with subepidermal pigment glans containing amongst others gossypol.

Increasing our knowledge on the various defense mechanisms in wild cotton may eventually help to develop ways to increase their effectiveness in cultivated cotton, which may reduce the need for pesticides in cultivars. Application of such natural defenses could be part of an integrated pest management (IPM) approach and be combined with biocontrol strategies that already exist in IPM-based cotton cultivation. IPM aims to use existing knowledge on pest behavior, ecology and biology to define and apply different combined tactics throughout the year, in order to control and reduce pest populations, with the objective to reduce economic losses and increasing yield in the current and future crops. IPM employs, for instance, biopesticides, pheromones traps, trap crops, bird perches and specific ploughing practices (Williams & Wilson, 2016). "Rewilding" is also being considered as strategy to reduce the impact of pests. This may involve crossing crops with their wild relatives to reintroduce resistance traits that were lost during crop domestication (Stenberg *et al.*, 2015). This approach may be a particularly interesting in cotton in order to reduce the heavy reliance on pesticides.

In this thesis I address the possibility of exploiting enhanced resistance induction in cotton mediated by plant-produced volatiles. The thesis is inspired by the reported effects of cotton training (topping and pruning), which appears to induce plant defenses and increase crop yield (Renou *et al.*, 2011; Llandres *et al.*, 2018), as explained below.

VOC-mediated plant-plant signaling

VOCs emitted by herbivore and pathogen challenged plants, but also by mechanically damaged plants can be perceived by neighboring plants. This interaction has been shown to prime the direct and indirect plant defense responses in receiving plants (Heil & Karban, 2010). This one-way signaling between emitter and receiver plants is considered as eavesdropping (Karban *et al.*, 2003), whereby the receiving plant actually exploits the VOCs that the damaged tissues of the emitting plant sends as signals to its healthy parts to alert these tissues to possible incoming attack (Heil & Silva Bueno, 2007).

One of the first studies suggesting airborne plant-plant signaling was published by Baldwin and Schultz (1983). In their experiments, they observed that poplar ramets (*Populus x euromericana*) had increased concentrations and rates of synthesis of phenolic compounds 52 hours after mechanical damage was applied. Interestingly, undamaged plants sharing the same enclosure with damaged plants exhibited similar increases, suggesting that airborne cues originating from damaged plants might induce physiological changes in neighboring plants (Baldwin & Schultz, 1983). The same paper also showed similar results in sugar maple (*Acer saccharum*) where tannins and phenolics levels were upregulated in both damaged and intact neighboring plant. Increased levels of tannins have been also shown to act as anti-defoliate agent against giraffes (*Giraffa camelopardalis*) in South Africa (Furstenburg & van Hoven, 1994). Interestingly giraffes were observed to only feed on one in ten acacia trees, avoiding feeding on trees downwind to already utilized trees (Furstenburg, 1991). The author suggests that the VOC ethylene which is emitted by utilized plants is dispersed by wind and activates tannin biosynthesis in intact leaves. In another study, Farmer & Ryan (1990) showed how sagebrush, *Artemisia tridentata*, when placed in chambers together with tomato plants, induced accumulation of proteinase inhibitors through activation of defensive genes in the neighbor tomato. This airborne leaf to leaf signaling between two different plant species is likely due to the VOC methyl-jasmonate, which in the same paper was shown to activate proteinase inhibitors in tobacco, alfalfa and tomato plants. A study by Zeringue (1987) showed changes in cotton leaf chemistry, namely increased heliocide levels, when the plant was exposed to VOCs from a fungi-infested plant or to pure myrcene.

The mechanisms that are implicated in plant-plant signaling are not yet fully understood, but the perception of HIPVs by maize plants is known to trigger the expression of jasmonic acid (JA)-inducible genes, and results in decreased herbivore damage on the receiving plant (Ton *et al.*, 2006). For some plant species it is also known which specific HIPVs are mainly

responsible for the observed priming in neighboring plants (Arimura *et al.*, 2000; Erb *et al.*, 2015). For instance, Erb *et al.* (2015) identified the aromatic compound indole as the key priming volatile for maize plants and that it leads to the production of stress hormones in receiving maize plants. VOC-mediated plant-plant signaling has also been shown to be effective against microbial pathogens (i.e. bacteria and viruses), targeting, in this case, salicylic acid (SA)-genes. Indeed, the emission of methyl salicylate and nonanal upon SAR (Systemic Acquired Resistance) reduced symptoms in exposed neighboring plants (Shulaev *et al.*, 1997; Yi *et al.*, 2009). In fact, SAR is mediated by SA signaling, which is known to increase the expression of pathogenesis-related proteins (PR) and phytoalexins resulting in resistance against biotrophic pathogens (Loon, 1997; Hammerschmidt, 1999; Durrant & Dong, 2004).

Other evidence for plant-plant signaling comes from studies on wild tobacco (*Nicotiana attenuata*) and sagebrush (*Artemisia tridentata*) (Karban & Baxter, 2001; Karban *et al.*, 2003, 2004). In the case of sagebrush, mechanical damage results in volatile emissions that elicit a defense state in neighboring plants and make them less palatable to generalist herbivores. These molecular signals can also be population specific. Indeed Moreira *et al.* (2016) found that *Phaseolus lunatus* exposed to VOCs emitted by same-population damaged plants, suffered less damage compared to plants exposed to VOCs emitted from another population, suggesting that sensitivity depends on the degree of genetic relatedness. Plant-plant signaling also occurs between trees, as Dolch & Tscharntke (2000) described in their study on the effect of artificial alder defoliation.

Hence, the occurrence and evolution of airborne plant signaling has been described for a variety of systems, but the mechanisms behind this phenomenon and, in most cases, the responsible volatile compounds still need to be unraveled. Better knowledge of crop specific mechanisms that are involved in these interactions may contribute to the formulation of sustainable pest management strategies.

State of the art

Cotton plants change the quantities and the composition of the volatiles that they emit when attacked by chewing herbivores (Loughrin *et al.*, 1994; McCall *et al.*, 1994). This modification of volatile emissions can have an impact on the ecological community that is associated with the plant, for instance, by deterring other herbivores or attracting natural enemies. The induced volatiles can also prime and/or induce defenses in the plant itself and its neighbors, as discussed in the plant-plant signaling section above. Although this type of associational resistance and how it has been affected by domestication has so far been poorly studied for cotton, it has great potential for the development of pest control methods. In an early study, Bruin *et al.* (1992) showed that, when infested by herbivorous mites, cotton seedlings produce VOCs that attract predatory mites. The same study also reported on decreased oviposition by herbivorous mites on un-infested cotton plants that had been exposed to the VOCs from infested neighboring plants. Two further studies also found that cotton VOCs can interfere with oviposition behavior. Hagenbucher *et al.* (2013) observed that wild ancestral cotton *G. raimondii* provided associational resistance to domesticated *G. hirsutum*, resulting in lower oviposition on plants in groups that contained a mix of the two species as compared to groups with only domesticated *G. hirsutum* plants. Importantly, Zakir *et al.* (2013) showed a significant reduction in oviposition by S. *littoralis* moths on undamaged plants that had been placed nearby herbivore-damaged cotton plants under both field and laboratory conditions. In terms of inducible defense responses McAuslane *et al.* (1997) had already shown that feeding by *S. exigua* in cotton plants induces a systemic increase in the production of pigment glands in newly grown leaves, resulting in higher levels of toxic and deterrent terpenoid aldehydes (e.g. gossypol and heliocides), reducing herbivory in these distal parts of the plant.

The sequence of studies that actually motivated this PhD thesis were performed by Alain Renou and collaborators in Senegal and Mali (CIRAD: Centre de coopération international en recherche agronomique pour le développement and IER: Institut d'economie rurale), who mainly used a cultivated cotton variety (STAM59A) (Renou *et al.*, 2011; Téréta, 2015; Llandres *et al.*, 2018). Their studies showed how training (i.e. topping and pruning) cotton plants resulted in enhanced resistance against cotton bollworm (*Helicoverpa armigera*, Hübner) and increased yields to levels comparable to those of plots that were treated with classical pesticides. Interestingly, Téréta's thesis (2015), showed that not only trained plants had benefitted from the treatment, but also neighboring plants, suggesting that volatile signaling between trained plants and intact neighboring had taken place (Fig.2).

The above-mentioned cotton studies highlight the potential of VOCs in protecting neighboring plants; however, none of them address the responsible mechanism and/or compounds behind the observed effects. In fact, from the results it cannot be determined whether the observed reduction in oviposition by *H. armigera* moths on neighboring plants was due to the induction of defenses, to the direct effect of the VOC emitted by the source plant or by both these factors. That the latter is possible and is illustrated by Rodriguez-Saona *et al.* (2001), who observed that exogenous methyl-JA treatment, which attracts natural enemies, also induces the emission of the same volatile compounds that are emitted by herbivore-damaged cotton, again showing the potential of applying VOCs to induce plants defenses and resistance. The effects of methyl-JA treatments were also tested in a field experiment and resulted in increased emissions of limonene and (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) (Williams *et al.*, 2017).

To my knowledge no study has yet addressed the mechanisms that are involved in signaling among cotton plants and the physiological changes that take place in neighboring plants. The overall objective of this thesis was to unravel these VOC-mediated plant signaling mechanisms. In a series of laboratory experiments we exposed cotton plants to different natural and synthetic VOC treatments. We then used the receiver plants to measure VOC emissions, production of secondary metabolites and hormone levels, relative defense-related genes expression, as well as caterpillar feeding preferences. The ultimate goal of this project was to provide new knowledge on signaling among cotton plants that might contribute to the development of novel sustainable environmentally friendly pest control strategies.

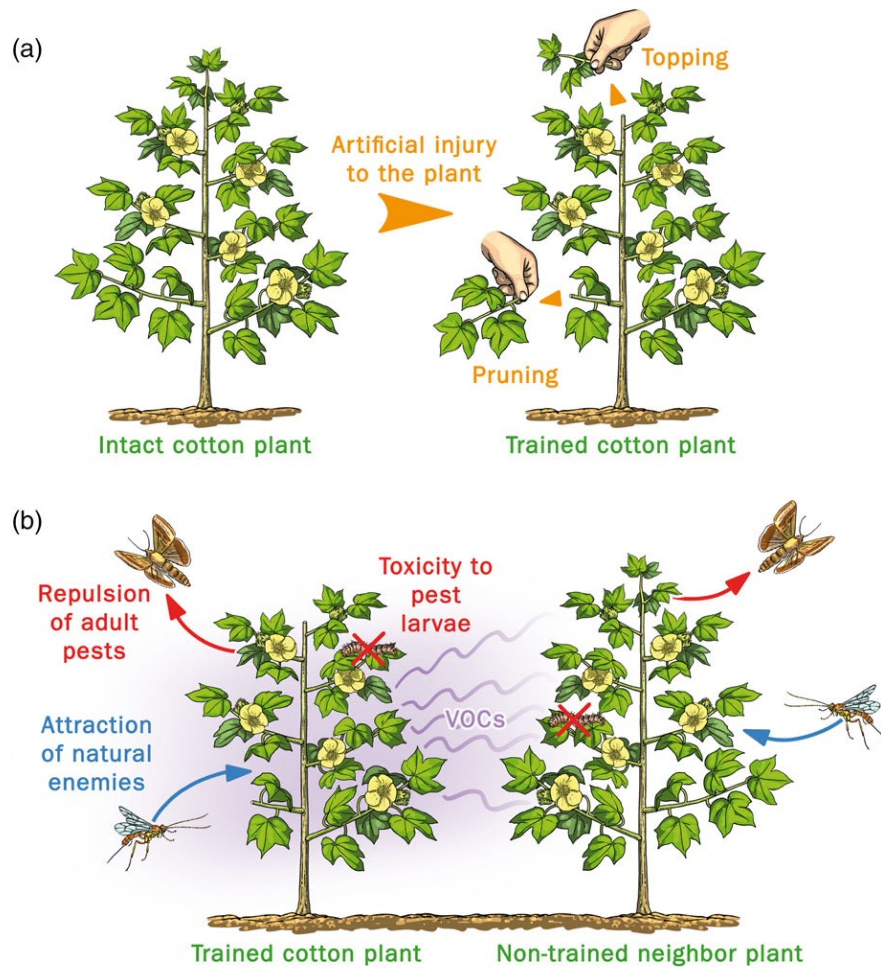

Figure 2. "Schematic representation of cotton plant training including topping (removal of the terminal bud of the main stem) and pruning (removal of apical points of vegetative and fruiting branches). (B) Effects of cotton training on herbivores and natural enemies through the activation of plant resistance strategies in trained and non-trained neighboring plants. VOCs, volatile organic compounds.» (Llandres *et al.*, 2018).

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Chapter 1: Caterpillar-induced cotton volatiles activate multiple defenses in neighboring cotton plants

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// Abstract

The research conducted, including the rationale: Herbivore-induced plant volatiles (HIPVs) are known to attract natural enemies and repel herbivores, but also play a key role in plant-plant signaling. Recent field evidence suggests that HIPVs from cotton plants are particularly potent in inducing resistance in neighboring cotton plants, which may have application potential. We conducted a series of molecular, chemical, and behavioral assays to test if exposure to cotton HIPVs indeed trigger defense responses in conspecific plants.

Methods: Cotton plants were exposed for 48h to volatiles from either a caterpillar-infested plant or from an intact control plant. Defense gene expression, concentrations of cotton defense compounds (gossypol and heliocides), emissions of volatiles, and caterpillar feeding preferences were measured for the two exposure treatments.

Key results: Compared to control plants, HIPV-exposed plants contained higher levels of gossypol, released larger quantities of volatiles and showed increased expression of defense genes. Moreover, in case of feral cotton, caterpillars fed less on new leaf tissues of HIPV-exposed plants.

Main conclusion: The results reveal that HIPVs from cotton plants can directly induce resistance, rather than just prime defenses as is known for other plants. The striking physiological changes caused by HIPVs are in line with preliminary field evidence suggesting that volatile-mediated signaling has potential to be applied against insect pests on cotton.

Key Words: (5-8, alphabetical)

Gossypium hirsutum (Cotton), HIPVs, Plant Defenses, Plant-Plant Signaling, *Spodoptera* spp.

Introduction

Cotton plants (*Gossypium hirsutum*) under attack by chewing herbivores markedly change the quantities and composition of the volatile blend that they emit (Loughrin *et al.*, 1994; McCall *et al.*, 1994; Röse *et al.*, 1996). This modification of volatiles may modulate the plant's ecological community by repelling herbivores and attracting their natural enemies (Röse *et al.*, 1998; Zakir *et al.*, 2013a). Considering the large quantities of volatiles that are involved, we can also expect that herbivore-induced volatiles (HIPVs) from cotton can affect resistance of neighboring plants against herbivores, as shown for other plants (Heil & Karban, 2010). The first studies demonstrating volatile mediated plant-plant interactions were met with scepticism (Baldwin & Schultz, 1983; Farmer & Ryan, 1990; Bruin *et al.*, 1992), but the phenomenon is now accepted as common and ecologically relevant (Heil & Karban, 2010). Studies by Karban and others provided evidence for volatile-mediated interactions among cotton plants (Karban, 1986, 1993; Bruin *et al.*, 1992; Omer *et al.*, 2001), which has been further elaborated with elegant cage experiments by Peter Anderson research group (Zakir *et al.*, 2013a,b). The one-way interaction between emitter and receiver plants is considered a form of eavesdropping on volatiles that serve as within plant signals (Karban *et al.*, 2003). The volatiles from the challenged plant parts are perceived by healthy parts of the same plant, which then mobilize their direct and indirect defenses (Heil & Silva Bueno, 2007). The effect on neighboring plants is normally that they enter a primed state, as opposed to directly inducing defense (Ton *et al.*, 2006). Primed plants do not yet activate their defense genes, nor do they increase the production of the defense compounds, but they show greatly enhanced defense gene expression and more rapidly produce defense compounds once they are actually attacked themselves (Conrath *et al.*, 2006; Martinez-Medina *et al.*, 2016).

Priming was first reported and studied for plant-pathogen interactions, which mainly involve the salicylic acid pathway (Mauch-Mani *et al.*, 2017) and is considered a cost-effective way to get ready for an incoming attack (van Hulten *et al.*, 2006). This physiological strategy to boost plant immunity against pathogens may also be achieved through plant to plant priming via inducible volatiles, such as methyl salicylate and nonanal, which are typically released upon pathogen infection and have been found to cause reduced symptoms in exposed neighboring plants (Shulaev *et al.*, 1997; Yi *et al.*, 2009).

In the case of defenses against arthropods, numerous studies have reported on the role of plant-plant signaling (e.g. Baldwin and Schultz 1983; Dolch & Tscharntke, 2000; Karban & Baxter, 2001; Karban *et al.*, 2003; Heil and Silva Bueno, 2007). Several field studies have

revealed that the signals involved may be specific (Karban et al., 2004; Moreira *et al.*, 2016). Farmer et al. (1990) were the first to identify airborne methyl jasmonate as a potent elicitor of defenses against insects. Since this seminal paper a number of different volatile compounds have been implicated in inducing or priming of neighboring plants. So-called green leaf volatiles, which are released by virtually all plants immediately upon tissue damage (Turlings & Erb, 2018), were found to trigger defense priming or induction in several studies (Bate & Rothstein, 1998; Farag & Paré, 2002; Kost & Heil, 2006; Sugimoto *et al.*, 2014), whereas the aromatic HIPV indole has been found to be essential for the priming of maize plants (Erb *et al.*, 2015).

Plant-plant signaling among cotton plants was first studied by Bruin *et al.* (1992) who found that, when infested by herbivorous mites, cotton seedlings not only produce volatiles that attract predatory mites, but also that these volatiles cause a decrease in oviposition by herbivorous mites on neighboring plants. Zakir *et al.* (2013b) found a significant reduction in oviposition by *Spodoptera littoralis* (Lepidoptera, Noctuidae) on undamaged plants nearby caterpillar-damaged cotton plants under both field and laboratory conditions. These studies highlight the potential of applying HIPVs to boost cotton resistance. Furthermore, Rodriguez-Saona *et al.* (2001) observed that the application of exogenous methyl jasmonate induces the emission of the same volatile compounds as released by herbivore-damaged cotton, highlighting the VOCs potential to directly induce defenses in neighboring plants. *Spodoptera exigua* caterpillars (Lepidoptera, Noctuidae) feeding on cotton were also shown to induce other plant parts to systemically increase the number of pigment glands and the content of toxic and deterrent terpenoid aldehydes therein (e.g. gossypol and heliocides), thus reducing herbivory in those parts (McAuslane *et al.*, 1997).

Further evidence of VOCs mediated signaling among cotton plants is provided by field studies performed in Senegal and Mali by Téréta, Llanders and colleagues (Cirad & IER). Their study showed that topping, i.e. the manual removal of the apical part of flowering cotton plants, results not only in significantly reduced bollworm infestation on the plant itself (Renou *et al.*, 2011), but also on neighboring plants, when compared to untreated control plants (Téréta, 2015; Llandres *et al.*, 2018). These field data suggest that volatile released from topped cotton plants trigger strong defense responses in neighboring plants. The apparent dramatic increase in resistance suggests that it is not merely due to priming, but rather a volatile-mediated induction of cotton defenses. The current study was conducted to test this hypothesis. By exposing healthy cotton plants to the volatiles of caterpillar-infested plants we assessed whether cotton HIPVs directly induce defense in neighboring plants. As comparison, control

plants were exposed to the volatiles from healthy intact plants. The exposed plants were used to measure the relative expression of defense related genes, the content of the defense compounds gossypol and heliocides, volatile emissions, and caterpillar feeding preference.

Materials and methods

Plants and insects

Cotton seeds (*Gossypium hirsutum*, the cultivated variety "STAM59A" and a feral population from Puerto Escondido, Mexico) were scratched on sandpaper, then gently poked with a needle and placed on moist cotton (24h-48h, 28°C, dark) to accelerate and enhance the germination process. Subsequently, seed were sown in plastic pots (height: 8.5cm; diameter: 6cm) containing commercial soil (Profi Substrat Soil, Einheitserde, Germany). Seedlings were grown in phytotrons (Growbanks, 25°C, 16h:8h light:dark) and watered every other day. Four-true-leaves-plants were used for the experiments. Larvae of *Spodoptera exigua* and *Spodoptera frugiperda* were reared on artificial diet (Frontier Scientific Services, Newark, USA). Late first and early second instar larvae were used for the experiments.

VOCs exposure

To study the effects HIPVs on neighboring plants, receiver plants were exposed for 48h either to VOCs from *Spodoptera spp.* infested plant (HIPVs Exposed) or to VOCs from a control, un-infested plant (Control). Source and receiver plants were introduced in glass vessels (Verre & Quartz Technique SA, Switzerland) and connected through a Teflon tube. In case of source infestation treatment, 10 caterpillar larvae were dropped into the vessel onto the plant. Charcoal-purified and humidified air at 1L/min was pushed into the source plant vessel (Fig. 1a, b).

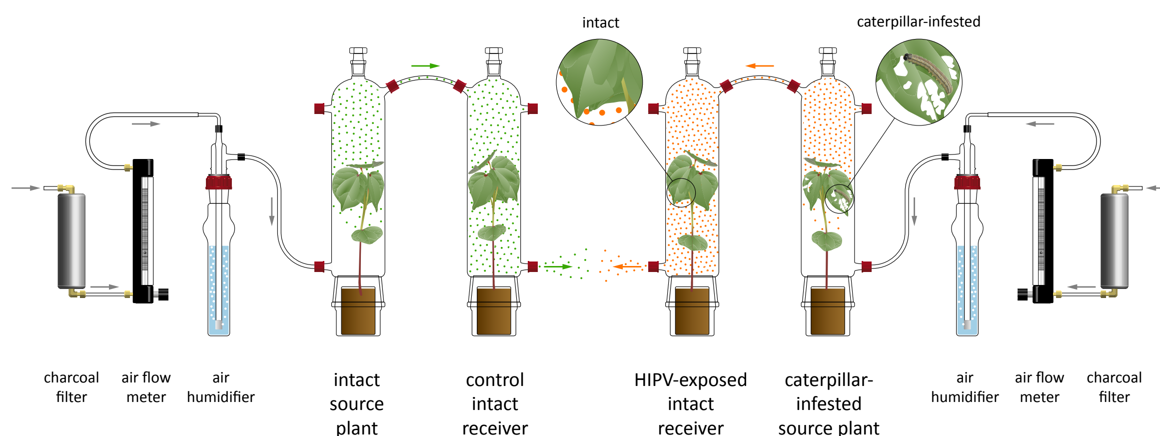

Figure 1. VOCs exposure system. All ports besides inflow and outflow were sealed either with caps or parafilm, one-way airflow occurred through Teflon tubes. (a) Control treatment, (b) HIPVs treatment (Illustration by Thomas Degen).

Gossypol and heliocides extraction and analysis

First and fourth leaves were excised and wrapped in aluminum foil and frozen in liquid nitrogen right at the end of the 48h VOCs exposure. The aluminum envelopes containing leaves were stored at -80°C until extraction procedure. For the extractions, frozen leaves were ground into a fine powder in a mortar filled with liquid nitrogen. Circa 50±5mg of powder were then transferred into a frozen 1.5 mL Eppendorf tube. Next, 80 µL acetonitrile and 4-6 glass beads (1.25-1.65 mm diameter) was added to each tube. The mixture was vortexed and then homogenized for 3 minutes at 30 Hz using a beadmill (Retsch MM 300, Haan, Germany). The samples were subsequently centrifuged for 5 minutes at 14000 rpm. The recovered supernatant was subjected to a second centrifugation step to obtain a totally limpid solution. Finally, 80 µL of the supernatant was recovered and transferred into 2 mL glass vial (BGB, Germany) for analysis with a High Performance Liquid Chromatograph (HPLC) coupled to a DAD detector set at 288 ± 2 nm (HP1100, Agilent Technologies, Santa Clara, CA, USA) as described by Glauser *et al.*, (2013). A 1.5 µl aliquot of each sample was injected onto an Extent-C18 column (2.1 x 150mm, 5µm) (Agilent Technologies). The flow rate was held constant at 0.5 mL/min and the temperature was kept at 30°C. The mobile phase A consisted of 0.05% formic acid in water and the mobile phase B of 0.05% formic acid in acetonitrile. The following gradient was used: 35-90% B in 20 min, 90-100% B in 1 min, holding at 100% for 3 min followed by re-equilibration at 35% B for 6 min. Gossypol and heliocides H1 to H3 were identified by their retention time. Quantification was done by calculating a calibration equation obtained by linear regression from five calibration points in gossypol equivalents. The experiment was repeated four times, using four different blocks of plants, during 4 different weeks, using *S. exigua* or *S. frugiperda* (each twice) to infest source plants.

Volatiles collection and analyses

Plants were placed in custom-made glass vessels (VTQ SA, Switzerland) and VOCs were collected at a rate of 1L/min inflow and 0.9L/min outflow during 2 hours through filters containing 25mg of 80/100 mesh Hayesep-Q adsorbent (Sigma, Switzerland), as adapted from Chappuis et al. (unpublished) and Turlings *et al.* (1998). Subsequently, filters were eluted with 100µL dichloromethane, the elute was then spiked with 10 µL internal standards solution (n-octane and nonyl-acetate, [20 ng/µL] each). Samples were analyzed on a gas chromatograph (GC Agilent 6890N) coupled to a mass spectrometer detector (MSD Agilent 5973). A 2 µL aliquot of each sample was injected in pulsed splitless mode onto an Agilent HP-5MS column

(30 m length x 250 µm diameter and 0.25 µm film thickness). After injection, temperature was maintained at 40°C for 3 min, increased to 100°C at a rate of 8°C per min and subsequently to 200°C at a rate of 5°C per min followed by a post run of 3 min at 250°C. Helium was used as carrier gas and kept at constant flow of 1.1mL/min. Compounds were subsequently identified by comparing their mass spectra with those from the NIST mass spectral library or from PBM Quicksearch library (U.S. Department of Commerce). Authentic standards were used for confirmation of identities based on retention times. Compounds were quantified by comparing peak areas (total ion counts) with those of internal standards.

We first collected VOCs from the two types of intact receiver plants (i.e. feral and cultivated) straight after the 48h exposure. Subsequently we infested the same plants with 10 *S. exigua* larvae for 24h and then performed a second collection. The experiment was performed twice on two different blocks of plants during two different weeks (N=6).

Caterpillar preference

Second and fourth leaves from both Control and HIPVs treatments were paired (i.e leaf, 2nd leaf vs 2nd leaf, 4th leaf vs 4th leaf) and placed in single petri dishes on top of moistened filter-papers. Three 1st to 2nd instar *S. exigua* caterpillars were released at the same distance from the two leaves and allowed to move freely and feed on the leaves. The position of the caterpillars was recorded at several time points during the first 24 hours. The experiment was performed three times with three different blocks of plant during three different weeks (N=9).

Caterpillar preference was further assessed by measuring the final leaf area consumption. Pictures of the leaves were taken, imported on the computer, and the consumed area was quantified using Adobe Photoshop.

Gene expression measurements

For the gene expression measurements, we used the same plant material as used for gossypol and heliocides analyses. The frozen powder of 1st and 4th leaves obtained at end of the 48h VOCs exposure was used to isolate total RNA using the GeneJET Plant Purification Mini Kit (Thermo Scientific) according to the manufacturer's instructions. The complete DNA removal was performed by using the RNase-Free DNase Set (QIAGEN). Each total RNA sample (500 ng) was reverse transcribed using the GoScript™ Reverse Transcription System (Promega). Real time qPCR was performed on the Rotor-Gene™ 6000 (Corbett Research) using GoTaq® qPCR Master Mix (Promega). *GhACT4* (GenBank accession no. AY305726) was used as an internal standard to normalize cDNA concentrations. The relative expression of eight defense-

related genes were analyzed. The genes were selected based on their role in cotton defense (VOCs and/or terpenoids synthesis) and primer's availability. The primers used for qPCR and the respective genes are listed in Supporting Information Table T1. Three independent biological replicates were analyzed in each experiment for each treatment. Expression levels were calculated by using the ΔΔCt method (Schmittgen & Livak, 2008). The experiment was repeated four times, for four different blocks of plants, during four different weeks, using *S. exigua* or *S. frugiperda* (each twice) to infest source plants.

Statistical analyses

Statistics ($P<0.05$) and graphs were performed and computed using the program R and its complementary console R-studio (http://www.rstudio.com). Gossypol and heliocides content, VOC emissions and relative gene expression were analyzed through ANOVA fitting different linear models. Some models were subject to logarithmic correction to meet normality assumptions. The choice test results were analyzed for each single timepoint and factor combination through generalized linear model with binomial distribution, P-values were adjusted with *fdr* correction to cope with repeated measures.

Results

HIPVs induce increase in gossypol content in intact neighboring plants

Gossypol content analysis showed significant effects of treatment (F=6.408, P<0.05, Fig. 2a) and leaf age (F=253.567, P<2e-16, Fig. 2b), with higher levels in leaves that had been exposed to HIPVs compared to control leaves and in higher levels in younger tissue (4th Leaf) compared to older tissue (1st leaf). The cotton genotype (Feral or Cultivated) did not differ significantly and were pooled for further comparisons. Heliocides analysis revealed the same significant leaf effect and a similar trend of increased content in HIPVs-exposed plants compared to controls (S.I. Fig. S1). The possible differential induction effect of caterpillar species used to infest source plants (*S. exigua* or *S. frugiperda*) was pre-tested in a separate model, and no significant effect was found. Therefore, caterpillar species was excluded as factor from the final model.

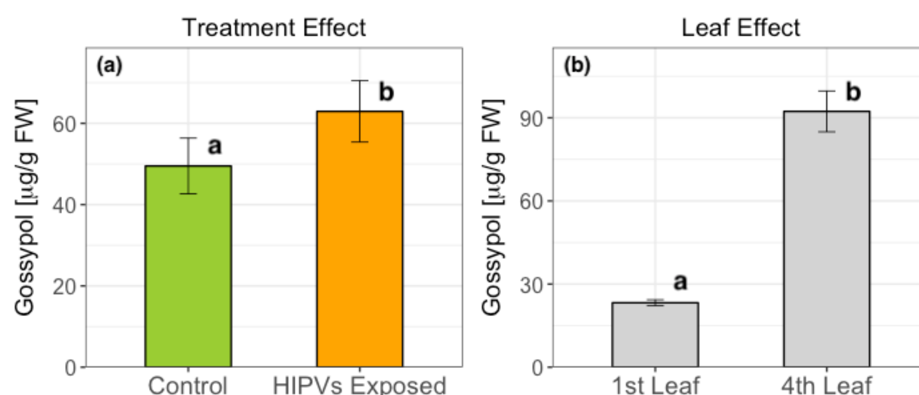

Figure 2. Gossypol content in cotton leaves (mean ± standard error). Different letters indicate significant differences (ANOVA). (a) Treatment Effect: control (N=44) vs HIPVs Exposed (N=44), pooled factors: caterpillar species, cotton variety and leaf age (b) Leaf Effect: 1st Leaf (N=46) vs 4th Leaf (N=42), pooled factors: caterpillar species, cotton variety and treatment.

HIPVs induce higher VOCs emissions in intact neighboring plants

Plants that had been exposed to HIPVs emitted higher total amounts of VOCs than control plants (F=6.6086, P<0.05, Fig. 3), independent of cotton variety. VOCs were collected a second time from the same plants after they had been damaged by *S. exigua* larvae for 24h. The caterpillars caused a considerable increase in emitted volatiles, with the cultivated plants releasing significantly more than the feral plants (F=16.6742, P<0.001, Fig. 3), but no effect of HIPV exposure was observed.

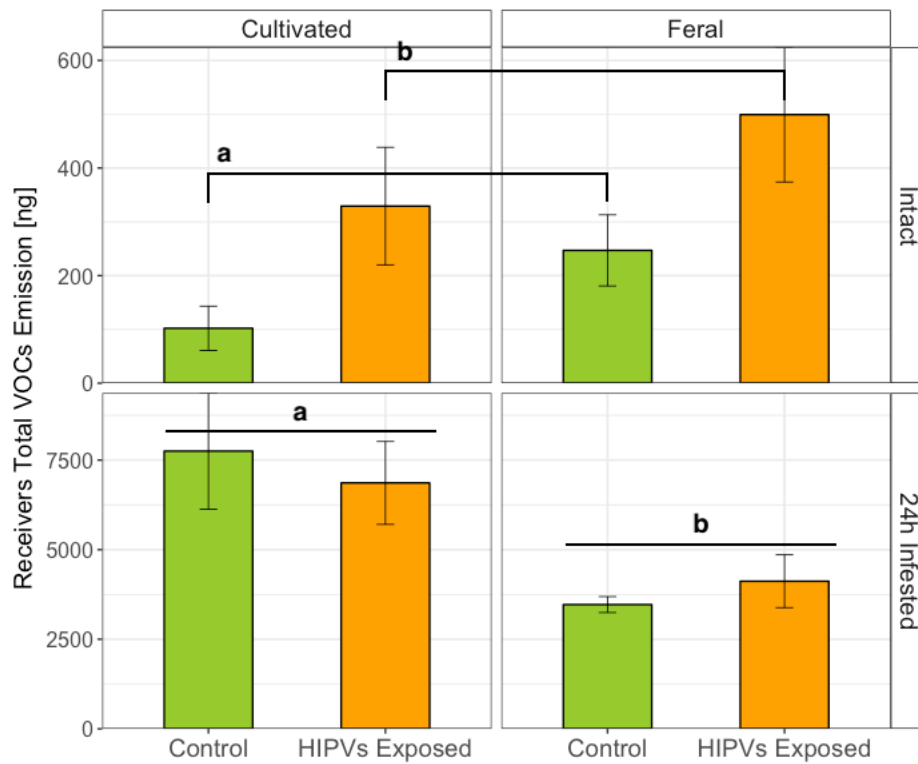

Figure 3. Total VOC emissions (mean ± s.e.) by receiver plants over two hours depending on exposure treatment (Control vs HIPVs Exposed), collection (from intact or 24h infested receivers) and variety (Cultivated vs Feral). ANOVA were run for intact and 24h infested plants separately. Intact plants: significant effect of exposure (ANOVA, N=6). 24h infested plants: significant variety effect (ANOVA, N=6).

Intact plants that were exposed to HIPVs tend to emit more benzaldehyde, DMNT ((3E)-4,8-dimethyl-1,3,7-nonatriene) and TMTT ((E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene) than intact control plants (Fig. 4) (ANOVA DMNT emitted by Feral plants, F=7.75, P=0.02). For the plants that had been damaged for 24h by *S. exigua* larvae we found that cultivated variety released significantly more (*Z*)-3-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenylacetate, all green leafy volatiles, than the feral plants, independent of the exposure treatment (Fig. 5, S.I. Tab. 3). This difference is likely due to the fact that the larvae tended to consume more leaf tissue of the cultivated plants during the 24h of infestation (S.I. Fig. S2).

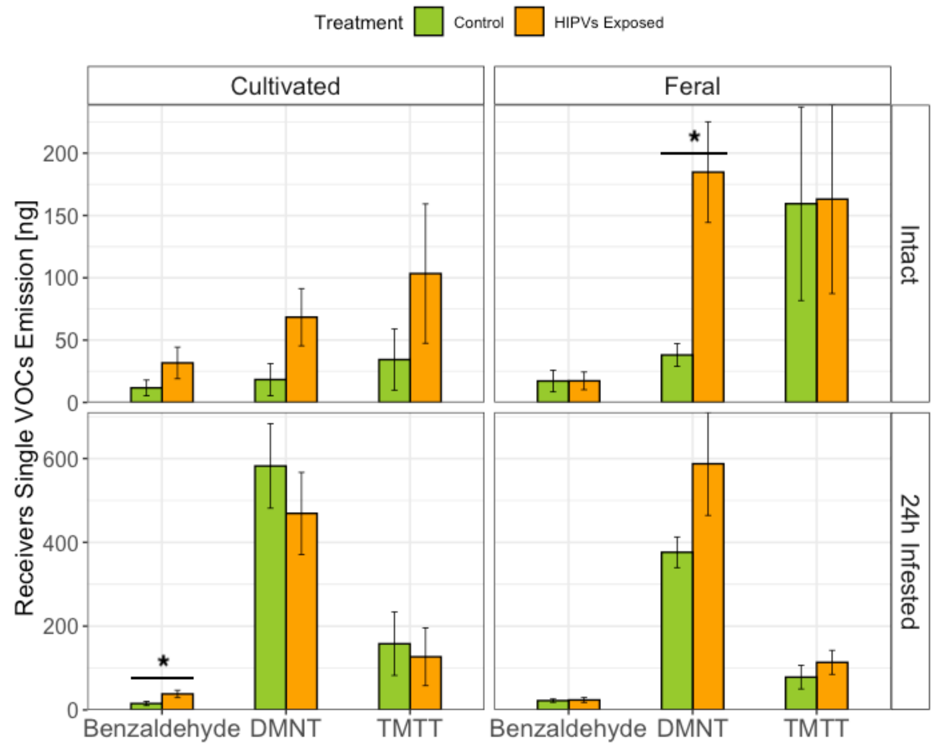

Figure 4. Emissions of single inducible VOCs (benzaldehyde, DMNT, TMTT) (mean ± s.e.) over two hours by the receiver plants exposed to either constitutive VOCs (control) or HIPVs. The bars reflect the quantities released by receiver plants before caterpillar infestation and after 24h of infestation (green = control, orange = HIPV-exposed) for both cultivated (left) and feral plants (right). Asterisks (*) indicate significant difference between the two treatments (ANOVA DMNT emitted by Feral Intact: $F=11.5$, $P=0.008$; ANOVA Benzaldehyde emitted by Cultivated 24h infested plants: $F=7.75$, $P=0.02$). $N=6$ for each bar.

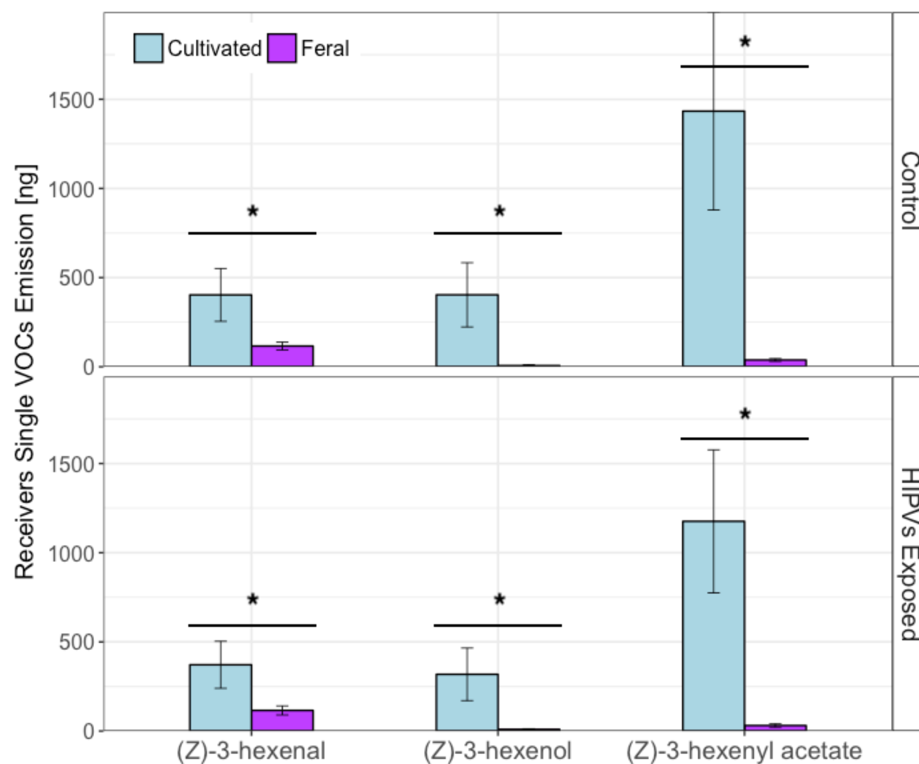

Figure 5. Emissions of single green leaf volatiles (GLVs) (mean ± s.e.) over two hours by receiver plants that were damaged by caterpillars for 24h (control and HIPV-exposed plants; blue = Cultivated, purple = Feral). Asterisks (*) indicate significant differences between the two genotypes (P<0.05, ANOVA, N=6).

Spodoptera exigua larvae avoid tissue of HIPV-exposed neighboring plants

In general, *S. exigua* larvae showed a preference for control leaves when offered alongside leaves from HIPV-exposed plants in petri dishes. This preference was most evident for leaves from feral plants and for the 4th (youngest) leaf (Fig. 6). For cultivated plants the preference for control leaves was less clear, and only significant for the 4th leaf at one of the measured time points (Fig. 6). Reduced appetite for HIPV-exposed plants was also reflected in trends in total leaf area consumed at the end of the assay, with a significant difference for the 4th leaf feral comparison (P<0.01, S.I. Fig. S3)

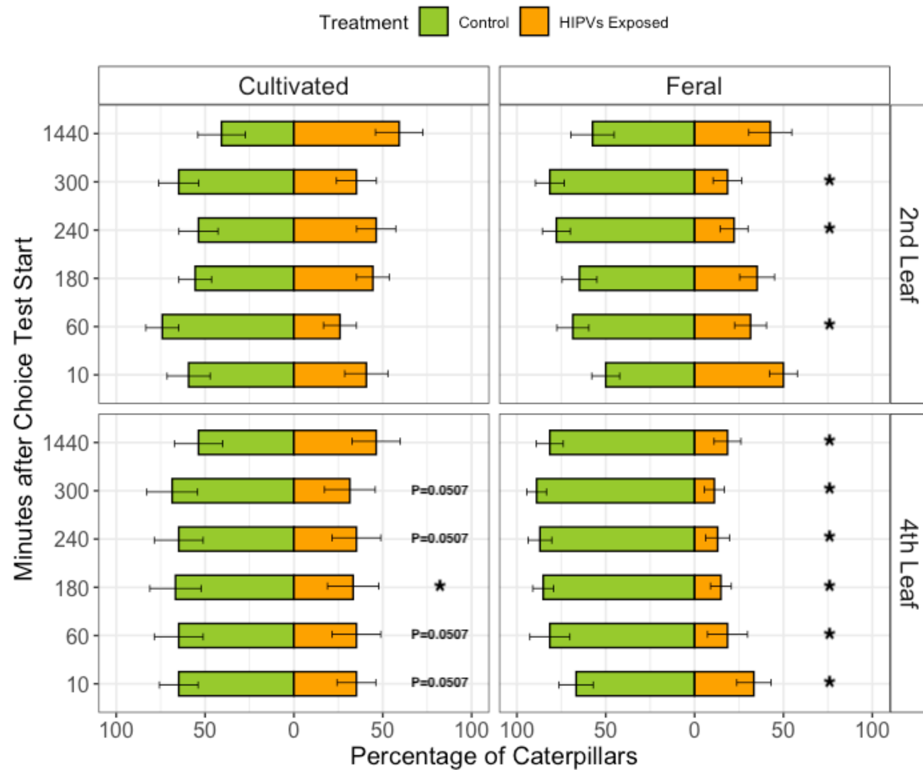

Figure 6. Preference of *S. exigua* larvae choosing between leaves from differently exposed receiver plants (green = Control, orange = HIPVs Exposed) (mean ± s.e.) at different timepoints, separated for cotton genotype (Cultivated vs Feral) and leaf age (2nd vs 4th). Asterisks (*) indicate significant preferences for control leaves at specific timepoints (Binomial GLM with fdr correction, N=9)

HIPV-exposure induces higher expression of defense-related genes in intact neighboring plants

Cotton genes involved in VOC production (*GhTPS12*, *GhTPS14*, *GhCYP82L1*, *GhCYP82L2*) and gossypol production (*Cad1A*, *Cdn1C3*, *CYP706B1*, *FPS1*) were tested for their relative expression. For both type of genes we found significant higher expression in the HIPV-exposed plants, compared to those that were exposed to volatiles from intact plants (Fig. 7, Fig. 8, S.I. Tab. T2), with considerable gene-specific variability among pooled factors, i.e. leaf age and cotton type (i.e. feral or cultivated). The possible differential effect of caterpillar species used to infest source plants was tested in a separate model, and no significant effect was found. Therefore, the factor caterpillar species was excluded from the final model.

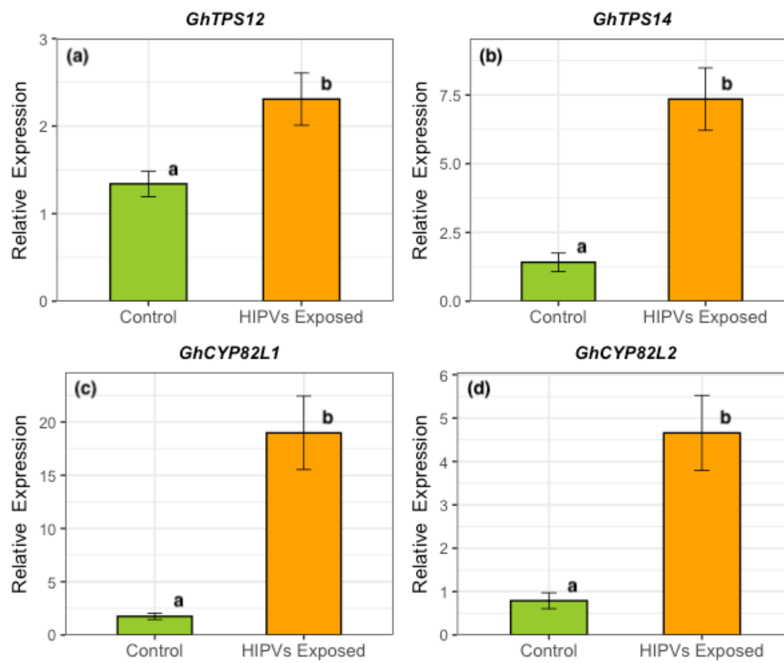

Figure 7. Relative expression (mean ± s.e.) of VOC-related genes (*GhTPS12*, *GhTPS14*, *GhCYP82L1*, *GhCYP82L2*). Different letters indicate significant treatment effects (Control vs HIPV-exposure) within each gene (ANOVA, N=46-48). Pooled factors: caterpillar species, cotton genotype and leaf age.

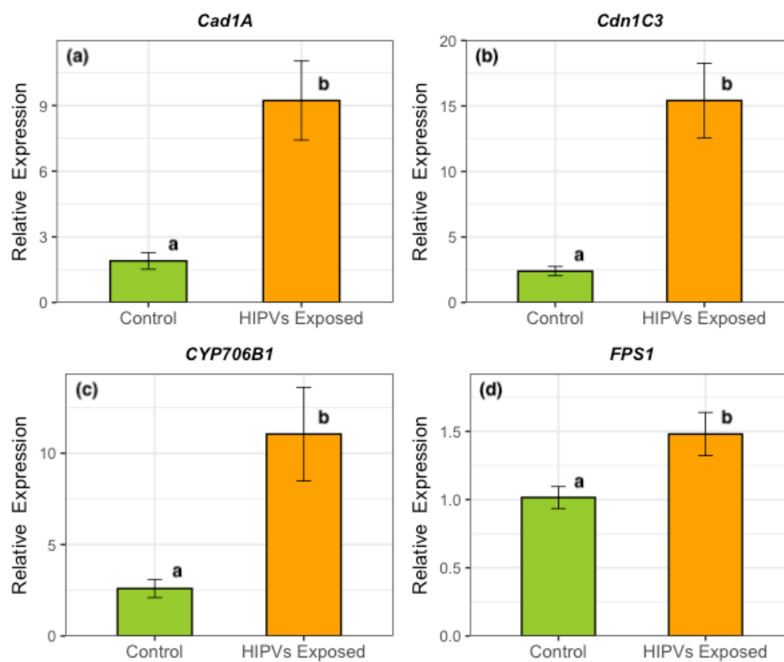

Figure 8. Relative expression (mean ± s.e.) of gossypol-related genes (*Cad1A*, *Cdn1C3*, *CYP706B1*, *FPS1*). Different letters indicate significant treatment effects (Control vs HIPV-exposure) per gene (ANOVA, N=46-48). Pooled factors: caterpillar species, cotton genotype and leaf age.

Discussion

The ecological relevance and application potential of plant-produced VOCs are major topics of interest in the field of chemical ecology (Dicke & Baldwin, 2010; Heil, 2014; Pickett & Khan, 2016; Turlings & Erb, 2018). Cotton HIPVs are known to play a key role in multitrophic interactions (Bruin *et al.*, 1992; McCall *et al.*, 1993; Röse *et al.*, 1998; De Moraes *et al.*, 1998). There is also recent field evidence that cotton volatiles are involved in plant-plant interactions. Specifically, the traditional practice of topping (manual removal of the apical part of flowering cotton plants) has been shown to increase insect-resistance in neighboring plants at a level that may have the same protective effect as pesticides (Téréta, 2015; Llandres *et al.*, 2018). The current study was conducted to gain insight into the molecular and chemical mechanisms involved in this unusually effective plant-plant interaction, and to confirm that, under controlled conditions, the volatiles from damaged plants indeed induce resistance against insect herbivores in neighboring plants.

Plants that were exposed to HIPVs showed increased levels of gossypol compared to control plants (i.e. plants exposed to volatiles from intact plants), in particular in the fourth leaf (Fig. 2a). Levels of heliocides, one of the other defense compounds in cotton pigment glands (Nazarova *et al.*, 1981) showed a similar, but non-significant trend (S.I. Fig. S1). The increases fit the observed general up-regulation of genes involved in gossypol production in the same HIPV-exposed plants (Fig. 8). These findings imply that cotton plants, when exposed to HIPVs, increase their direct defenses, as they do in response to direct caterpillar infestation (McAuslane *et al.*, 1997).

We also tested the relative expression of genes related to VOCs production, usually activated under direct herbivore attack. Also in this case, we found a general up-regulation in intact plants that had simply been exposed to HIPVs for 48h (Fig. 7). Such changes in gene expression have so far only been described in cotton plants under direct attack by caterpillars (Liu *et al.*, 2017; Zebelo *et al.*, 2017; Huang *et al.*, 2018), but also for cotton plants with symbiotic rhizobacteria (Zebelo *et al.*, 2016). Also matching the gene expression results, we found increased total VOCs emissions by intact receiver plants that had been exposed to HIPVs compared to controls, independent of cotton genotype (Fig. 3). This increase was mainly due to inducible volatiles (i.e. DMNT and TMTT) that are usually released by cotton only hours after caterpillar attack, as opposed to several cyclic terpenoids that are released immediately upon damage (Loughrin *et al.*, 1994; McCall *et al.*, 1994).

Surprisingly, when the exposed plants were subjected to caterpillar attack themselves, we no longer found a difference in VOC emissions between HIPV-exposed plants and control plants. These results imply that in cotton HIPV exposure does not prime, but directly induce receiver plants. This is contrary to what has for instance been found for maize (Engelberth *et al.*, 2004; Ton *et al.*, 2006; Erb *et al.*, 2015). As was the case for the healthy exposed plants, cultivated plants emitted higher amounts in comparison to feral plants (Fig. 3). This can be explained by somewhat higher leaf area consumption found on cultivated plant, independent of the exposure treatment (S.I. Fig. S2). In contrary to findings by Loughrin *et al.*, 1995, which showed that a commercial cotton release seven times less HIPVs compared to a naturalized line, this cultivated variety (i.e. STAM59A) emits more VOCs than the compared feral variety and confirms its signaling ability already suggested by Téréta (2015).

Plant-plant interactions mediated by volatiles have been reported for several plant species including cotton and confirmed in the last four decades (Baldwin & Schultz, 1983; Karban, 1986; Farmer & Ryan, 1990; Bruin *et al.*, 1992; Omer *et al.*, 2001; Heil & Karban, 2010; Zakir *et al.*, 2013b,a). Karban (1986) showed that cotton resistance against spider mites can be induced by early herbivore attack or mechanical damage. Bruin *et al.* (1992) reported that cotton defenses are improved after exposure to VOCs from infested conspecific plants. Omer *et al.* (2001) documented that cotton resistance against multiple pests could be induced by application of jasmonic acid (JA). Zakir *et al.* (2013b) showed that cotton HIPVs provide associational resistance to neighboring plants resulting in reduced oviposition by moths. All the above-mentioned studies on cotton confirmed cotton defenses to be improvable by means of volatiles but none of them unraveled the mechanisms behind the observed phenomenon. Our study provides first insights in the physiological changes in intact neighboring plants right after exposure to HIPVs (i.e. gossypol level, genes expression, VOCs emission).

That the observed physiological changes indeed enhance insect resistance in HIPV-exposed cotton plants was confirmed with the larval choice-tests. *S. exigua* larvae clearly preferred young leaves of control plants to leaves of plants that had been exposed to HIPVs (Fig. 6), likely due to the induction of defensive metabolites (e.g. gossypol, Fig. 2a). The effect of HIPV exposure on the larval preferences was strongest for the young leaves of feral plants (S.I. Fig. S3). This is in line with the theory that in cultivated plants breeding for specific agronomic traits goes at the cost of defense traits, which are readily maintained in wild plants due to natural selection (Chen *et al.*, 2015a,b). The results suggest that this is the case for HIPV-induced defenses in cotton, but this will need to be confirmed by testing multiple cotton varieties and wild cotton populations.

The measured gossypol/heliocides levels, as well as the results from the feeding assays show higher investment in defense of young tissue (4th leaf) compared to old tissue (1st leaf). This nicely supports the “optimal defense theory”, which asserts that plants invest more resources in defending parts that have highest fitness value (Eisenring *et al.*, 2017).

The choice test (Fig. 6) and the quantification of leaf consumption (S.I. Fig. S3) show that herbivores tend to avoid and feed less on HIPV-exposed plants. This nicely lines-up with the findings of Téréta (2015) which in his thesis reported that plants that had been exposed to VOCs from conspecific topped neighboring plants had lower herbivore infestations later in the season. Our results provide further evidence and novel insight into volatile-mediate interactions among cotton plants and reveal several molecular and chemical changes that take place in receiver plants. Indeed, we could show that HIPVs exposure can directly induce in receiver plants higher VOC emissions, increased production of toxic secondary metabolites (i.e. gossypol) as well as the upregulation of genes related to both VOCs and gossypol production.

These findings contribute to a better understanding of plant defense responses in natural and agricultural ecosystems and may also lead to the implementation of new specific pest control strategies. Future work should focus on the identification of the volatile compounds that trigger the observed physiological changes in neighboring plants. Eventually, these specific VOCs may be applied as natural elicitors to enhance pest resistance in cultivated cotton cultivars and thus reduce pesticide use in the world’s “dirtiest” crop together with already existing techniques such as cotton training for induced defenses (topping & pruning) in the context of an IPM program (Llandres *et al.*, 2018).

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Supporting Information

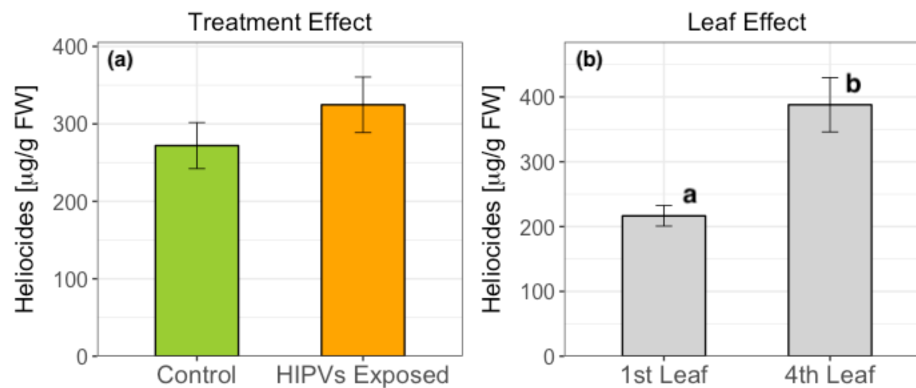

Figure S1. Heliocides content (in gossypol equivalents) (mean ± s.e.) in cotton leaves. Different letters mean significant difference (ANOVA). (a) Treatment Effect: control (N=44) vs HIPV-Exposed (N=44), pooled factors: caterpillar species, cotton variety and leaf number. (b) Leaf Effect: 1st Leaf (N=46) vs 4th Leaf (N=42), pooled factors: caterpillar species, cotton variety and treatment (ANOVA: F=10.62, P<0.01).

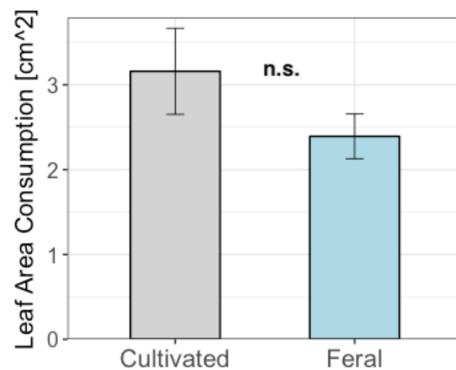

Figure S2. Leaf area consumption [cm^2] (mean ± s.e.) on 24h infested plants divided by domestication status. Pooled factor: exposure treatment (ANOVA: F=1.19. P=0.29).

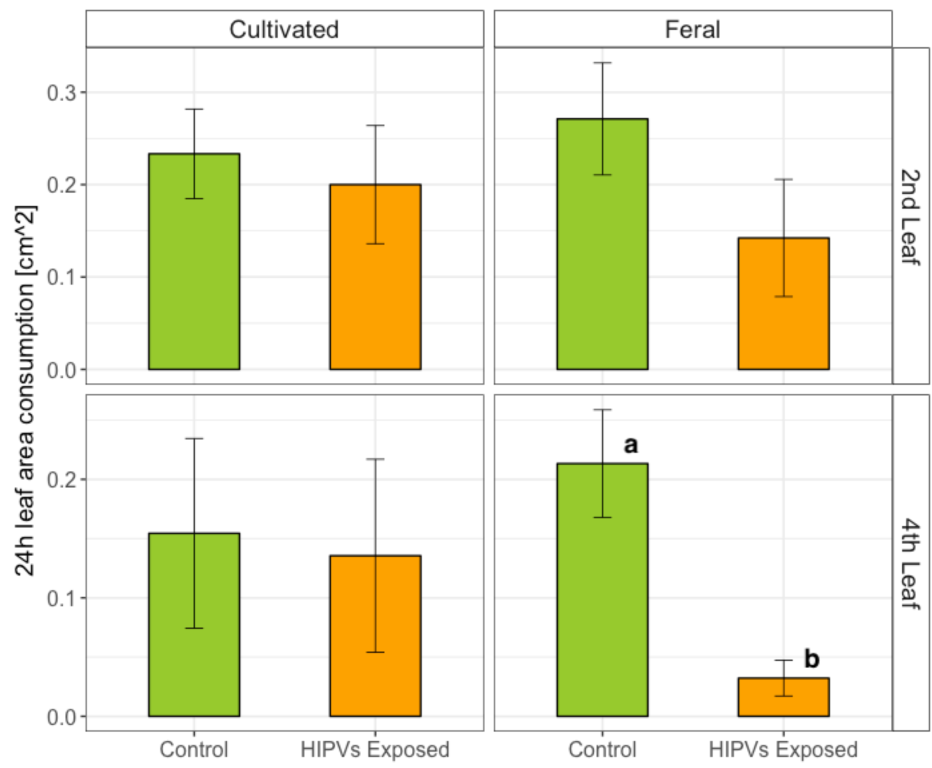

Figure S3. Leaf area consumption [cm^2] (mean ± s.e.) after 24h feeding in single petri dishes (choice test). Different letters mean significant difference in the single square ($P<0.01$, paired Wilcoxon Test).

Table T1. Genes Information

Gene ID	GenBank Acc. No.	Gene Family	Function	Forward Primer Sequence	Reverse Primer Sequence	Reference
Cad1-A	Y18484	(+)-δ-cadinene synthase (CDNS; referred to as CAD); *CAD1-A* subfamil	*CAD1* family genes regulate the first step in gossypol biosynthesis by catalyzing the cyclization of farnesyl diphosphate (FPP) to (+)-δ-cadinene via a nerolidyl diphosphate intermediate.	ATAAGGATGAAATGCGT	GAAGCTTGGTAAAGTTC	Zebelo et al. 2017
Cdn1-C3	AF174294	(+)-δ-cadinene synthase (CDNS; referred to as CAD); *CAD1-C* subfamil	*CAD1* family genes regulate the first step in gossypol biosynthesis by catalyzing the cyclization of farnesyl diphosphate (FPP) to (+)-δ-cadinene via a nerolidyl diphosphate intermediate.	AACTCAAAAACGCCACC	TAGTCGGAATCGAAGGG	Zebelo et al. 2017
CYP706B1	AF332974	Cytochrome P450 monooxygenase	*CYP706B1* regulates the second step in gosspol biosynthesis by catalyzing the hydroxylation of (+)-δ-cadinene to form 8-hydroxy-(+)-δ-cadinene.	GCAAGCCAATTGATTTT	GCACCGGGAAAATATCA	Zebelo et al. 2017
FPS1	KF871071	Farnesyl diphosphate synthase	*FPS1* catalyze FPP which is a precursor for a structurally diverse class of terpenoides including gossypol.	GGAAACCAGACACTGCC	ACACTGCTTGCACTGGTT	Zebelo et al. 2017
GhCYP82L1	KY247144	Cytochrome P450; CYP82L subfamily	homoterpene (DMNT and TMTT) biosynthesis	ATTCTCTGGTAACGAGT	AACACTGATTACTGAGTC	Liu et al. 2017
GhCYP82L2	KY247145	Cytochrome P450; CYP82L subfamily	homoterpene (DMNT and TMTT) biosynthesis	ATTCTCCGGTAACGATTA	AACATTGATTATTGAGTC	Liu et al. 2017
GhTPS12	KJ957818	Terpene Synthase	In vitro: *GhTPS12* produce nerolidol when incubated with FPP. In	GGTTCGGTTCAGGGAAT	CGAAGGATTGTAGCAGT	Huang et al. 2017
GhTPS14	KX963376	Terpene Synthase	In vitro: *GhTPS14* accepted both FPP and GGPP to produce (E)-nerolidol and (E,E)-geranyllinalool, respectively. Nerolidol and geranyllinalool are the precursors of DMNT and TMTT, respectively. Furthermore, *GhTPS14* produce (E)-β-ocimene and linalool when incubated with GPP.	GAACTGACCACCCTCAC	CGCCTCCACAAACCATGA	Huang et al. 2017
GhACTIN4	AY305726	Actin	Housekeeping Gene	TTGCAGACCGTATGAGC	ATCCTCCGATCCAGACAC	Zebelo et al. 2017

Table T2. Exposure treatment effect ANOVA table of defensive genes

Gene	F-value	P-value
GhTPS12	11.18	<0.005
GhTPS14	50.75	<0.0001
GhCYP82L1	75.96	<0.0001
GhCYP82L2	58.363	<0.0001
Cad1A	34.24	<0.0001
Cdn1C3	61.123	<0.0001
CYP706B1	35.94	<0.0001
FPS1	14.58	<0.0005

Table T3. Cotton variety effect ANOVA table of different VOCs

VOC	Treatment	F-value	P-value
(*Z*)-3-hexenal	Control	6.44	0.032
(*Z*)-3-hexenol	Control	8.11	0.019
(*Z*)-3-hexenyl acetate	Control	10.42	0.01
(*Z*)-3-hexenal	HIPVs Exposed	8.94	0.015
(*Z*)-3-hexenol	HIPVs Exposed	6.38	0.032
(*Z*)-3-hexenyl acetate	HIPVs Exposed	11.91	0.007

Chapter 2: A comparison of herbivore-induced plant volatiles and defense traits of wild *Gossypium hirsutum* from different populations.

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Abstract

The research conducted, including the rationale: It is increasingly evident that herbivore-induced plant volatiles (HIPVs) play a key role in the interactions of plants with their biotic environment. They are known to directly affect herbivore performance and behavior, can attract natural enemies of herbivores, and they are involved in plant-plant interactions. In cotton plants, it has been shown that HIPVs induce resistance against insect herbivores in intact neighboring plants. In this study we compared the HIPVs emission and other defensive traits of several wild cotton populations in order to identify genotypic differences in both emitter and receiver plants.

Methods: Receiver cotton plants were exposed for 48h to volatiles from plants (same genotype) that were infested with larvae of the moth *Spodoptera exigua*. Defense gene expression, concentrations of cotton defense compounds (gossypol and heliocides) and emissions of volatiles were measured and compared for the source and receiver plants. Some other plant parameters such as size, number of leaves, leaf area and leaf area consumed by the caterpillars were measured on source plants at the end of the exposure treatment.

Key results: One plant population (i.e. SA) was less damaged by *S. exigua* and caterpillars developed slower on these plants. Two other populations (i.e. HC and CO), when infested with caterpillars, emitted high amounts of indole which interestingly is known to prime maize defenses. Gene expression analysis did not show patterns that could be correlated with the other results of this study.

Main conclusion: The experiments show interesting trends of differences in insect damage and indole emissions among plant genotypes. The genotypes that have higher expression of these traits are good candidates to be used in further experiments aimed at improving the efficiency of plant defenses and plant-plant interactions in cotton. Due to limited seed availability, low germination rate and the logistical limitations of the exposure system, the number of replicates was low, which decreased the confidence of trait and variability estimates.

Introduction

In this study we compared several wild cotton populations from the Yucatan peninsula of Mexico in terms of HIPV emissions and responses to these emissions. The comparisons also included the two genotypes used in the first chapter (i.e. feral from Puerto Escondido and STAM59A). The aim of this study was to screen the wild cotton genotypes for strong volatile emitters and highly inducible receivers. We may expect that wild populations possess better defenses than cultivated plants. It has been proposed that through artificial selection humans have modified non-target traits in crops due to the existence of trade-offs with production traits, which has resulted in "Domestication syndromes" (Meyer *et al.*, 2012; Chen *et al.*, 2015a). More specifically, modern crops have generally been selected for greater yields possibly at the cost of defense efficiency, thus increasing their susceptibility to pests (Welter & Steggall, 1993; Gepts, 2004; Chen *et al.*, 2015a,b), although specific defenses against specialist pests may have been maintained or even improved (Gaillard *et al.*, 2018) . Darwin (1859), already alluded to the fact that domesticated plants may no longer be viable in the wild on their own (Gepts, 2004). To test if wild cotton indeed emits more volatiles in response to herbivore damage and possibly responds stronger to these volatiles we infested cotton plants with larvae of the moth *Spodoptera exigua* (Hübner, Lepidoptera: Noctuidae). Originally from Southeast Asia, *S. exigua* is a cosmopolitan pest that feeds on numerous crop plants, including cotton, and has developed multiple insecticide resistance (Powell Cobb & Bass, 1975; Brewer *et al.*, 1990; Greenberg *et al.*, 2001; Ahmad *et al.*, 2018). In a laboratory setup we exposed cotton plants of 12 different genotypes to volatiles emitted by *S. exigua*-infested plants from the same genotype during 48 hours and took several measurements on both source and receiver plants. The aim of this experiment was to screen for genotypes that are strong emitters or highly inducible receivers. We found trends of lower leaf consumption in two genotypes of the same wild population and higher indole emissions by 4 other genotypes from 2 different populations.

Methods

Plants and insects

Cotton seeds (*Gossypium hirsutum*) of twelve different genotypes collected in the Yucatan peninsula in Mexico (see Tab.1) were scratched on sandpaper, gently poked with a needle and placed on moist cotton (24h-48h, 28°C, dark) to accelerate and increase the germination process. Subsequently, the seeds were sown in plastic pots (height: 8.5cm; diameter: 6cm) containing commercial soil (Profi Substrat Soil, Einheitserde, Germany). Since only about one hundred seeds of each wild genotype were available, we planted only 10 seeds per genotype per week from September to November 2018. Seedlings were grown in phytotrons (CLF Growbanks, 25°C, 16h:8h light:dark) and watered every other day. When available, pairs of four to five-week old plants were used for each experiment. We used plants with at least three true leaves expanded and tested a maximum 12 pairs per week. *Spodoptera exigua* was reared by adapting the method described by Maag *et al.* (2014). Eggs were collected from the cage with moths (30cm x 30cm x 30cm) and placed in plastic boxes (13.5cm x 15cm x 5cm) with artificial diet (F9219B, Frontier Scientific Services, Newark, USA). One week later hatched larvae were individually transferred in multi-wells tray (RT32W, Frontier Scientific Services, Newark, USA) and supplied with artificial diet. Trays were left untouched until pupation. Pupae were then transferred into plastic boxes (13.5cm x 15cm x 5cm) and wrapped with moist paper towel until adult emergence. Adults were then transferred into a cage (30cm x 30cm x 30cm) and sustained with water and water-honey solution. Dead adults were constantly removed. The whole cycle occurred at room temperature (8h:16h dark/light). First to second instar larvae reared on artificial diet were used for the experiments.

Table 1: List of the different cotton genotypes used in the study. For wild genotypes, in the "Label" column, the 2-letters code (e.g. SA) stands for the population, while the number represents the individual plant label. Cultivated and Feral genotypes are the same as used in chapter 1.

Label	**Full Name**	**Origin**	**Coordinates [LAT/LON]**	**Domestication Status**
SA1	Sal-1	Yucatan (Mexico)	20.935425/-90.366062	Wild
SA9	Sal-9	Yucatan (Mexico)	20.935569/-90.266016	Wild
CH8	Chapo2-8	Yucatan (Mexico)	21.019404/-90.323185	Wild
CH3	Chapo2-13	Yucatan (Mexico)	21.014736/-90.324633	Wild
CA1	Cam-1	Yucatan (Mexico)	20.99822/-90.337157	Wild
CA4	Cam-4	Yucatan (Mexico)	20.997283/-90.337614	Wild
HC1	Hol-Chit 1	Yucatan (Mexico)	21.602866/-88.053191	Wild
HC9	Hol-Chit 9	Yucatan (Mexico)	21.602557/-88.050506	Wild
CO1	Coloradas-1	Yucatan (Mexico)	21.608945/-87.96131	Wild
CO8	Coloradas-8	Yucatan (Mexico)	21.58574/-87.894249	Wild
ST	STAM 59A	Senegal	NA	Cultivated
PU	Puerto Escondido	Oaxaca (Mexico)	15.883557/-97.108137	Feral

Figure 1. Map of the several locations of cotton from Mexico. In blue are the wild populations from the Yucatan peninsula, in green the feral genotype from Puerto Escondido (Oaxaca). The ST genotype is not represented here since is a commercial line.

Experimental procedure

Source and receiver plants of the same cotton variety were placed in glass vessels (Verre & Quartz Technique SA, Switzerland) that were pairwise connected via Teflon tubes. Groups of 10 *Spodoptera exigua* larvae were weighed and then dropped on the source plants. A charcoal-purified and humidified airflow of 1L/min was pushed from the vessel with the source plant to the vessel with the receiver plant, in order to expose the latter to HIPVs during 48h. During the last 2 hours of the exposure, VOCs were collected from source bottles and analyzed as explained below (VOCs Collection and Analysis). At the end of the exposure (and VOCs collection), receiver vessels were removed in order to keep the receiver plants "HIPVs-free" for at least 45 minutes before further proceedings. At the same time, source vessels containing plants and caterpillars were removed from the system. Caterpillars were collected, counted and re-weighed as a group in order to calculate their average relative growth rate [(final weight - initial weight)/initial weight]. The size of the source plants was measured and then leaves were excised, counted, placed on a scaled paper and photographed in order to quantify total leaf area and the surface consumed by the caterpillars with Adobe Photoshop. The 3rd leaf of each plant was collected and individually wrapped in aluminum foil and frozen in liquid nitrogen right after the photoshoot. Aluminum envelopes with leaves were stored at -80°C until the extraction procedures (Gene expression; Gossypol & Heliocides).

After the HIPV-free period, intact receiver plants were placed in clean glass vessels and VOCs were collected and analyzed as explained below (VOCs Collection and Analysis). At the end of these collections, the receiver plants were also removed from the system and their leaves processed as described above for the source plants. All the data were analyzed with ANOVA and Tukey Posthoc using the program R ($P<0.05$).

Gossypol and heliocides extraction and analysis

Approximately 50±5mg of the frozen leaf powder that was obtained as described above were transferred into a frozen 1.5 mL Eppendorf tube. Then, 80 µL acetonitrile and 4-6 glass beads (1.25-1.65 mm diameter) were added to each tube. The mixture was vortexed and then homogenized for 3 minutes at 30 Hz using a beadmill (Retsch MM 300, Haan, Germany). Next, samples were centrifuged for 5 minutes at 14000 rpm. The recovered supernatant was subjected to a second centrifugation step to obtain a totally limpid solution. Finally, the supernatant was recovered and transferred into 2 mL glass vial (BGB, Germany) for HPLC analysis (Glauser *et al.*, 2013). An Ultra High Performance Liquid Chromatograph (UHPLC) coupled to a DAD

detector set at 288 ± 2 nm (Ultimate 3000 Dionex, Thermo Fisher Scientific, MA, USA) was used to analyze a 5 µl aliquot of each sample, which was injected onto an ACQUITY UPLC® BEH C18 column (2.1 x 100mm, 1.7µm) (Waters, MA, USA). The flow rate was held constant at 0.45 mL/min and the temperature was kept at 30°C. The mobile phase A consisted of 0.05% formic acid in MilliQ water (18 Ω) and the mobile phase B of 0.05% formic acid in acetonitrile (HiPerSolv, VWR Chemicals®, France). The following gradient was used: 45-90% B in 8 min, 90-100% B in 0.5 min, holding at 100% for 2.5 min followed by re-equilibration at 45% B for 3.5 min. Gossypol and heliocides (grouped H1 to H3) were identified by their retention time. Quantification was done by calculating a calibration equation obtained by linear regression from six calibration points (5 to 250 µg/mL) in gossypol equivalents.

Gene expression measurements

Frozen leaves were ground into a fine powder in a mortar filled with liquid nitrogen. Leaf total RNA was isolated using the GeneJET Plant Purification Mini Kit (Thermo Scientific) according to the manufacturer's instructions. The complete DNA removal was performed by using the RNase-Free DNase Set (QIAGEN). Each total RNA sample (500 ng) was reverse transcribed using the GoScript™ Reverse Transcription System (Promega). Real time qPCR was performed on the Rotor-Gene™ 6000 (Corbett Research) using GoTaq® qPCR Master Mix (Promega). *GhACT4* (GenBank accession no. AY305726) was used as an internal standard to normalize cDNA concentrations. The relative expression of 8 defense-related genes was quantified. The primers used for qPCR and the respective genes are provided in Table 4. Three to four independent biological replicates were performed from each genotype, with the exception of CA4 and CH3, where only 2 and 3 technical replicates respectively were analyzed. Relative expression levels were calculated using the ΔΔCt method (Schmittgen & Livak, 2008), comparing expression relative to that of PU genotype, as it was constantly present in each of the experimental blocks. Single gene expression of each of the 12 genotypes was analyzed on the same plate in order to allow direct comparison. Due to design limitation source and receiver had to be analyzed separately on different plates and therefore are not directly comparable. Plant material was exactly the same as used for the gossypol and heliocides analyses.

VOCs collections and analysis

Filters containing 25mg of 80/100 mesh Hayesep-Q adsorbent (Sigma, Switzerland) were connected to the source bottle and VOCs were collected during 2h by pulling air through the filters at a rate of 0.9L/min, while the air pushed into the vessel was increased form 1 L/min to 1.2 L/min in order to continue to deliver air to the vessel with the receiver plant. Subsequently, filters were eluted with 100µL dichloromethane and samples were stored at -80°C. The elutes were spiked with a 5 µL internal standard solution (n-octane and nonyl-acetate, 20 µg/µL each) before they were analyzed on a gas chromatograph (Agilent 6890) coupled to a mass selection detector (Agilent MSD 5973). A 2 µL aliquot of each sample was injected in pulsed spitless mode onto an Agilent HP-5MS column (30 m length x 250 µm diameter and 0.25 µm film thickness). After injection, temperature was maintained at 40°C for 3 min, increased to 100°C at a rate of 8°C per min and subsequently to 200°C at a rate of 5°C per min followed by a post run of 3 min at 250°C. Helium was used as carrier gas and kept at constant flow of 1.1mL/min. Compounds were subsequently identified by comparing their mass spectra with those from the NIST mass spectral library or from PBM Quicksearch library (U.S. Department of Commerce). Compounds were quantified by comparing their peak areas with those of the internal standards. After the 48h exposure time, receiver plants were each placed in a clean glass vessel (VTQ SA, Switzerland) and VOCs were collected with an air flow of 1L/min into the vessel, and 0.9L/min pulled through Hayesep-Q filters for 2 hours. Samples were processed as described above.

Statistical analyses

Statistics ($P<0.05$) and graphs were performed and computed using the program R and its complementary console R-studio (http://www.rstudio.com). Data were analyzed through ANOVA fitting different linear models. Some models were subject to logarithmic correction to meet normality assumptions.

Results and discussion

Plants from wild populations were growing slower as judged by their height compared to the feral and the cultivated genotypes also used in chapter 1 (Fig. 2). The feral genotype reached to a larger leaf area on average than all the others at the time of the experiments (Fig. 3). Some genotypes seemed to be less palatable to *S. exigua* (i.e. SAs), as expressed by low consumption (Fig. 5) low relative growth rate of the caterpillars (Fig. 6), probably due to their high gossypol levels (Fig. 8). Indeed, gossypol levels were negatively correlated with the leaf area consumed by caterpillars across all the genotypes (Fig. 9) confirming its deterrent role against *S. exigua* (McAuslane *et al.*, 1997). Due to the missing control (intact, non-exposed plants), no direct inference on possible VOCs-mediated induction of defense traits can be done. Still, the heliocides levels of SAs were the highest in receiver plants, while they were similar among genotypes in source plants, indirectly suggesting a possible strong defense induction in SA population (Fig.10). Some other genotypes emitted more VOCs when infested (Fig. 11), mainly due to indole which was detected in high amounts (i.e. HCs and COs, Fig. 12). Exposure to high doses of indole by some of the varieties (HC and CO) did not directly consistently result in stronger defense gene expression or higher gossypol and heliocides levels in receiver plants. Indole is an essential herbivore-induced volatile priming signal in maize (Erb *et al.*, 2015). We suggest that these wild cotton plants could only be primed and will show better and faster defenses only upon subsequent herbivore challenge.

The expression of 8 different genes was measured in both source and receiver plants in the 4th leaf of all the different cotton varieties (Tab. 2, Tab. 3). No clear conclusions can be drawn from this analysis, even though we could sometimes observe an increased expression of genes related to VOCs production in receiver plants (i.e. *GhCYP82L1*, *GhCYP82L2*, *GhTPS14*) in some genotypes that showed high total VOCs emission from source plants (i.e. HC9 and CO1).

Further inquiries on SA, HC and CO varieties are necessary in order to complete this story. For instance, survival *of S. exigua* caterpillars could be tested on source and receiver plants. Furthermore, gene expression and secondary metabolites levels could be measured on receiver plants after a further infestation period as well, in order to test them for possible priming responses. Plant hormones levels (e.g. JA and SA) and the expression of the related genes could also be tested in infested source, intact receiver and infested receiver plants for

extended information on cotton defense strategies. Cross-varieties exposure experiments could also be carried out.

We decided to stop this subproject of my thesis at this point due to limited seed amounts and extremely low germination rate (not reported) which both were a major impediment.

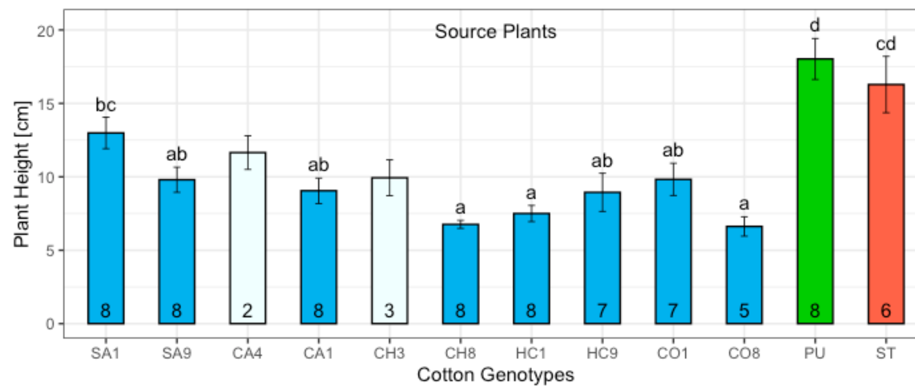

Figure 2. Plant height [cm] (mean ± s.e.) circa 1 month after sowing (at least 3rd leaf stage) of different cotton genotypes at the end of the 48 hours exposure procedure. In blue and light blue wild genotypes from Yucatan, in green feral genotype/population from Puerto Escondido (Mexico), in red cultivated variety (STAM59a). Light blue genotypes (with only few replications) were not included in the statistical analysis (ANOVA & Tukey Posthoc). The number in the bars corresponds to the number of replicates for each genotype. Different letters indicate significant differences (ANOVA: $F=14.81$, $P<0.001$).

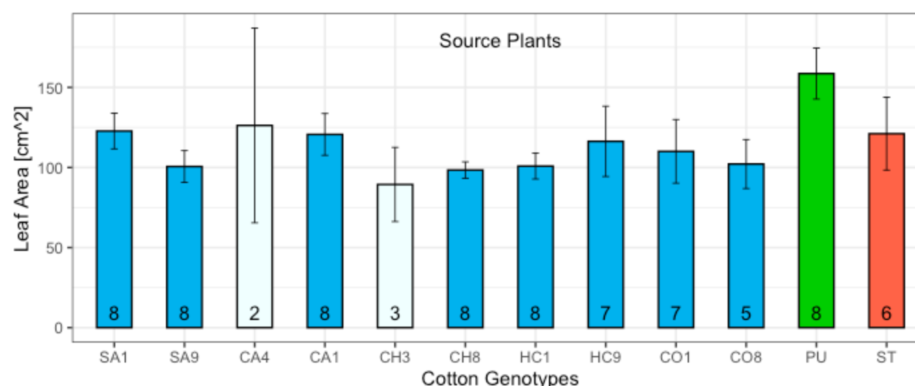

Figure 3. Leaf area [cm^2] (mean ± s.e.) circa 1 month after sowing (at least 3rd leaf stage) of different cotton genotypes at the end of the 48 hours exposure procedure. In blue and light blue wild genotypes from Yucatan, in green feral genotype/population from Puerto Escondido (Mexico), in red cultivated variety (STAM59a). Light blue genotypes (with only few replications) were not included in the statistical analysis. The number in the bars corresponds to the number of replicates for each genotype. ANOVA did not show any significant differences ($F=1.61$, $P=0.13$).

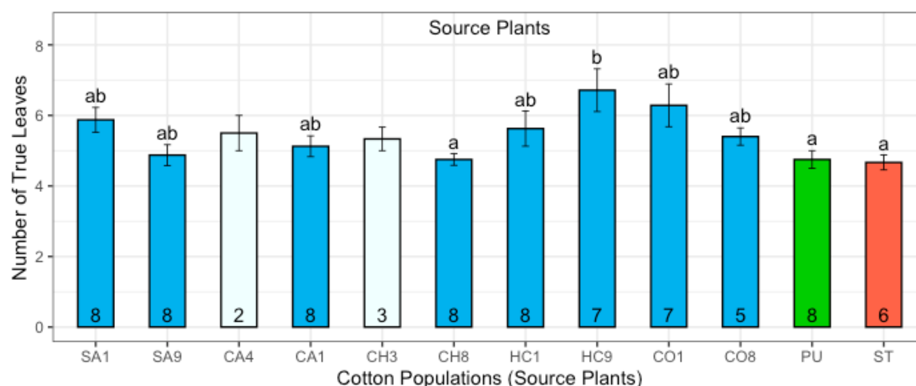

Figure 4. Number true leaves (mean ± s.e.) circa 1 month after sowing of different cotton genotypes at the end of the 48 hours exposure procedure. In blue and light blue wild genotypes from Yucatan, in green feral genotype/population from Puerto Escondido (Mexico), in red cultivated variety (STAM59a). Light blue genotypes (with only few replications) were not included in the statistical analysis (ANOVA & Tukey Posthoc). The number in the bars corresponds to the number of replicates for each genotype. Different letters indicate significant differences (ANOVA: F=3.31, P<0.01)

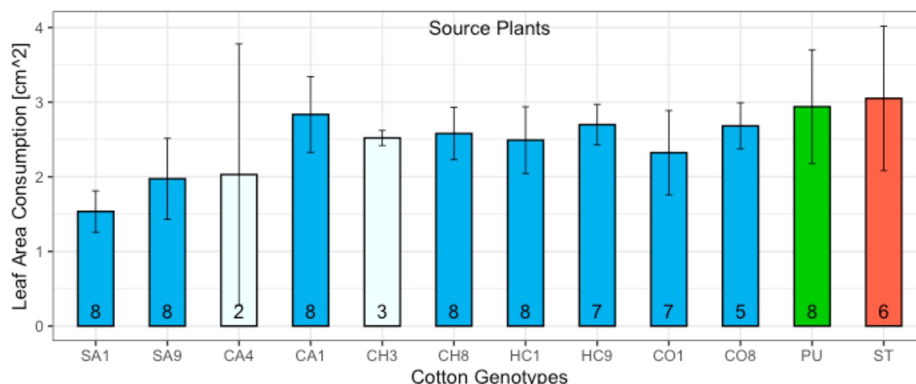

Figure 5. Leaf area consumption [cm^2] (mean ± s.e.) by *S.exigua* caterpillars during the 2 days of exposure treatment on different cotton genotypes. In blue and light blue wild genotypes from Yucatan, in green feral genotype/population from Puerto Escondido (Mexico), in red cultivated variety (STAM59a). Light blue genotypes were not included in the statistical analysis. The number in the bars corresponds to the number of replicates for each genotype. ANOVA did not show any significant differences (F=0.80, P=0.61).

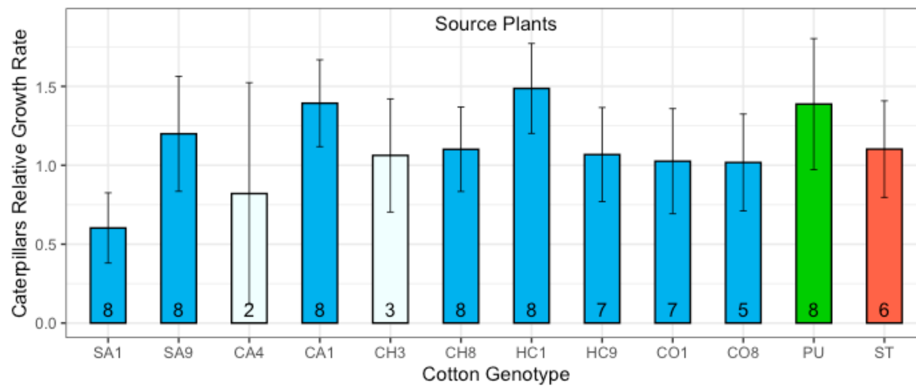

Figure 6. Relative growth rate (mean ± s.e.) of *S. exigua caterpillars* on different cotton genotypes during the 2 days exposure treatment. In blue and light blue wild genotypes from Yucatan, in green feral genotype/population from Puerto Escondido (Mexico), in red cultivated variety (STAM59a). Light blue populations were not included in the statistical analysis. The number in the bars corresponds to the number of replicates for each genotype. ANOVA did not show any significant differences (F=0.86, P=0.56).

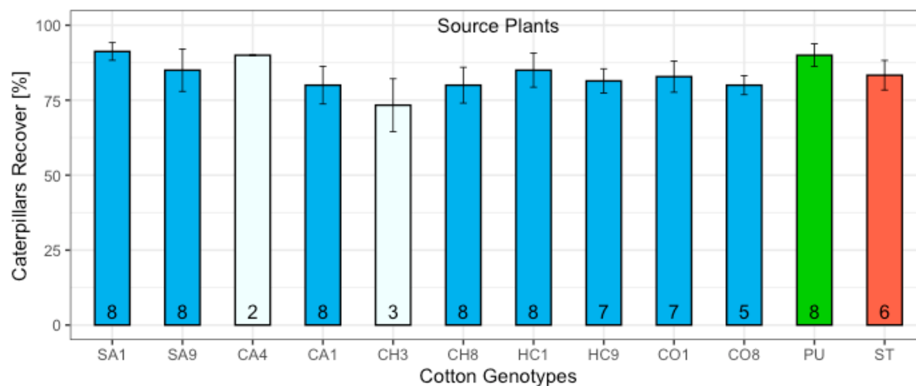

Figure 7. Percentage of *S. exigua* caterpillars recovered (mean ± s.e.) on different cotton genotypes after 2 days exposure treatment (10 caterpillars were released at the beginning of the infestation). In blue and light blue wild populations from Yucatan, in green feral population from Puerto Escondido (Mexico), in red cultivated variety (STAM59a). Light blue populations (with only few replications) were not included in the statistical analysis. The number in the bars corresponds to the number of replicates for each genotype. ANOVA did not show any significant differences (F=0.6, P=0.79).

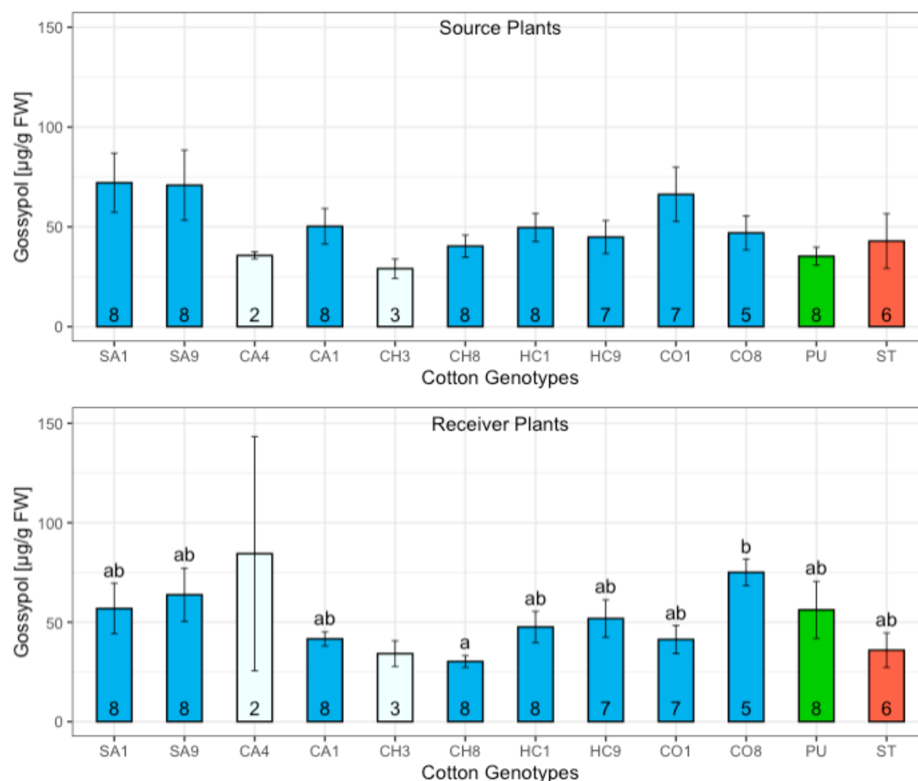

Figure 8. Gossypol level [µg/g FW] (mean ± s.e.) in 3rd leaf of different cotton genotypes. In blue and light blue wild genotypes from Yucatan, in green feral genotype/population from Puerto Escondido (Mexico), in red cultivated variety (STAM59a). Light blue populations were not included in the statistical analysis. The number in the bars corresponds to the number of replicates for each genotype. On top, source plants measurements, on bottom receiver plants measurements. Statistical analysis was performed separately for source and receiver plants (ANOVA & Tukey Posthoc). Source plants did not show any significant differences (ANOVA: $F=1.52$, $P=0.16$). In receiver plants graph different letters indicate significant differences (ANOVA: $F=2.23$, $P<0.05$).

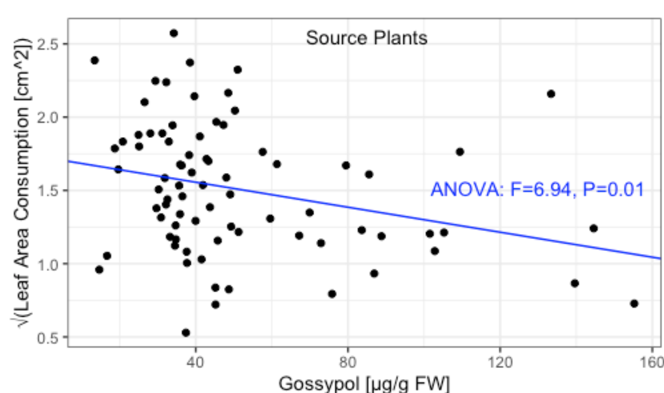

Figure 9. Squared root of leaf area consumption (LAC, cm^2) versus gossypol levels (µg/g FW) in source plants. A linear mixed model with plant genotypes as random factor was fitted and the ANOVA resulted in a significant gossypol effect ($F=6.94$, $P=0.01$). The same analysis was done for the squared root of LAC versus Heliocides but no significance was found (ANOVA: $F=0.1$, $P=0.75$). Models with relative growth rate versus gossypol or heliocides could not be fitted due to non-normal distribution.

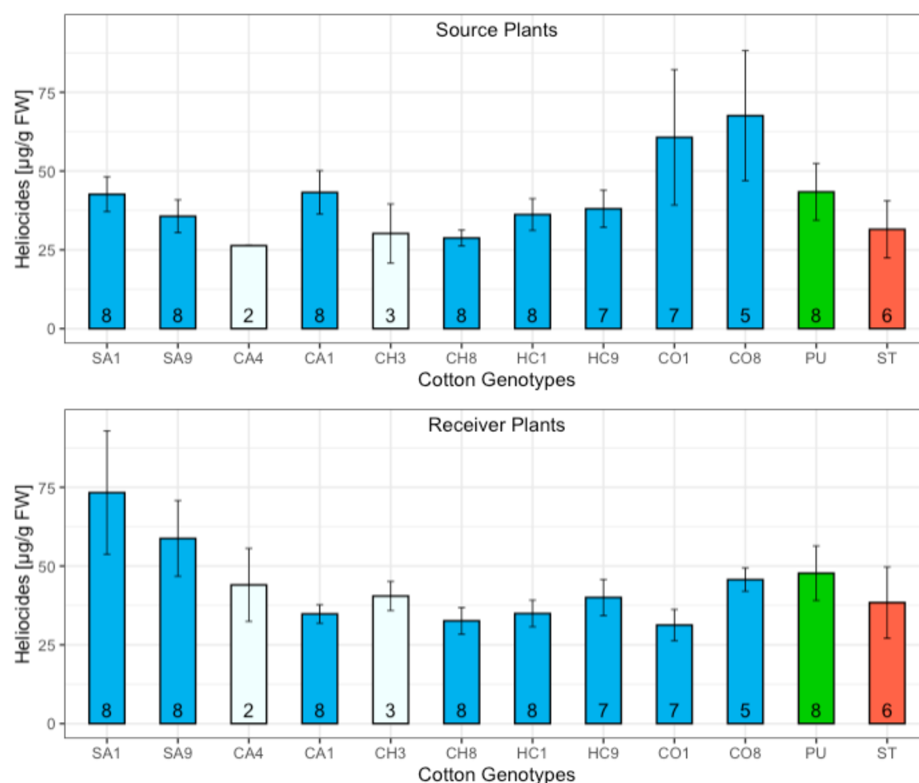

Figure 10. Heliocides level [µg/g FW] (mean ± s.e.) in 3rd leaf of different cotton genotypes. In blue and light blue wild genotypes from Yucatan, in green feral genotype/population from Puerto Escondido (Mexico), in red cultivated variety (STAM59a). Light blue populations were not included in the statistical analysis. The number in the bars corresponds to the number of replicates for each genotype. On top, source plants measurements, on bottom receiver plants measurements. Statistical analysis was performed separately for source and receiver plants and did not show any significant differences (ANOVA Source: $F=1.14$, $P=0.35$, ANOVA Receiver: $F=1.73$, $P=0.1$).

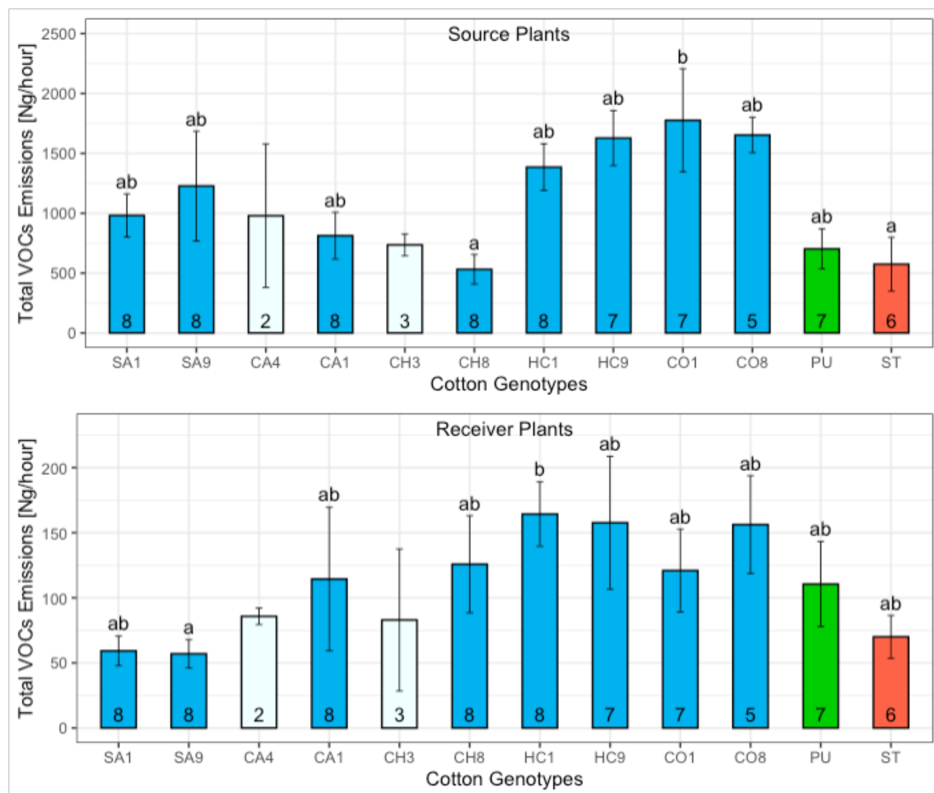

Figure 11. Total VOCs emissions (mean ± s.e.) by different cotton genotypes. In blue and light blue wild populations from Yucatan, in green feral variety from Puerto Escondido (Mexico), in red cultivated variety (STAM59a). Light blue populations were not included in the statistical analysis. The number on the bar's base correspond to number of replicates for each genotype. On top, infested source plants measurements, on bottom intact receiver plants measurements. Statistical analysis was performed separately for source and receiver plants (ANOVA & Tukey Posthoc). Different letters indicate significant differences within the same graph (ANOVA source: F=3.56, P<0.01, ANOVA receiver: F=2.53, P<0.05)

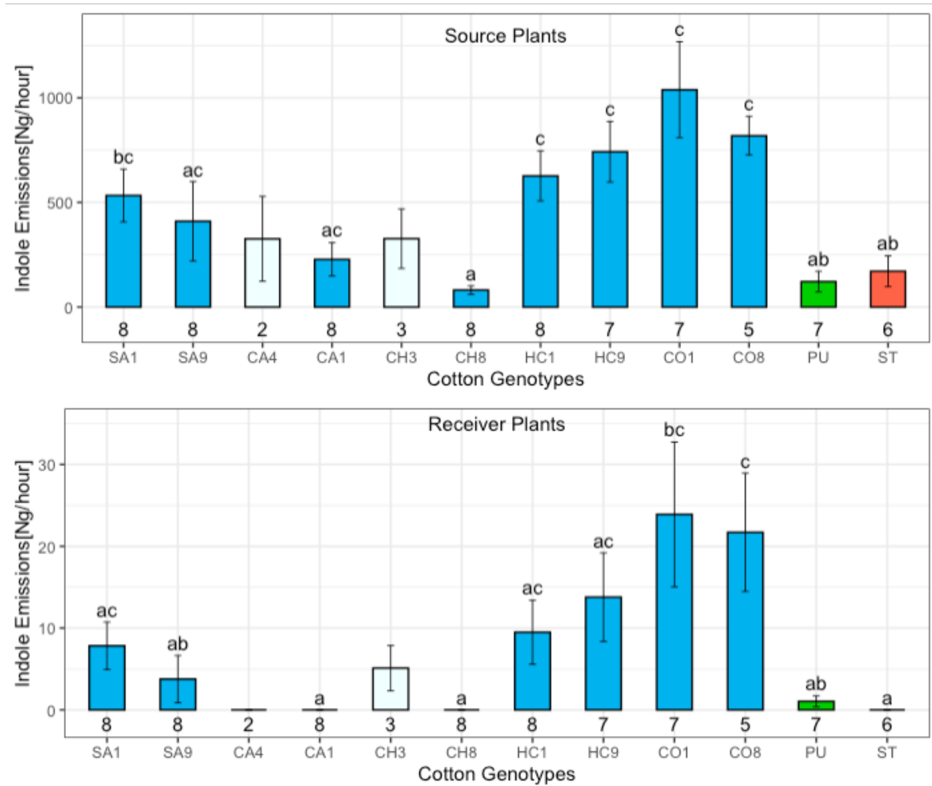

Figure 12. Indole emissions (mean ± s.e.) by different cotton genotypes. In blue and light blue wild populations from Yucatan, in green feral variety from Puerto Escondido (Mexico), in red cultivated variety (STAM59a). Light blue populations were not included in the statistical analysis. The number in the bars base corresponds to the number of replicates for each genotype. On top, infested source plants measurements, on bottom intact receiver plants measurements. Statistical analysis was performed separately for source and receiver plants (ANOVA & Tukey Posthoc). Different letters indicate significant differences within the same graph (ANOVA source: $F=7.26$, $P<10^{-6}$, ANOVA receiver: $F=5.62$, $P<10^{-4}$)

Table 2: Relative gene expression (mean ± s.e.) of 8 genes in each genotype (source plants).

Source	*GhCYP82L1*	*GhCYP82L2*	*GhTPS12*	*GhTPS14*	*CAD1A*	*Cdn1C3*	*CYP706B1*	*FPS1*
SA1	0.91 ± 0.31	0.84 ± 0.14	1.27 ± 0.46	0.68 ± 0.24	1.04 ± 0.42	1.18 ± 0.46	1.03 ± 0.15	1.19 ± 0.22
SA9	0.9 ± 0.3	0.86 ± 0.3	1.85 ± 0.43	0.93 ± 0.27	2.67 ± 1.77	1.94 ± 0.99	1.59 ± 0.81	1.61 ± 0.55
CA4	1.6 ± 0.06	1.08 ± 0	3.02 ± 0.01	0.61 ± 0.01	0.19 ± 0.01	1.09 ± 0.02	0.65 ± 0.02	3.05 ± 0
CA1	0.42 ± 0.2	0.72 ± 0.12	5.84 ± 2.05	0.99 ± 0.2	2.48 ± 0.61	2.85 ± 0.81	1.31 ± 0.48	2.87 ± 0.81
CH3	0.52 ± 0.03	1.09 ± 0.04	1.37 ± 0.05	1.93 ± 0.02	4.18 ± 0.34	6.59 ± 0.18	4.92 ± 0.12	4.19 ± 0.13
CH8	0.89 ± 0.33	1.66 ± 0.68	4.98 ± 1.19	1.31 ± 0.63	0.79 ± 0.44	3.7 ± 1.75	1.52 ± 0.42	3.22 ± 1.65
HC1	1.37 ± 0.54	0.56 ± 0.03	0.68 ± 0.44	1.96 ± 0.74	0.26 ± 0.12	0.47 ± 0.21	0.18 ± 0.06	1.59 ± 0.26
HC9	1.89 ± 0.45	2.4 ± 0.39	2.16 ± 1.16	2.44 ± 0.74	2.11 ± 1.29	1.82 ± 1.03	1.59 ± 0.67	1.39 ± 0.4
CO1	0.93 ± 0.43	1.57 ± 0.63	1.86 ± 0.45	2.27 ± 0.66	1.13 ± 0.55	2.16 ± 1.04	1.91 ± 1.14	1.53 ± 0.26
CO8	0.48 ± 0.14	0.87 ± 0.11	0.5 ± 0.1	2.84 ± 0.53	1.19 ± 0.63	1.9 ± 0.84	0.32 ± 0.08	2.05 ± 0.57
PU	1.26 ± 0.53	1.11 ± 0.31	1.39 ± 0.69	1.35 ± 0.53	1.27 ± 0.41	1.3 ± 0.4	1.03 ± 0.15	1.08 ± 0.26
ST	0.64 ± 0.15	1.03 ± 0.17	1.48 ± 0.93	1.91 ± 0.64	2.09 ± 0.58	1.96 ± 0.48	0.7 ± 0.14	2.21 ± 0.75

Table 3: Relative gene expression (mean ± s.e.) of 8 genes in each genotype (receiver plants).

Receiver	*GhCYP82L1*	*GhCYP82L2*	*GhTPS12*	*GhTPS14*	*CAD1A*	*Cdn1C3*	*CYP706B1*	*FPS1*
SA1	0.6 ± 0.19	2.77 ± 0.72	1.81 ± 0.24	0.3 ± 0.1	0.88 ± 0.38	1.12 ± 0.48	1.06 ± 0.34	1.34 ± 0.14
SA9	0.64 ± 0.12	2.99 ± 0.89	2.02 ± 0.45	0.91 ± 0.57	10.27 ± 6.53	7.88 ± 4.4	3.19 ± 1.88	1.28 ± 0.36
CA4	0.5 ± 0.31	0.92 ± 0.01	0.91 ± 0.02	0.11 ± 0.06	0.99 ± 0	4.69 ± 0.66	3.72 ± 0.15	1.35 ± 0.35
CA1	0.62 ± 0.2	2.42 ± 0.78	4.22 ± 1.73	0.22 ± 0.05	3.92 ± 1.36	3.83 ± 1.31	2.52 ± 0.94	1.35 ± 0.19
CH3	0.41 ± 0.11	3.54 ± 2.96	1.37 ± 0.53	2.15 ± 1.94	4.14 ± 3.04	5.14 ± 3.72	1.46 ± 0.18	1.71 ± 0.43
CH8	2.05 ± 0.64	3.12 ± 1.56	6.67 ± 3.08	1.44 ± 1.09	2.23 ± 1.3	6.76 ± 3.1	1.83 ± 0.59	1.32 ± 0.36
HC1	1.67 ± 0.58	3.76 ± 1.35	0.83 ± 0.37	1.11 ± 0.47	3.58 ± 3.45	3.34 ± 3.13	3.37 ± 2.44	1.04 ± 0.29
HC9	23.82 ± 12.21	14.67 ± 6.45	1.06 ± 0.13	4.2 ± 2.27	3.43 ± 2.63	2.66 ± 1.92	4.13 ± 1.97	1.44 ± 0.51
CO1	1.75 ± 0.77	21.16 ± 4.44	5.46 ± 1.38	4.54 ± 1.81	0.44 ± 0.28	0.61 ± 0.37	2.05 ± 0.09	0.8 ± 0.26
CO8	1.25 ± 0.18	6.62 ± 1.22	1 ± 0.42	2.26 ± 0.32	3.85 ± 2.84	3.01 ± 2.07	1.67 ± 0.84	0.69 ± 0.05
PU	1.17 ± 0.37	1.04 ± 0.17	1.12 ± 0.25	1.01 ± 0.07	1.19 ± 0.44	1.24 ± 0.52	1.25 ± 0.44	1.05 ± 0.19
ST	1.16 ± 0.62	6.16 ± 2.73	1.05 ± 0.26	2.13 ± 0.52	0.93 ± 0.77	1.3 ± 1.14	0.44 ± 0.23	0.75 ± 0.34

Table 4: Information on the identity and function of the genes studied.

Gene ID	GenBank Acc. No.	Gene Family	Function	Forward Primer Sequence	Reverse Primer Sequence	Reference
Cad1-A	Y18484	(+)-δ-cadinene synthase (CDNS; referred to as CAD); *CAD1-A* subfamil	*CAD1* family genes regulate the first step in gossypol biosynthesis by catalyzing the cyclization of farnesyl diphosphate (FPP) to (+)-δ-cadinene via a nerolidyl diphosphate intermediate.	ATAAGGATGAAATGCGT	GAAGCTTGGTAAAGTTC	Zebelo et al. 2017
Cdn1-C3	AF174294	(+)-δ-cadinene synthase (CDNS; referred to as CAD); *CAD1-C* subfamil	*CAD1* family genes regulate the first step in gossypol biosynthesis by catalyzing the cyclization of farnesyl diphosphate (FPP) to (+)-δ-cadinene via a nerolidyl diphosphate intermediate.	AACTCAAAAACGCCACC	TAGTCGGAATCGAAGGG	Zebelo et al. 2017
CYP706B1	AF332974	Cytochrome P450 monooxygenase	*CYP706B1* regulates the second step in gosspol biosynthesis by catalyzing the hydroxylation of (+)-δ-cadinene to form 8-hydroxy-(+)-δ-cadinene.	GCAAGCCAATTGATTTT	GCACCGGGAAAATATCA	Zebelo et al. 2017
FPS1	KF871071	Farnesyl diphosphate synthase	*FPS1* catalyze FPP which is a precursor for a structurally diverse class of terpenoides including gossypol.	GGAAACCAGACACTGCC	ACACTGCTTGCACTGGTT	Zebelo et al. 2017
GhCYP82L1	KY247144	Cytochrome P450; CYP82L subfamily	homoterpene (DMNT and TMTT) biosynthesis	ATTCTCTGGTAACGAGT	AACACTGATTACTGAGTC	Liu et al. 2017
GhCYP82L2	KY247145	Cytochrome P450; CYP82L subfamily	homoterpene (DMNT and TMTT) biosynthesis	ATTCTCCGGTAACGATTA	AACATTGATTATTGAGTC	Liu et al. 2017
GhTPS12	KJ957818	Terpene Synthase	In vitro: *GhTPS12* produce nerolidol when incubated with FPP. In	GGTTCGGTTCAGGGAAT	CGAAGGATTGTAGCAGT	Huang et al. 2017
GhTPS14	KX963376	Terpene Synthase	In vitro: *GhTPS14* accepted both FPP and GGPP to produce (E)-nerolidol and (E,E)-geranyllinalool, respectively. Nerolidol and geranyllinalool are the precursors of DMNT and TMTT, respectively. Furthermore, *GhTPS14* produce (E)-β-ocimene and linalool when incubated with GPP.	GAACTGACCACCCTCAC	CGCCTCCACAAACCATG	Huang et al. 2017
GhACTIN4	AY305726	Actin	Housekeeping Gene	TTGCAGACCGTATGAGC	ATCCTCCGATCCAGACAC	Zebelo et al. 2017

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Chapter 3: Constitutive vs inducible cotton volatiles: which ones trigger defenses in neighboring cotton plants?

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Abstract

The research conducted, including the rationale: Recent evidence suggests that herbivore-induced plant volatiles (HIPVs) from cotton plants are particularly potent in inducing resistance in neighboring plants, which may have application potential. HIPVs can be divided into groups based on chemical properties, synthesis and, most importantly, into two groups based on their time of release. The firsts group, that is mainly composed by constitutive volatiles, is released immediately upon damage (fresh damage), whereas the second group, that is mainly composed by inducible volatiles, is only emitted after more than a day of continuous damage (old damage). In this study we tested the hypothesis that inducible volatiles may be mainly responsible for defense induction in neighbor plants.

Methods: In laboratory experiments, we compared receiver cotton plants that were exposed for 48h to volatiles emitted by *S. exigua*-infested cotton plants with only fresh damage or plants with old damage. This resulted in two types of volatile exposures, either to a group composed mainly of constitutive volatiles, or to a group composed mainly of inducible volatiles. We then compared defense gene expression, concentrations of cotton defense compounds (gossypol and heliocides), emissions of volatiles, as well as hormone levels and caterpillar preference. Plants exposed to pure air were also included in each experiment as control.

Key results: Plants exposed to volatiles of plants with old damage contained higher levels of Jasmonic Acid (JA) and JA-Isoleucine (JA-Ile) compared to plants that had been exposed to volatiles of plants with fresh damage or to control plants. Similar differences were also found for the expression of several genes related to gossypol and volatile production. In the preference test the caterpillars avoided plants that had been exposed to volatiles of plants with old damage.

Main conclusion: This study largely supports our hypothesis that inducible volatiles, mainly emitted by plants with old damage, have a key role in cotton plant-plant signaling, and directly trigger defense mechanisms in intact neighboring plants.

Introduction

Volatile organic compounds (VOCs) play important roles in the multitrophic interactions between plants and their biotic environment, resulting in various ecologically relevant responses such as attraction, repulsion, defense induction or priming and associational resistance (Heil & Silva Bueno, 2007; Dicke & Baldwin, 2010; Clavijo McCormick *et al.*, 2012; Zakir *et al.*, 2013b; Téréta, 2015; Llandres *et al.*, 2018; Erb, 2018; Turlings & Erb, 2018). Of particular interest are herbivore-induced plant volatiles (HIPVs), which are often released in exceptionally large quantities after herbivory by insects (War *et al.*, 2011). HIPVs can specifically attract natural enemies, i.e. predators and parasitoids (Turlings & Wäckers, 2004; Clavijo McCormick *et al.*, 2012), and discourage conspecific herbivore females from visiting and ovipositing on already infested plants (Moraes *et al.*, 2001). HIPVs also play a role in within-plant and between-plant signaling. For instance, Heil & Silva Bueno (2007) showed for wild lima bean plants that exposure to HIPVs leads to increased production of extrafloral nectar, an indirect plant defense.

For cotton (*Gossypium*, Linnaeus 1753), our model plant, the results from several studies indicate that HIPV-exposure affects multitrophic interactions (Karban, 1986, 1993; Bruin *et al.*, 1992; Omer *et al.*, 2001; Zakir *et al.*, 2013b,a; Téréta, 2015; Llandres *et al.*, 2018), including enhanced resistance against herbivore attack. Zakir *et al.* (2013b) reported a significant reduction in oviposition by *Spodoptera littoralis* (Lepidoptera, Noctuidae) on undamaged cotton plants nearby caterpillar-damaged conspecific plants under both laboratory and field conditions. Llandres *et al.* (2018) recently reviewed studies that show how cotton plants that are “trained” (i.e. pruned and topped) become more resistant to insect attack and that this is also the case for neighboring plants. Indeed, in a field trial conducted in Senegal, cotton bollworm infestation was greatly reduced in both trained plants and their neighbors, suggesting a signaling role of VOCs (Téréta, 2015). These findings motivated our first study (Chapter 1) on the topic, and we provided the first experimental evidence of HIPV-mediated physiological changes that lead to enhanced defenses in receiver plants.

VOCs are generally divided in different groups based on their biosynthesis, function and chemical properties (Takabayashi *et al.*, 1991; Dudareva *et al.*, 2013). Cotton VOCs can be divided in two major groups based on the timing of their release and whether they are stored in the plant (i.e. constitutive) or *de novo* synthetized (i.e. inducible). This distinction results from observations made by Loughrin *et al.* (1994), who monitored the diurnal cycle of cotton HIPVs. They found that the composition of the volatile blend shifted between the early and the

later stage of infestation. At the early stage (i.e. the first day of infestation), the volatile blend was mainly characterized by green leafy volatiles (GLVs) and several cyclic terpenoids (e.g. α-pinene, α-humulene, myrcene and caryophyllene), which are constitutively stored in the plant (Elzen *et al.*, 1985). In the later stages (i.e. the third day of infestation, 2 days after the start), when plants had old damage, the volatile blend was mainly composed of inducible *de novo* synthesized acyclic terpenes (e.g. (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene) and the aromatic heterocyclic compound indole. The constitutive volatiles are most likely stored in pigment glands (Elzen *et al.*, 1985) and released when these glands are ruptured, whereas the newly synthesized HIPVs are released throughout the leaves.

Both constitutive and inducible plant volatiles have been shown to play several specific roles in multitrophic interactions. GLVs are normally immediately released upon damage as a result of tissue breaking (Arimura *et al.*, 2009). They are mainly released from the damaged site and are known to attract natural enemies (Halitschke *et al.*, 2007) and, when perceived by other plants, can induce the expression of defense-related genes in several plant species (Matsui, 2006). The sesquiterpene caryophyllene, which is also immediately released by damaged cotton plants, is also known to attract natural enemies (Xiao *et al.*, 2012) and can even kill bacterial pathogens (Huang *et al.*, 2012). Of the inducible VOCs, indole is toxic to caterpillars and can prime neighboring plants for enhanced defenses (Erb *et al.*, 2015; Veyrat *et al.*, 2016), whereas (E)-β-ocimene, linalool and (E)-4,8-dimethyl-1,3,7-nonatriene have been found to attract predatory mites (Dicke *et al.*, 1990) and can elicit defense genes in lima bean leaves (Arimura *et al.*, 2000).

The dynamics of HIPVs released by cotton plants make it a highly suitable model to study the specific roles of constitutive versus inducible volatiles in plant-plant interactions. Because the inducible compounds are *de novo* synthesized in response to continuous insect attack (Loughrin et al., 1994; Paré and Tumlinson, 1997), these volatiles should be most reliable in informing neighbors of potential dangers. Thus, we hypothesized that these volatiles are of key importance in inducing specific defense responses. To test this hypothesis, we conducted a series of experiments in which we exposed cotton plants to volatiles from freshly damaged cotton plants or from plants with older damage. After exposure several defense-related biochemical and molecular parameters were measured and compared to intact pure air-exposed control plants. Defense gene expression, concentrations of cotton defense compounds (gossypol and heliocides), emissions of volatiles, as well as hormone levels were tested. A

caterpillar preference test was also performed in order to observe the choice of *Spodoptera exigua* among the three differently treated plants.

This series of experiment represent a further step towards our ultimate goal to identify biologically active VOCs that might be used in the field as part of an integrated pest management strategy.

Methods

Plants and insects

Cultivated cotton seeds (*Gossypium hirsutum,* variety STAM59A, kindly provided by Dr. Thierry Brévault) were soaked in water for 24h at 27°C and subsequently sown in plastic trays containing commercial soil (Profi Substrat Soil, Einheitserde, Germany) until germination. The variety STAM59A is the commercial line that was used in the field studies that revealed plant-plant interactions (Téréta, 2015; Llandres *et al.*, 2018), which we confirmed under laboratory conditions (Chapter 1). After germination, small seedlings were transferred to plastic pots (height: 8.5cm; diameter: 6cm) and placed in phytotrons (Growbanks, 28°C, 16h:8h light:dark) and watered every other day. Healthy plants with 4 true leaves were used for the experiments. We adapted the method described by Maag *et al.* (2014) to rear *Spodoptera exigua*. Eggs were collected from a cage with moths (30cm x 30cm x 30cm) and placed in plastic boxes (13.5cm x 15cm x 5cm) with artificial diet (F9219B, Frontier Scientific Services, Newark, USA). One week later, hatched larvae were individually transferred to a multi-wells tray (RT32W, Frontier Scientific Services, Newark, USA) and supplied with artificial diet. Trays were left untouched until pupation, pupae were then transferred into plastic boxes (13.5cm x 15cm x 5cm) and wrapped with moist paper towel until adult emergence. Adults were then transferred into a cage (30cm x 30cm x 30cm) and fed with water and water-honey solution. Dead adults were frequently removed from the cage. The whole cycle occurred at room temperature (8h:16h dark/light). Second instar, larvae from the same batch were used for all the experiments conducted in the same week.

Exposure treatment procedure

Receiver cotton plants were exposed during 48h to two different groups of HIPVs. The first consisted of HIPVs emitted by source plants during the first 24h of caterpillar-infestation (mainly constitutive volatiles, "*fresh*", 0h to 24h). The second exposure group was with HIPVs emitted from source plants during the second 24h of infestation (mainly inducible volatiles, "*old*", 24h to 48h). The *fresh* treatment mainly include VOCs that are immediately emitted upon leaf damage, while the *old* treatment also include VOCs that are de novo synthesized and only emitted after continued damage (Loughrin *et al.*, 1994; Paré & Tumlinson, 1997). In order to obtain these distinct exposure treatments we carefully timed the infestations and switched source bottles after the first 24h (Fig. 1). Control plants were individually placed in bottles with

connected pure airflow during 48h, at the same time that treated receiver plants were exposed to the fresh or old emissions (1L/min). Immediately after they were used for exposure, the source plants were processed. Leaves were individually cut off at the leaf base, placed on a scaled paper and scanned to measure leaf area and to determine leaf area consumed (Adobe Photoshop). Receiver plants were processed either right at the end of the 48h exposure treatments or later at the end of the VOC collection, depending on the replicate. Control plants were processed directly after the 48h clean air exposure. The exposure treatment procedure was performed five times in five different weeks with five different blocks of plants. Three blocks were dedicated to the gossypol, heliocides, phytohormones and gene expression analysis in receiver plants, the other two blocks were dedicated to the analysis of VOCs emission by receiver plants. VOCs emission by source plants was monitored during different time periods over the five blocks.

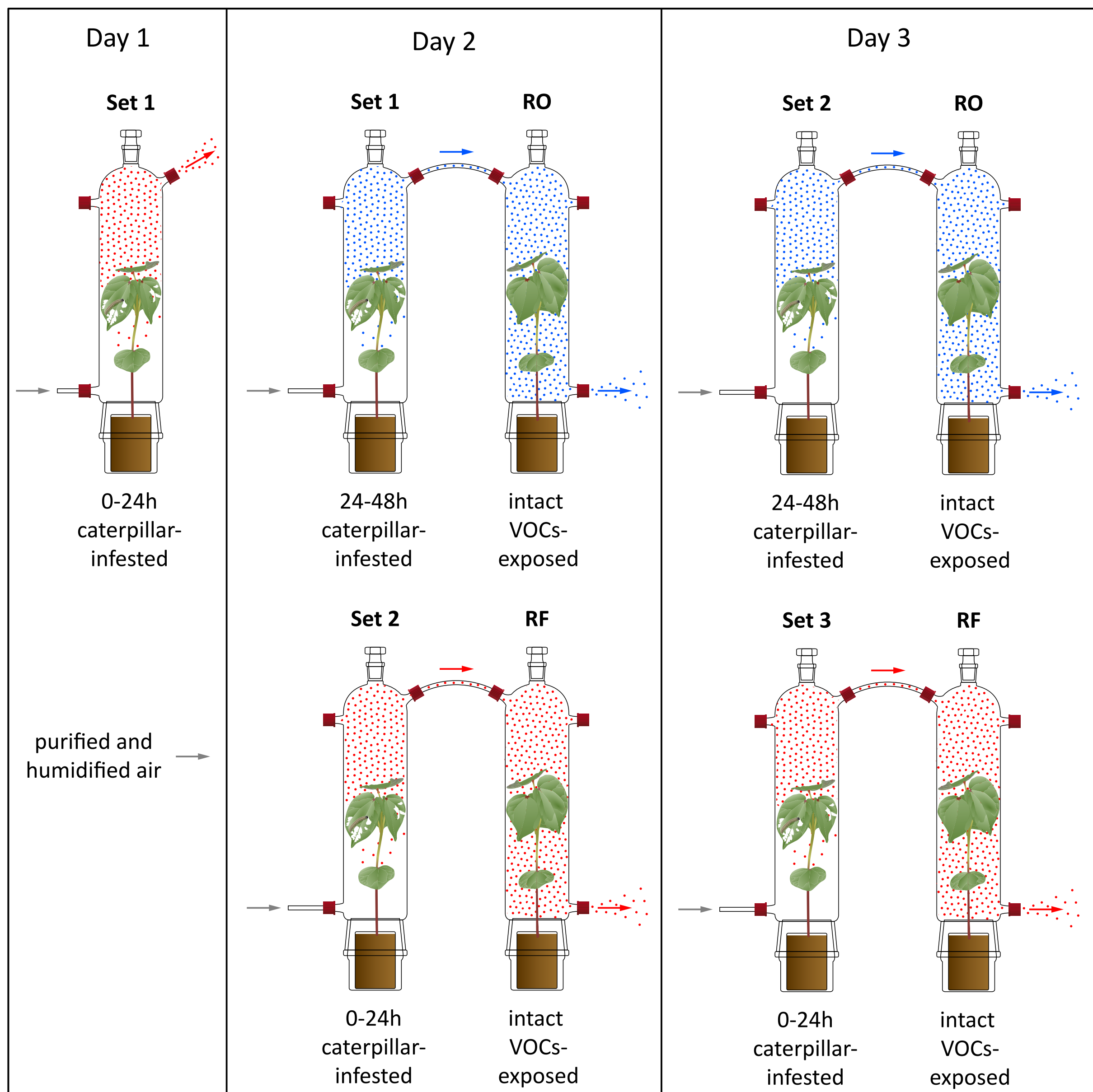

Figure 1: On Day 0 a first set of source plants was placed into glass vessels and infested with ten 2nd instar *S. exigua.* On Day 1 a second set of source plants was placed into glass vessels and infested with ten 2nd instar *S. exigua.* A set of receiver plants were also placed in glass vessels and left un-infested. In each case an airflow of 1L/min was applied to prevent a buildup of volatiles in the vessels. Half of the receiver plants (named RO: "Receiver Old") were connected to the first set of source plants, that had already been infested for 24h (now displaying old damage), the other half (named RF: "Receiver Fresh") were connected to the newly prepared second set of source plants (starting to display fresh damage). Again, an airflow of 1L/min was applied to the connected vessels, and the old and fresh exposure treatments was initiated. On Day 2, the first set of source plants was disconnected and processed. The second set of source plants (now displaying old damage) were connected to RO plants. A third set of source plants was placed into glass bottles, infested with to 2nd instar *S.exigua* and connected to the RF plants, ensuring that these plants were exposed only to volatile blend of plants with fresh damage, during the entire 48h period.

VOCs collection and analysis

The VOCs of source plants were collected at four different timepoints during the 48h exposure treatments in order to provide a picture of VOCs dynamics and to confirm the patterns indicated by Loughrin et al. (1994). VOCs were collected 4 to 6 hours, 22 to 24 hours, 28 to 30 hours and 46 to 48 hours after the start of the infestation over 5 different blocks. VOCs were also collected from receiver plants after the 48h exposure treatment, but only in two blocks where receiver leaf material was not used for the analyses of defense metabolites, gene expression, or in larval choice tests. In those cases, at the end of the exposure treatment, receiver plants were removed from their vessels and left for 30 minutes. The intact receiver plants were then placed in clean glass vessels and VOCs were first collected for 2h in order to determine possible treatment-dependent direct induction of VOCs. Subsequently the plants were infested with 10 *S. exigua* larvae and VOCs were collected for 4 to 6 hr and 22 to 24 hr after the start of the infestation in order to detect possible treatment-dependent priming effects on VOC production. For the initial volatile collections, filters containing 25mg of 80/100 mesh Hayesep-Q adsorbent (Sigma, Switzerland) were connected to the source bottles and the air from the vessels was pulled through each filter for 2h at a rate of 0.7 L/min, while the inflow of purified humid air was set at 1.2 L/min in order to continue deliver air to the neighboring connected receiver plant. Subsequently, filters were eluted with 100µL dichloromethane and samples were added with 10 µL internal standards solution (n-octane and nonyl-acetate, 20 µg/µL each). Samples were stored at -80°C until they were analyzed on a gas chromatograph (Agilent 7890B) coupled to a mass spectrometer detector (Agilent 5977B). A 1.5 µL aliquot of each sample was injected in pulsed spitless mode onto an Agilent HP-5MS column (30 m length x 250 µm diameter and 0.25 µm film thickness). After injection, temperature was maintained at 40°C for 3 min, increased to 100°C at a rate of 8°C per min and subsequently to 200°C at a rate of 5°C per min followed by a post run of 3 min at 250°C. Helium was used as carrier gas and kept at constant flow of 1.1 mL/min. Compounds were subsequently identified by comparing their mass spectra with those from the NIST mass spectral library and comparisons with the analyses of authentic standards. Compounds were quantified based on a comparison of their peak areas with those of the internal standards.

Gossypol and heliocides extraction and analysis

At the end of the 48h exposure treatments, in replicates where receiver plants were not used for VOCs collections, receiver plants and control plants were processed for chemical and

molecular analyses. For each plant, the 3rd true leaf was frozen in liquid nitrogen and stored at -80°C until further processing. The frozen leaves were ground into a fine powder in a mortar filled with liquid nitrogen. Circa 50±5mg of powder were transferred into a frozen 1.5 mL Eppendorf tube. Then, 80 µL acetonitrile and 4-6 glass beads (1.25-1.65 mm diameter) were added to each tube. The mixture was vortexed and then homogenized for 3 minutes at 30 Hz using a beadmill (Retsch MM 300, Haan, Germany). Next, samples were centrifuged for 5 minutes at 14000 rpm. The recovered supernatant was subjected to a second centrifugation step to obtain a totally limpid solution. Finally, the supernatant was recovered and transferred into a 2 mL glass vial (BGB, Germany) for analysis (Glauser *et al.*, 2013), using an Ultra High Performance Liquid Chromatograph (UHPLC) coupled to a Diode Array Detector (DAD) set at 288 ± 2 nm (Ultimate 3000 Dionex, Thermo Fisher Scientific, MA, USA). A 5 µl aliquot of each sample was injected onto an ACQUITY UPLC® BEH C18 column (2.1 x 100mm, 1.7µm) (Waters, MA, USA). The flow rate was held constant at 0.45 mL/min and the temperature was kept at 30°C. The mobile phase A consisted of 0.05% formic acid in MilliQ water (18 Ω) and the mobile phase B of 0.05% formic acid in acetonitrile (HiPerSolv, VWR Chemicals®, France). The following gradient was used: 45-90% B in 8 min, 90-100% B in 0.5 min, holding at 100% for 2.5 min followed by re-equilibration at 45% B for 3.5 min. Gossypol and heliocides (grouped H1 to H3) were identified by their retention time. Quantification was done by calculating a calibration equation obtained by linear regression from six calibration points (5 to 250 µg/mL) in gossypol equivalents. The same leaf materials were also used for gene expression analysis and hormone profiling.

Plant hormone profiling

The level of Jasmonic Acid (JA), Jasmonic Acid-Isoleucine (JA-Ile), Salicylic acid (SA), and abscisic acid (ABA) were measured. The extraction and analysis procedure are fully described in Glauser *et al.* (2014).

Gene expression measurements

Leaf material from the third true leaf was prepared as described above. The thus obtained powder was used for total RNA extraction using the GeneJET Plant Purification Mini Kit (Thermo Scientific) according to the manufacturer's instructions. The complete DNA removal was performed by using the RNase-Free DNase Set (QIAGEN). Each total RNA sample (500 ng) was reverse transcribed using the GoScript™ Reverse Transcription System (Promega).

Real time qPCR was performed on the Rotor-Gene™ 6000 (Corbett Research) using GoTaq® qPCR Master Mix (Promega). *GhACT4* (GenBank accession no. AY305726) was used as an internal standard to normalize cDNA concentrations. The relative expression of twelve defense-related genes was analyzed; the primers used for qPCR and the respective genes are listed in Supporting Information (S.I. Tab. 3). Relative expression levels were calculated using the ΔΔCt method, comparing the relative expression to controls (Schmittgen & Livak, 2008). Three independent analyses on the three blocks were conducted, and results were pooled for ANOVA statistics.

Spodoptera exigua choice test

To determine if larvae of *S. exigua* showed a specific preference for plants from the different exposure treatments, we conducted a choice experiment. At the end of the 48h exposure, leaf discs (diameter: 2.8cm) were cut from the 4th leaf of each treatment plant (i.e. control, fresh and old) and placed together equidistant from each other in five to six petri dishes (diameter: 15cm) depending on plants availability. One *S. exigua* caterpillar (2nd-3rd instar) was placed in the middle of the 3 leaf discs and its movement was monitored. We recorded the first choice (i.e. the first leaf touched) plus hourly timepoints up to five hours after caterpillar release. These results would reveal whether the exposure treatments have a direct effect on caterpillar behavior. We expected to observe a preference for the control treatment due to possible defense induction in HIPV-exposed plants.

Statistical analyses

We used the program R and its complementary console R-studio (http://www.rstudio.com) to compute statistics and create graphs. We performed ANOVA and Tukey Post hoc tests fitting different linear models including block random factor for gossypol, heliocides and phytohormones content, source VOCs emission and gene expression. Log or squared root transformations were applied when necessary to meet normality assumptions on dependent variable distribution. Considering receiver VOCs emissions, we performed t-test on each comparison of each block and collection period. The choice test was analyzed via Fisher's exact test.

Results

Source plant volatiles

Total volatiles emissions by infested source plants continuously increased over the 48h exposure treatment (Fig. 2, ANOVA: $F=33.16$, $P<0.001$). Single Volatiles analysis showed that constitutive compounds such as α-humulene, β-caryophyllene and α-pinene were already emitted right at the start of the infestation (Fig. 3d,e,f), while inducible compounds such as indole , DMNT ((*E*)-4,8–dimethyl–1,3,7-nonatriene) and linalool were virtually absent at the beginning, but their emissions increased considerably with time after initial infestation (Fig. 3a,b,c). Constitutive compounds α-pinene, β-caryophyllene and α-humulene did not show any significant difference in emission for the first 3 collection periods. A table of typical plant volatiles collected from source plants as well as typical chromatograms for each of the collection period are attached as supporting information (S.I. Tab. 1 & S.I. Fig.1).

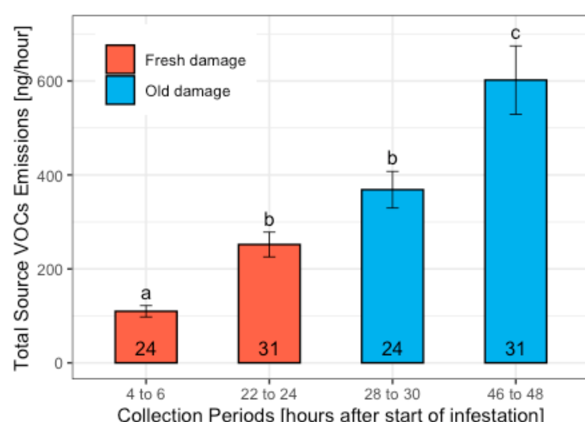

Figure 2: Total volatile emissions (ng/h) (mean ± s.e.) collected from source plants during different periods of the ongoing infestation. At the bottom of the bars are the number of single plant replicates is given. Different letters indicate significant differences (Tukey Post-hoc).

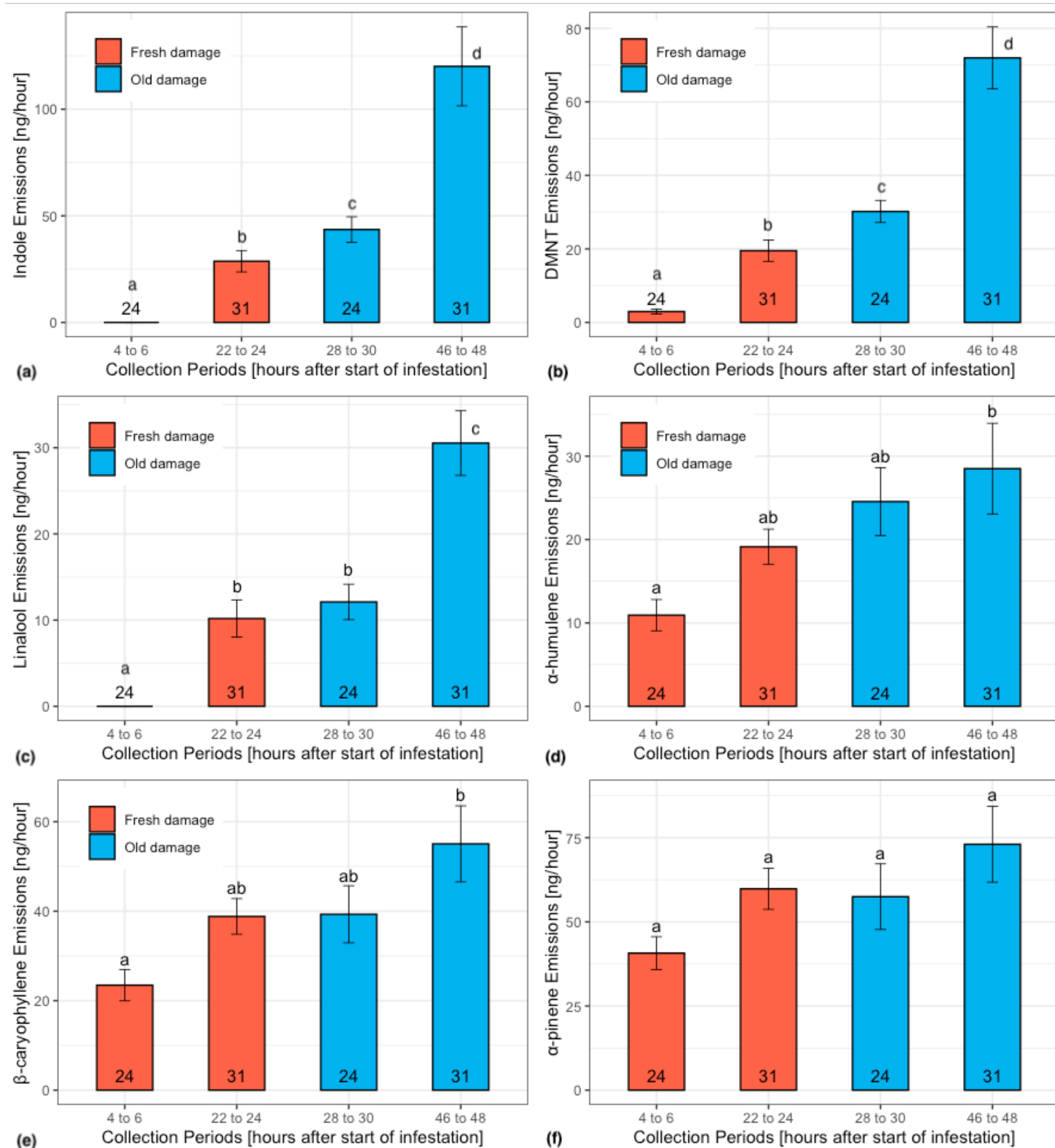

Figure 3: Emission of single volatile compounds (ng/h) (mean ± s.e.) collected during different periods of the ongoing infestation. At the bottom of the bars the number of single plant replicates is given. Different letters indicate significant differences within the same graph. Top four graphs stats were performed even if the data did not pass the Shapiro-Test of normality due to the presence of zeros or few outliers.

Receiver plant volatiles

Total VOCs emission by receiver plants after the exposure treatments was measured in two different replicates, in the first (B1) plants that had been exposed to volatile blend of plants with old damage (i.e. RO) showed a not significant trend of higher total emission rates compared to plants that had been exposed to volatile blend of plants with fresh damage (RF)

(Fig. 4, B1) (t-test; intact: P=0.17, infested 4-6h: P=0.09, infested 22-24h, P=0.59). For the second replicate, this difference was no longer found and the two treatments resulted in similar VOCs emissions by receiver plants (Fig. 4) (t-test; intact: P=0.96, 4-6h: P=0.67, 22-24h, P=0.52).

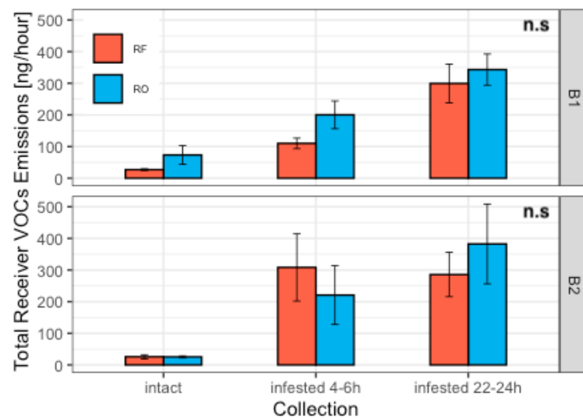

Figure 4: Total volatile emission (ng/h) (mean ± s.e.) collected from differently treated receiver plants (i.e. RF and RO) under different conditions (intact plants, 4 to 6 hours and 22 to 24 hours after the start of the infestation). N=6 for each bar. The measurements were taken from two different blocks of plants (B1 and B2).

Gossypol and heliocides

Gossypol and heliocides levels in the 3rd leaf were similar for all the treatments, and no significant differences were found (ANOVA gossypol: $F=0.77$, $P=0.47$; ANOVA heliocides: $F=0.01$, $P=0.98$) (Fig. 5).

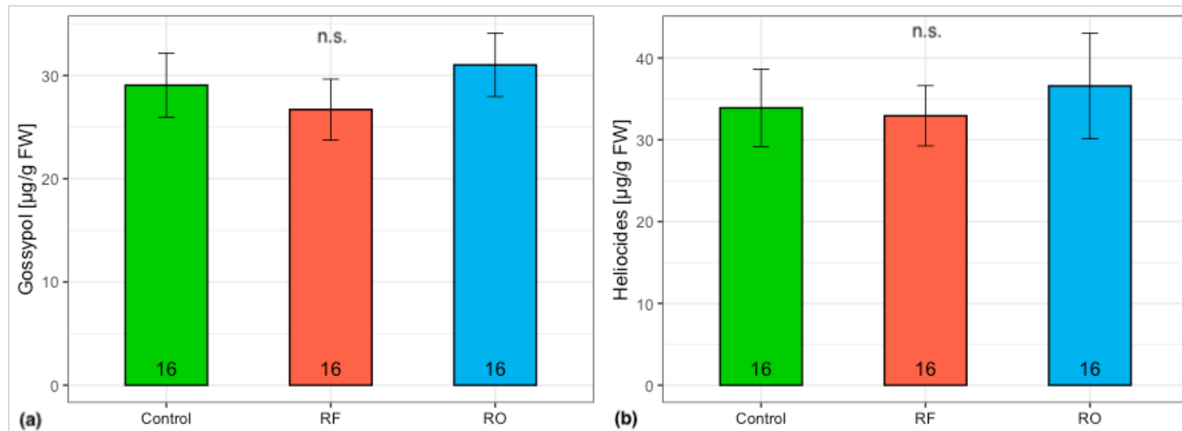

Figure 5: Gossypol and heliocides levels (µg/g fresh weight) (mean ± s.e.) in the 3rd leaf of control plants (green), plants exposed to volatile blend of plants with fresh damage (RF, red) and plants exposed to volatile blend of plants with old damage (RO, blue). The numbers on the bottom of the bars are the number of single plant replicates.

Plant hormone profiling

Levels of jasmonic acid (JA) and JA-Ile were significantly higher in 3rd leaves of plants exposed to volatile blend of plants with fresh damage (RO, blue) as compared to control plants and plants exposed to volatile blend of plants with fresh damage (RF, red) (ANOVA JA: $F=15.24$, $P<0.001$; ANOVA JA-Ile: $F=6.67$, $P<0.01$) (Fig. 6a,b). Salicylic acid (SA) levels showed a minor, non-significant treatment effect (ANOVA: $F=2.11$, $P=0.13$), with control plants having slightly higher levels compared to both RF and RO (Fig. 6c). Abscisic acid (ABA) levels showed no difference among treatments (ANOVA: $F=0.33$, $P=0.72$) (Fig. 6d).

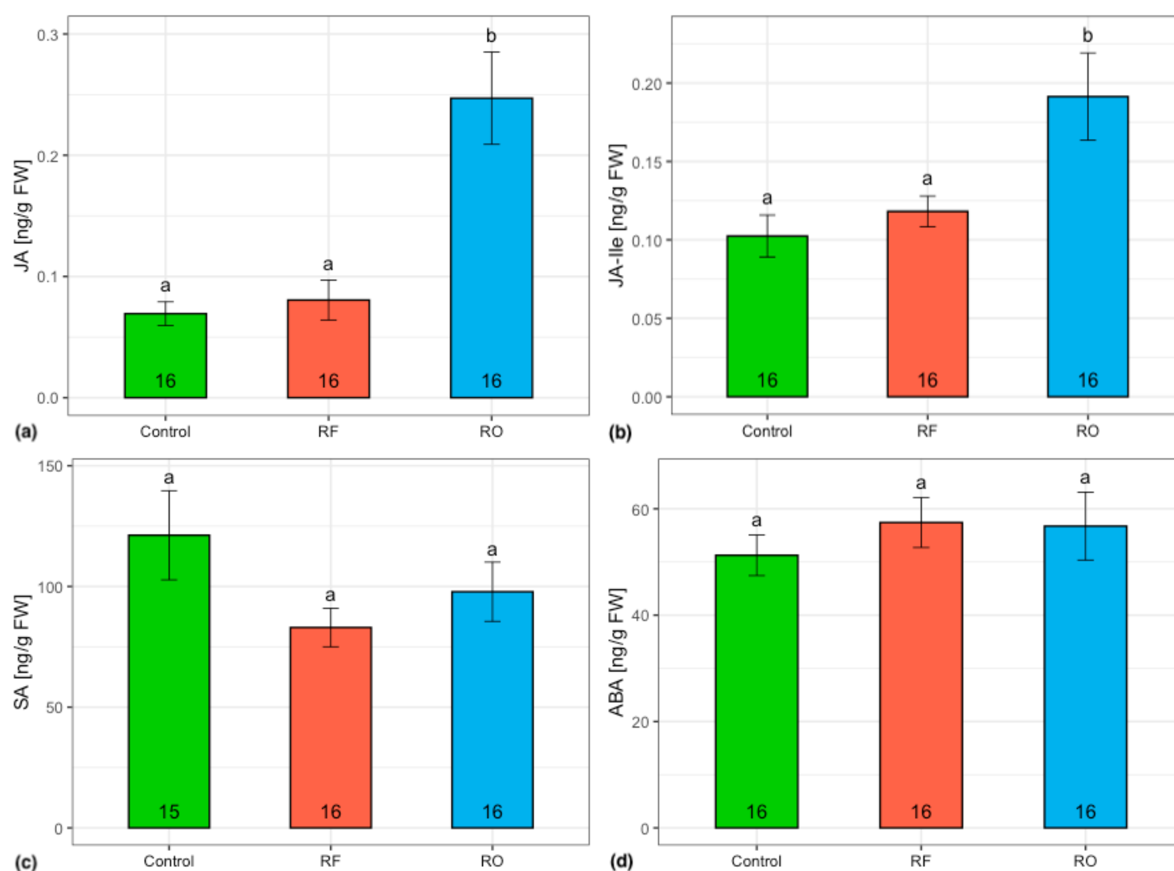

Figure 6: Levels of four major plant hormones (ng/g fresh weight) (mean ± s.e.) (i.e. JA, JA-Ile, SA and ABA) in control plants (green), plants exposed to volatile blend of plants with fresh damage (RF, red) and plants exposed to volatile blend of plants with old damage (RO, blue). At the bottom of the bars the number of single plant replicates is given. Different letters indicate significant differences within the same graph (Tukey post-hoc).

Spodoptera exigua choice test

Spodoptera exigua caterpillars showed a clear pattern of avoiding leaves that had been exposed to the Old VOCs (Fischer's exact test: P=0.001). Indeed, two hours after the start of this preference test, none of the larvae were found on the Old-exposed leaves (Fig. 7).

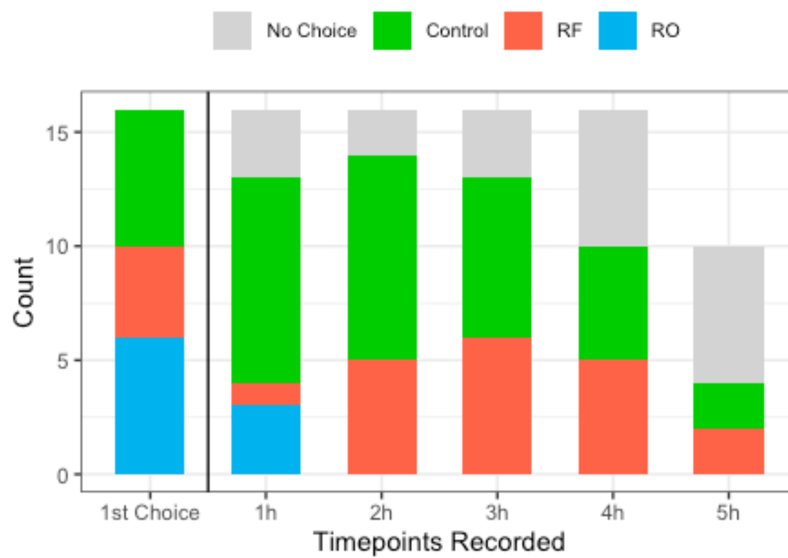

Figure 7: Number of caterpillars in touch with the specific leaves at a given time point over 5 hours among three treatments: Control (green), RF (red), and RO (blue). The results of 3 different blocks were pooled (N=16). In one replicate we did not take the "5h" timepoint.

Gene expression measurements

The expression of three different genes (i.e. *Cad1A*, *Cdn1C3* and *CYP706b*) was significantly upregulated in plants that had been exposed to volatile blend of plants with old damage (i.e. RO) compared to control plants and plants exposed to volatile blend of plants with fresh damage (i.e. RF) (ANOVA *Cad1A*: $F=5.9$ $P=0.005$; ANOVA *Cdn1C3*: $F=8.61$, $P<0.001$; ANOVA *CYP706b*: $F=4.57$, $P=0.016$) (Fig. 8a,b,c). Two other genes that are implicated in VOC production (i.e. *GhCYP82L1* and *GhCYP82L2*) tended to be more expressed VOC-exposed plants compared to control plants (Fig. 8d,e), whereby the expression of *GhCYP82L2* was significantly higher in RO plants when compared to controls (ANOVA: $F=4.07$, $P=0.024$). Another gene related to VOC production (*GhTPS14*) was suppressed by both exposure treatments compared to controls (ANOVA: $F=7.34$, $P=0.0017$) (Fig. 8f). Genes related to plant hormone production did not show any difference among treatments (figures not shown). The relative expression data for all the tested genes are presented in the supporting information (S.I. Tab. 2)

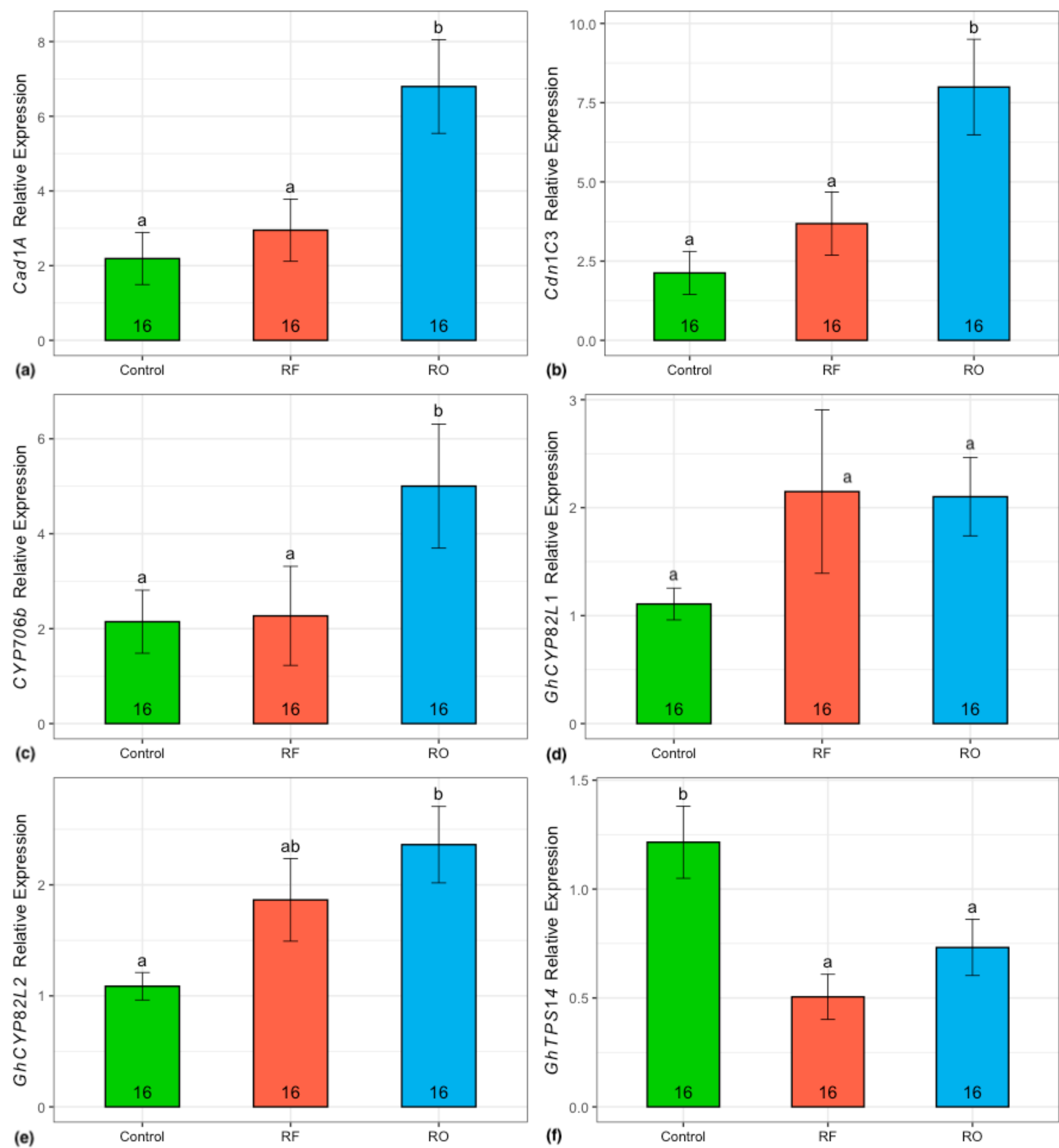

Figure 8: Relative expression of multiple defense-related genes (mean ± s.e.) in the third leaf of control plants (green), plants exposed to volatile blend of plants with fresh damage (RF, red) and plants exposed to volatile blend of plants with old damage (RO, blue). At the bottom of the bars the number of single plant replicates is given. Different letters indicate significant differences within the same graph (Tukey post-hoc). Results from three independent experiments were pooled.

Discussion

The objective of this study was to determine the relative importance of constitutive and inducible volatiles that are released by cotton in response to caterpillar feeding for defense induction in neighboring plants. Since the inducible compounds are produced and emitted in higher amounts in response to continuous insect attack (Loughrin *et al.*, 1994; Paré & Tumlinson, 1997) and are reliable indicators of occurring damage, we hypothesized that these volatiles are of key importance in the process of neighbor induction. In order to test our hypothesis, we exposed plants to groups of mainly constitutive or mainly inducible volatiles and we measured defense-related physiological changes in receiver plants. Most of the results suggest that, in accordance with our hypothesis, inducible VOCs play a more important role as alarm signals that alert cotton plants to incoming attack than constitutive VOCs.

The gossypol and heliocides analyses did not show any particular difference among RO, RF and control plants (Fig. 5). This result contradicts what was previously found and reported in Chapter 1: VOCs exposed plants had higher levels of these defense compounds compared to controls. On the other hand, the expression of three genes related to gossypol biosynthesis (i.e. *Cad1A*, *Cdn1C3*, *CYP706b*) was significantly upregulated in RO plants compared to RF and control plants (Fig. 8), similarly as found in Chapter 1, where these genes were also upregulated in VOCs exposed plant. Interestingly, Zebelo *et al.* (2017) showed that the exact same three genes are upregulated when *Spodoptera exigua* caterpillars feed directly on cotton. In our case, the mere exposure to volatiles of plants with old damage is enough to elicit a defense response similar to what is observed for directly infested plants. This implies that these volatiles are perceived by the receiver plant and play a key role in alerting them to the risk of herbivore attack. This perception apparently elicits the gene expression process, and it can be expected that the production of gossypol and heliocides increases, as was observed by Zebelo *et al.* (2017) for caterpillar-infested plants. The production is probably mainly in newly grown tissue (Chapter 1, Eisenring *et al.* ,2017), and would therefore not be detected in the third leaf. It might have been more appropriate to use the newly growing fourth leaf for the analyses, but we decided to reserve these for the caterpillar preference assays.

The direct defense induction was more evident from the hormone profiling analysis, which showed that that the third leaf of RO plants had significantly higher levels of JA and JA-Ile compared to RF and controls (Fig. 6a,b). JA is the main mediator of plant-chewing-herbivore interaction and is generally produced when plants are under attack by chewing insects or have been exposed to HIPVs (Browse, 2005; Dicke & Baldwin, 2010; Erb, 2018).

Clearly, the exposure to volatiles of plants with old damage increases JA and JA-Ile levels, whereas exposure to volatiles of plants with fresh damage does not.

The level of SA was higher in control plants compared to treated plants (Fig. 6c), confirming the crosstalk between the JA and SA signaling (Thaler *et al.*, 2012) . The expression of six genes related to JA and SA synthesis (three each) showed high variation and no clear trends (Tab. 2, supporting information). JA production related genes (i.e. *GhLOX1*, *GhAOS* and *GhOPR3)* are usually upregulated upon *Spodoptera exigua* damage (Zebelo *et al.*, 2016, 2017). On the other hand, SA production related genes are generally upregulated upon piercing-sucking damage or fungal infection and may be downregulated by crosstalk (Thaler *et al.*, 2012; Lv *et al.*, 2017; Miao *et al.*, 2019).

The analysis of VOCs emitted by intact and infested receiver plants gave different trends for the two replicates that were performed (Fig. 4). In the first replicate, RO plants generally emitted higher average quantities of VOCs compared to RF, whereas in the second replicate this trend was no longer present and the emissions were even. In this experiment, due to logistical constraints, we could not collect volatiles from intact and infested control plants at the same time as RO and RF plants and therefore no conclusions about direct induction or priming compared to control can be drawn. In case this will be tested in future we might expect to collect less volatiles from intact control plants than from intact RO plants (Chapter 1). Indeed, in Chapter 1, we found increased total volatile emissions by intact HIPV-exposed plants compared to controls. Interestingly, single compound analyses showed that this result was mainly due to inducible volatiles (i.e. DMNT and TMTT) that are usually released by cotton only several hours of ongoing caterpillar damage, as opposed to several green leafy volatiles and cyclic terpenoids that are released immediately upon damage (Loughrin *et al.*, 1994; McCall *et al.*, 1994). Furthermore, when HIPVs-exposed plants were subjected to caterpillar attack themselves, we no longer found a difference in VOC emissions between HIPV-exposed plants and control plants (Chapter 1). These results imply that in cotton HIPV exposure does not prime, but directly induce receiver plants.

The expression of genes related to VOC production showed some contrasting results, with *GhCYP82L1* and *GhCYP82L2* upregulated (Fig. 8d,e), while *GhTPS14* downregulated in treated plants compared to control plants (Fig. 8f). Interestingly, *GhCYP82L* genes, which are responsible for the production of (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), are also found to be upregulated in cotton plants that are attacked by *Helicoverpa armigera* caterpillars (Liu *et al.*, 2017). On the other

hand, no specific behavior has been described for GhTPS14, a gene related to the production of ocimene and linalool (Huang *et al.*, 2018).

In the choice test experiment, where caterpillars were offered the youngest (fourth) leaf of control, RF and RO plants, RO was clearly avoided by *S. exigua* (Fig.7). This result nicely confirms that plants that had been exposed during 48 hours to mainly inducible volatiles are better defended against these herbivores. This result suggests that under natural conditions, even if the adult moth will still lay eggs against odds on the exposed plant (Zakir *et al.*, 2013b,a), the larvae may not "like" the plant or perform well on it, and may eventually leave the plant or die. This hypothesis as well as oviposition behavior were not tested in this chapter but should be tested in a separate experiment.

In this study we compared defense responses of unexposed control plants with those of plants that were exposed to the volatiles that are emitted by plants under caterpillar attack, either emitted during the first 24 hours or emitted during the second 24 hours of infestation. It was confirmed that the two blends strongly differ; during the first 24 hours plants emitted mainly constitutive stored volatiles, while in the second 24 hours mainly inducible compounds were released (Fig. 3 & Tab. 1 supporting information), as has been reported by (Loughrin *et al.*, 1994; McCall *et al.*, 1994; Paré & Tumlinson, 1997). These results strongly support our hypothesis that the volatiles that are *de novo* synthesized in response to caterpillar feeding are key for the observed interactions among cotton plants (Chapter 1). However, we cannot exclude that some of the constitutive VOCs also played a role, as they are also part of the blend emitted by the plants with older damage (Fig. 3d,e,f & Tab.1, supporting information). Tests with synthetic versions of the different VOCs may possibly reveal the identity of the most important volatile compounds. Another constraint of the current experiments is that RO plants were exposed to larger total amounts of VOCs compared to RF (Fig. 2) and therefore we cannot exclude that the observed results were due to differences in quantity rather than quality of the two volatile blends. In the next chapter (i.e. 4), we used authentic standards (highly pure synthetic compounds) of some of the volatiles within each group in order to try to answer some of the remaining questions.

From the current study we can conclude that the inducible volatiles emitted by caterpillar-infested cotton plants indeed play a major role in the activation of different defense mechanisms in neighboring plants. The effect seems stronger than has been observed for other plants (Erb *et al.*, 2015), as it is not merely priming, whereby exposed plants merely respond stronger when they themselves are attacked (Conrath *et al.*, 2006; Ton *et al.*, 2006; Martinez-Medina *et al.*, 2016), but rather true induction, immediately enhancing resistance. This strong

effect on neighboring plants of volatiles emitted from damaged cotton plants explains the field observation of increased resistance against cotton pests after plant training (Renou *et al.*, 2011; Téréta, 2015; Llandres *et al.*, 2018). The eventual identification of the most bioactive VOCs may lead to their application as part of a novel integrated pest management strategy in cotton cultivation to induce defense in cotton plants.

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Supporting information

Table 1. Average emissions (± standard error) of volatile organic compounds by source plants at different periods of the caterpillar infestation.

Source VOCs [ng/hour]	**Collection Periods (hours after start of infestation)**			
	4 to 6	**22 to 24**	**28 to 30**	**46 to 48**
2-Hexenal	0.34 ± 0.24	1.9 ± 0.37	3.3 ± 0.74	5.64 ± 1.04
Z-3-hexenol	0.83 ± 0.39	5.14 ± 0.9	9.53 ± 1.73	7.27 ± 1.33
α-pinene	40.7 ± 4.86	59.8 ± 6.1	57.48 ± 9.76	73.04 ± 11.25
Benzaldehyde	22.55 ± 1.71	10.31 ± 0.92	9.92 ± 0.72	9.56 ± 1.06
β-pinene	5.14 ± 0.73	8.19 ± 0.88	7.99 ± 1.49	10.26 ± 1.61
β-myrcene	3.4 ± 0.92	7.9 ± 1.24	8.76 ± 2.05	11.25 ± 1.97
Z-3-Hexenylacetate	0.38 ± 0.27	12.14 ± 2.09	47.84 ± 7.66	22.94 ± 3.58
β-ocimene	0 ± 0	28.29 ± 6.74	45.43 ± 5.8	64.02 ± 7.75
Linalool	0 ± 0	10.18 ± 2.16	12.11 ± 2.06	30.54 ± 3.75
DMNT	2.97 ± 0.68	19.51 ± 2.89	30.19 ± 3	71.97 ± 8.43
Indole	0 ± 0	28.72 ± 4.98	43.59 ± 5.97	120.09 ± 18.51
β-caryophyllene	23.46 ± 3.5	38.83 ± 3.99	39.31 ± 6.38	55.05 ± 8.5
α-guaiene	0.12 ± 0.12	0.84 ± 0.37	0.98 ± 0.65	1.89 ± 0.97
β-farnesene	0 ± 0	0 ± 0	0 ± 0	22.62 ± 3.56
α-humulene	5.47 ± 0.94	9.56 ± 1.06	12.28 ± 2.03	14.26 ± 2.72
α-farnesene	0 ± 0	1.83 ± 0.5	25.18 ± 4.79	3.81 ± 0.99
γ-bisabolene	3.85 ± 0.97	7.52 ± 1.26	10.43 ± 2.38	10.03 ± 2.3
TMTT	0.61 ± 0.44	1.25 ± 0.34	4.23 ± 1.61	67.67 ± 23.3
Total	109.83 ± 12.06	251.92 ± 26.29	368.55 ± 39.01	601.88 ± 72.77

Table 2. Average relative expression ± standard error of several genes in differently treated plants

Gene	Control	RF	RO
GhLOX1	1.2 ± 0.21	0.81 ± 0.13	0.89 ± 0.08
GhAOS	1.16 ± 0.16	1.21 ± 0.21	1.36 ± 0.16
GhPR3	1.18 ± 0.17	1.22 ± 0.27	1.55 ± 0.23
GhPAL	1.11 ± 0.15	1.62 ± 0.48	2.06 ± 0.42
ICS1	1.14 ± 0.17	1.09 ± 0.1	1.13 ± 0.09
EDS1	1.12 ± 0.13	0.94 ± 0.14	1.32 ± 0.16
GhCYP82L1	1.11 ± 0.15	2.15 ± 0.76	2.1 ± 0.36
GhCYP82L2	1.09 ± 0.12	1.86 ± 0.37	2.36 ± 0.34
GhTPS14	1.22 ± 0.17	0.5 ± 0.1	0.73 ± 0.13
Cad1A	2.19 ± 0.7	2.95 ± 0.83	6.8 ± 1.25
Cdn1C3	2.13 ± 0.68	3.68 ± 1	7.99 ± 1.51
CYP706B1	2.15 ± 0.66	2.27 ± 1.04	5 ± 1.31

Table 3. Gene information.

Gene ID	GeneBank Acc. No.	Gene Family	Function	Forward Primer Sequence	Reverse Primer Sequence	Reference
GhLOX1	AF361893	Lipoxygenase	JA biosynthesis	GCCAAGGAGAGCTTCAAGAA	TAGGGGTACTTGGCAGAACCT	Zebelo *et al*. 2016
GhAOS	KM265129	Allene oxide synthase	JA biosynthesis	ATCATGTAATCCCCGAGTTCC	CCAGCTTGATCGTTAGCTGTC	Zebelo *et al*. 2016
GhOPR3	FB505932	12-oxo-phytodienoic acid reductase	JA biosynthesis	ATGTGACGCAACCTCGTTATC	CCGCCACTACACATGAAAGTT	Zebelo *et al*. 2016
GhPAL	JN032297	phenylalanine ammonia-lyase	SA biosynthesis	CGAGGAACAAAGCATTACAT	GTGGGAGACCGTTGTTGTAG	Lv *et al*. 2017
ICS1	PPS00849	isochorismate synthase	SA biosynthesis	GTCTTCAGCCACCTAATGGAC	GCTCTGGATTCACCTCTAGCAC	Miao *et al*. 2019
EDS1	JQ766941	enhanced disease susceptibility 1	SA biosynthesis	GCAGCAACAGCTCCTCTACCT	GGCAGACCAAGACGCTACAGA	Miao *et al*. 2019
GhCYP82L1	KY247144	Cytochrome P450; CYP82L subfamily	homoterpene (DMNT and TMTT) biosynthesis	ATTCTCTGGTAACGAGTT	AACACTGATTACTGAGTC	Liu et al. 2017
GhCYP82L2	KY247145	Cytochrome P450; CYP82L subfamily	homoterpene (DMNT and TMTT) biosynthesis	ATTCTCCGGTAACGATTA	AACATTGATTATTGAGTCGA	Liu et al. 2017
GhTPS14	KX963376	Terpene Synthase	production of DMNT, TMTT, (E)-β-ocimene and linalool	GAACTGACCACCCTCACTCA	CGCCTCCACAAACCATGAAT	Huang et al. 2017
Cad1-A	Y18484	(+)-δ-cadinene synthase (CDNS; referred to as CAD); CAD1-A subfamily	Gossypol biosynthesis	ATAAGGATGAAATGCGTCC	GAAGCTTGGTAAAGTTCCA	Zebelo et al. 2017
Cdn1-C3	AF174294	(+)-δ-cadinene synthase (CDNS; referred to as CAD); CAD1-C subfamily	Gossypol biosynthesis	AACTCAAAAACGCCACCAAC	TAGTCGGAATCGAAGGGATG	Zebelo et al. 2017
CYP706B1	AF332974	Cytochrome P450 monooxygenase	Gossypol biosynthesis	GCAAGCCAATTGATTTTGGT	GCACCGGGAAAATATCAGAA	Zebelo et al. 2017
Histone (H3)	AF024716	histone (H3) ribosomal mRNA	Housekeeping Gene	GAAGCCTCATCGATACCGT	CTACCACTACCATCATGGC	Zebelo *et al*. 2016

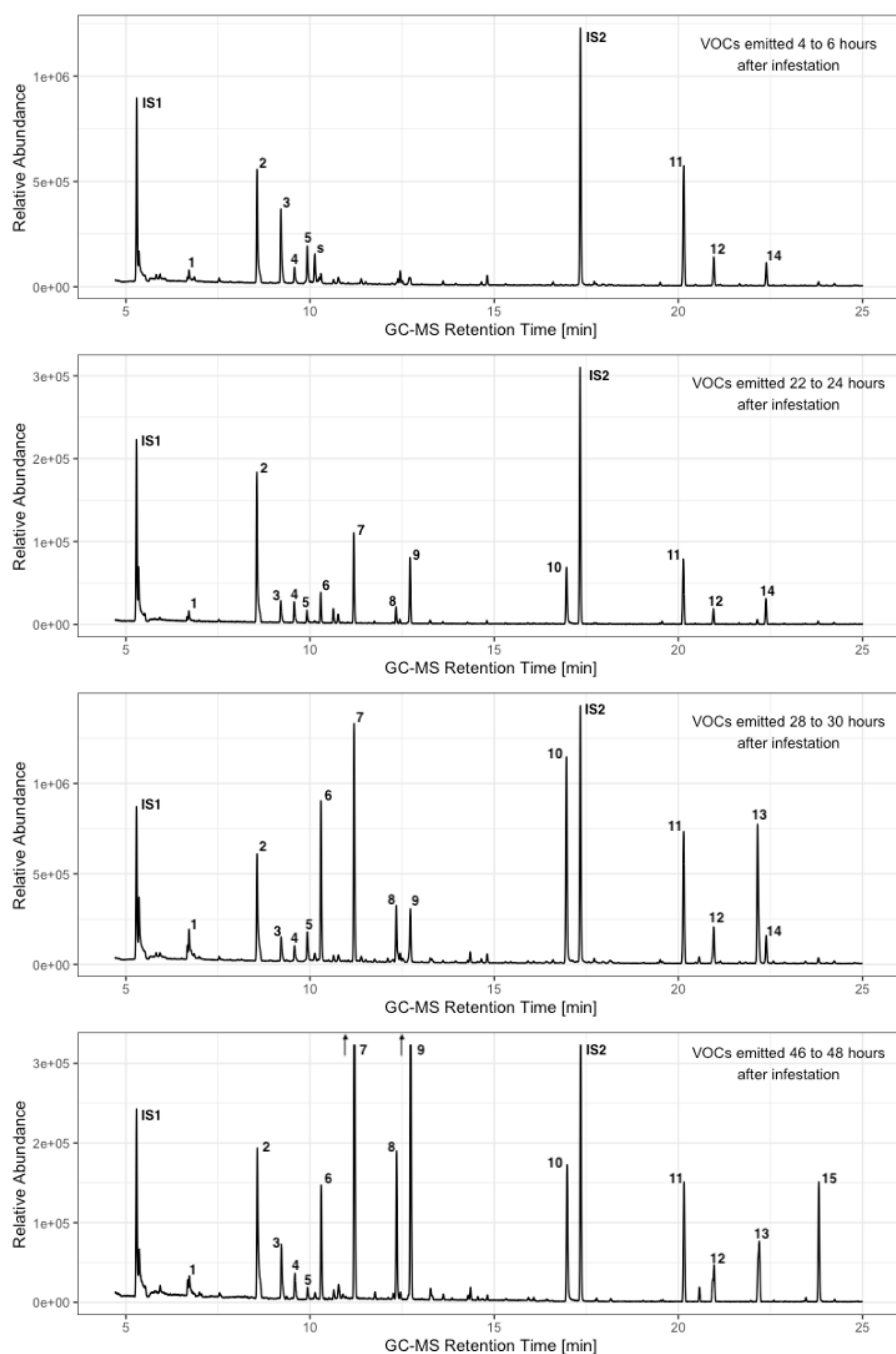

Figure S1. Typical chromatograms of different *S. exigua*-infested cotton plants collected during different time periods. IS1: n-octan, 1: (Z)-3-hexenol, 2: α-pinene, 3: benzaldehyde, 4: β-pinene, 5: β- myrcene, 6: (Z)-3-hexenylacetate, 7: β-ocimene, 8: linalool, 9: DMNT, 10: indole, IS2: nonyl-acetate, 11: β-caryophyllene, 12: humulene, 13: α-farnesene, 14: γ-bisabolene, 15: TMTT. In the last chromatogram peak 7 and peak 9 were higher than IS2 (↑) but were cut at the maximal abundance of IS2 in order to visually compare all four chromatograms.

Chapter 4: Which single or group of typical constitutive or inducible cotton volatiles trigger defenses in neighboring cotton plants?

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Abstract

Research rationale: In our previous studies we documented how herbivore-induced cotton volatiles induce physiological changes in intact exposed plants, resulting in increased defenses. Here we address the question of which volatiles are responsible for these changes, distinguishing between volatiles that are immediately released upon damage and truly inducible volatiles that are *de novo* synthesized and only emitted after at least one day of caterpillar feeding. To test this, we exposed intact cotton plants to different combinations of synthetic VOCs, that represent each of these two groups, and subsequently measured plant defense responses.

Methods: Intact cotton plants were exposed for 48h either to three constitutive VOCs (α-pinene, (*Z*)-3-hexenol, β-caryophyllene), three inducible VOCs (indole, (*E*)-4,8-dimethyl-1,3,7-nonatriene and linalool), or the full blend of all six compounds. Defense gene expression, concentrations of cotton defense compounds (gossypol and heliocides), and defense hormones levels were measured in the leaves of the differently treated plants and compared to control unexposed plants. In addition, some other treatment combinations, including exposures to single compounds, were performed.

Key results: No significant differences were found among all tested combinations. We observed some trends that still did not allow for the recognition of a clear pattern.

Main conclusion: In spite of the various experiments we could not determine single or groups of volatiles responsible for defense induction in neighboring cotton plants. We hypothesize that plants may require a full natural blend, a specific ratio or other non-tested compounds in order to directly induce defenses in neighboring plants.

Introduction

Treatments with chemical products have long been used to reduce crop damage and increase yield. Usually synthetic pesticides are applied, but they bring along health and environmental risks, and it is increasingly evident that sustainable alternatives are needed (Bhat *et al.*, 2019). This is particularly true for cotton, which is considered to be the "world's dirtiest crop", responsible for almost 25% of all pesticide use (Naranjo & Luttrell, 2008). The application of natural elicitors (Sobhy *et al.*, 2014) including volatile organic compounds (VOCS) (Turlings & Erb, 2018) figures among the alternatives being considered. Indeed VOCs, besides playing an important role in direct defense by repelling herbivores and in indirect defense of plants by attracting natural enemies of herbivores (Turlings & Wäckers, 2004; Zakir *et al.*, 2013b), have been shown to induce or prime the defenses of intact receiver plants (Heil & Karban, 2010; Ton *et al.*, 2006; Erb *et al.*, 2015). This appears to be particularly effective in cotton (Chapter 1; Zakir *et al.*, 2013a). Interestingly, some of these VOCs have been shown to singularly affect the defenses of exposed plants. One of the first observation of VOC-mediated interactions among plants was reported for cotton plants by Zeringue (1987). He found increased production of natural insecticidal terpenoid aldehydes (i.e. heliocides) in the leaves of myrcene-exposed plants. The same author also found that exposure to *trans*-2-hexenal can increase phytoalexins levels in wounded developing cotton bolls (Zeringue, 1992). Another active VOC is cis-jasmone that, when sprayed on intact bean plants, directly induces emissions of several VOCs including β-ocimene, which is involved in plant-parasitoid interaction (Birkett *et al.*, 2000). Interestingly, β-ocimene, as well as other inducible VOCs, namely (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), are singularly able to induce the expression of defensive genes (e.g. LOX, PAL and FSP) in lima beans (Arimura *et al.*, 2000). One of the most studied natural elicitor of VOC emissions is methyl-jasmonate (MeJA) (Farmer & Ryan, 1990; Rodriguez-Saona *et al.*, 2001; Preston *et al.*, 2001). For example, MeJA was found, similarly to *cis*-jasmone, to increase emissions of inducible terpenoids (e.g. β-ocimene, DMNT, TMTT and linalool) when sprayed on cotton plants (Rodriguez-Saona *et al.*, 2001). More recently Erb *et al.* (2015) described the key priming role of the aromatic inducible VOC indole in maize plants resulting in the production of stress hormones (i.e. jasmonate-isoleucine and abscisic acid).

All the above-mentioned volatile elicitors mainly target the jasmonic acid (JA) signaling pathway and are mostly involved in the defense against chewing herbivores. There are also elicitors that can specifically target the salicylic acid (SA) signaling pathway, which is involved in the defense against biotrophic pathogens and phloem-feeding insects (Thaler *et al.*, 2012). For instance, Shulaev *et al.* (1997) and Yi *et al.* (2009) showed how exposure to methyl-salicylate (Me-SA) and nonanal, respectively, can increase the expression of pathogenesis-related proteins (PRs) and increase plant resistance. The SA signaling pathway is also been targeted with synthetic elicitors (e.g. benzothiazole) developed by agro-tech firms (Sadras & Felton, 2010). To sum up, application of single natural or synthetic VOCs or blends of several VOCs have clear potential in plant protection (Kost & Heil, 2006).

In cotton (*Gossypium ssp.*), which is the plant model of this study, several observations of the putative or confirmed eliciting roles of VOCs have been reported (Zeringue, 1987, 1992; Bruin *et al.*, 1992; Zakir *et al.*, 2013b; Hagenbucher *et al.*, 2013; Llandres *et al.*, 2018, Chapter 1 and Chapter 3). Bruin *et al.* (1992) found that, when infested by herbivorous mites, cotton seedlings produce VOCs that attract predatory mites. The same study also reported on decreased oviposition by herbivorous mites on uninfested cotton plants that had been exposed to the VOCs from infested neighboring plants. Two further studies also found that cotton VOCs can interfere with oviposition behavior of a generalist herbivorous insect (i.e. *Spodoptera littoralis*). Hagenbucher *et al.*, (2016) observed that wild ancestral cotton *G. raimondii* provided associational resistance to domesticated *G. hirsutum*, resulting in lower oviposition on plants in groups that contained a mix of the two species as compared to groups with only domesticated *G. hirsutum* plants. Importantly, Zakir *et al.* (2013b) showed a significant reduction in oviposition by S. *littoralis* moths on undamaged plants that had been placed nearby herbivore-damaged cotton plants under both field and laboratory conditions. Each of these studies highlights the potential of VOCs in protecting neighboring plants; however, none of them address the responsible mechanism and/or compounds behind the observed effects. In previous experiments (Chapter 1 and Chapter 3) we specifically addressed this knowledge gap and observed that HIPVs can actually shape the physiology of intact receiver plants by activating defense responses. Furthermore, we found that the volatile blend of plants with older caterpillar damage, which is mainly composed by inducible volatiles is more potent than volatiles from fresh caterpillar damage in inducing responses in neighboring plants. This was reflected in higher production of JA and JA-Ile, stronger activation of gossypol related genes and sometimes higher VOCs emissions by exposed plants (Chapter 3). With this knowledge in mind, we here conducted a series of experiments to try to identify key VOCs that are

responsible for the changes in exposed plants, and to determine if the truly inducible VOCs are indeed the exclusive actors in this process.

In order to expose plants to controlled amounts of VOCs we opted for small vial dispensers recently used in several studies (Mérey *et al.*, 2011; Robert *et al.*, 2012; Hiltpold & Shriver, 2018; Hu *et al.*, 2019). Using this method, Mérey *et al.* (2011) could show that when synthetic GLVs are released in maize fields, the emission of sesquiterpenes by exposed plants increase. Hiltpold *et al.*, (2010) and Robert *et al.* (2012) demonstrated that dispensers also work below ground. The latter study showed that β-caryophyllene attracts corn rootworm larvae in a dose-dependent manner. More recently, Hiltpold & Shriver (2018) used these dispersers to demonstrate that birds may use HIPVs for prey location. Similar dispensers were also used to reveal the specific role of indole in the interactions among maize plants (Erb et al., 2015), as confirmed by Hu *et al.* (2019), who investigated how maize plants respond to two synthetic HIPV (i.e. *Z*-3-hexenylacetate, indole), again using dispensers. They found that *Z*-3-hexenyl-acetate directly induces gene expression, whereas *Z*-3-hexenylacetate and indole both together increased JA signaling, defense gene expression, production of defensive secondary metabolites and plant resistance when combined with simulated herbivory. Interestingly, the combined exposure to the two HIPVs was considerably more efficient.

In the current study we wished to determine which HIPVs play such key roles in the interactions among cotton plants. For our experiments we employed individual dispensers and combined them in three different groups based on their characteristics. The *constitutive* group, representing compounds that are immediately emitted when a cotton plant is damaged, consisted of (*Z*)-3-hexenol, α-pinene and β-caryophyllene. The *inducible* group, representing truly inducible compounds that are only emitted when cotton plants have been damaged for a longer period of time, was composed of linalool, DMNT and indole. The *mix* group included all six of the mentioned VOCs. These three different combinations of dispensers were used to expose intact cotton plants. Control plants were exposed to clean air. With these experiments we further tested our hypothesis, already stated in Chapter 3, that the truly inducible HIPVs are essential for the induction of enhanced defenses in exposed plants. The knowledge thus gained, could be a major step forward towards our ultimate goal of applying potent natural elicitors as part of an integrated pest management strategy in cotton.

Materials and methods

Plant material

Cotton seeds (*Gossypium hirsutum,* variety STAM59A) were soaked in water for 24h at 27°C and subsequently sown in plastic trays containing commercial soil (Profi Substrat Soil, Einheitserde, Germany) until germination. Germinated seeds were then transferred to plastic pots (height: 8.5cm; diameter: 6cm) filled with the above-mentioned commercial soil. Plants were grown in phytotrons (Growbanks, 28°C, 16h:8h light:dark) and watered every other day. Healthy plants with four true leaves were used for the experiments.

Dispensers

Several combinations of vials, capillary and VOCs at different concentrations were tested and selected (Tab. 1) ahead of the main experiments in order to try to reproduce dispenser release rates comparable to rates of caterpillar-infested plants (Tab. 2). The method was inspired by studies using similar dispensers (Mérey *et al.*, 2011; Robert *et al.*, 2012; Hu *et al.*, 2019). The definitive dispenser compositions are described in table 1. To prepare a VOC dispenser, glass wool was first heated in oven at 200°C for 3 hours in order to evaporate possible contaminants, and then 100mg was placed in an amber vial. Then, 2µL of a particular liquid VOC were dropped on the glass wool in the vial, or 20mg in case of solid indole, were directly placed in a vial without glass wool. Vials were then sealed with crimp or screw caps containing a rubber, Teflon-coated septum. A little hole was poked into the septum with a needle and a specific capillary was inserted through the hole, while avoiding contact with the glass wool. Furthermore, the dispensers were wrapped in parafilm in order to prevent possible leaks.

Table 1. Single VOC dispensers. _Definitions:_ DMNT: (E)-4,8-dimethyl-1,3,7-nonatriene. BGB: Amber vials (1.5mL, #080401-W 8-425) and screw caps (#070301 8-425) by BGB. Agilent: Amber vials (2mL, #5181-3376) and crimp caps (#5181-1210) by Agilent. TSP: Deactivated GCMS column, TSP FS-Tubing (P/M), 100µm ID, 375µm OD). Drummond: glass microcapillary by Sigma-Aldrich.

VOC	**Producer**	**Vial & Cap**	**Glass Wool**	**Capillary**	**Quantity**	**Purity %**
(*Z*)-3-hexenol	Sigma-Aldrich	Agilent, crimp cap	100mg	TSP (32mm)	2µL (50% in paraffin oil)	98.6
α-pinene	Sigma-Aldrich	Agilent, crimp cap	100mg	TSP (64mm)	2µL (10% in paraffin oil)	98.8
β-caryophyllene	Sigma-Aldrich	BGB, screw cap	100mg	Drummond (2µL, 32mm)	2µL (pure)	98.9
linalool	Sigma-Aldrich	Agilent, crimp cap	100mg	TSP (64mm)	2µL (pure)	97.5
DMNT	TRC Canada	Agilent, crimp cap	100mg	TSP (64mm)	2µL (pure)	100
indole	Sigma-Aldrich	BGB, screw cap	none	Drummond (2µL, 32mm)	20mg (powder)	100

At least three days after assembling the dispensers, their emissions were tested by placing them into glass vessels, trapping VOCs with Hayesep-Q filters (inflow: 1L/min, outflow, 0.7L/min) and extracting and analyzing them as explained below (VOCs collections and Analysis). Dispensers emitting at a release rate range comparable to that of plants were used to expose experimental plants, which means at least the minimum or maximum double of what had been previously detected in infested plants (Tab. 2). In some cases, most of the tested dispenser emissions were out of the range and we did not have enough dispensers to expose the plants. In that case, "off-range" dispensers were re-capped and re-sealed with parafilm tape and used "blindly" (i.e. without further testing their emission rates) to expose plants.

Table 2. Average plant release rate of single VOCs during different periods of *S. exigua* infestation (Hours after the start of the infestation, [ng/hour]). (Chapter 3).

Constitutive VOC	**4 to 6**	**22 to 24**
Z-3-hexenol	0.83 ± 0.39	5.14 ± 0.9
α-pinene	40.7 ± 4.86	59.8 ± 6.1
β-caryophyllene	23.46 ± 3.5	38.83 ± 3.99
Inducible VOC	**28 to 30**	**46 to 48**
Linalool	12.11 ± 2.06	30.54 ± 3.75
DMNT	30.19 ± 3	71.97 ± 8.43
Indole	43.59 ± 5.97	120.09 ± 18.51

Exposure procedure

Dispensers were distributed in different source bottles depending on treatment (*constitutive*: *(Z)*-3-hexenol, α-pinene and β-caryophyllene; *inducible*: linalool, DMNT and indole; *mix*: *Z*-3-hexenol, α-pinene, β-caryophyllene, linalool, DMNT and indole). In control treatments, no dispensers were added. Source vessels were connected via Teflon tubes to receiver vessels, which contained an intact cotton plant (Fig. 1). An airflow of 1L/min was maintained that first entered the source vessel and carried the volatiles to the receiver vessel with the intact cotton plant (Fig. 1). The exposure lasted 48 hours; 22 hours and 46 hours after the start of the exposure, the release rates of the compounds from the dispensers were measured by trapping the VOCs for two hours. The VOC collection procedures and subsequent analyses are explained below. At the end of the exposure period, the third and the fourth leaf of each plant were collected, frozen in liquid nitrogen and stored at -80°C until further processing. The fourth leaf was used for gossypol, heliocides and phytohormones analyses. The third leaf, which was bigger than the fourth, allowed us to perform the same set of analyses, as well as measurements of defensive genes expression (S.I. Tab.4).

The experiment was performed in 3 blocks. In block 2 some dispensers already used in block 1 were used again to expose plants. Indeed, in preliminary tests we found that three days after assembling the dispensers, the release rates stayed stable for at least two weeks (data not presented). For block 3, completely new dispensers were assembled.

VOCs collections and analyses

Filters containing 25mg of 80/100 mesh Hayesep-Q adsorbent (Sigma, Switzerland) were connected to the source vessels and VOCs were collected during 2h at a rate of 0.7L/min, while

the inlet flow was turned up to 1.2 L/min in order to continue delivery of the VOCs to the receiver vessel. Subsequently, filters were eluted with 100µL dichloromethane and samples were spiked with 10 µL internal standards solution (n-octane and nonyl-acetate, 20 µg/µL each). Samples were stored at -80°C until GC-MS analysis. Samples were analyzed on a gas chromatograph (Agilent 7890B) coupled with a mass spectrometer detector (Agilent 5977B). A 1.5 µL aliquot of each sample was injected in pulsed spitless mode onto an Agilent HP-5MS column (30 m length x 250 µm diameter and 0.25 µm film thickness). After injection, temperature was maintained at 40°C for 3 min, increased to 100°C at a rate of 8°C per min and subsequently to 200°C at a rate of 5°C per min followed by a post run of 3 min at 250°C. Helium was used as carrier gas and kept at constant flow of 1.1mL/min. Compounds were subsequently identified by comparing their mass spectra with those from the NIST mass spectral library. Compounds were quantified based on comparison of peak areas with those of the internal standards. This method is the same already described in Chapter 3 and Quijano-Medina et al. (in press).

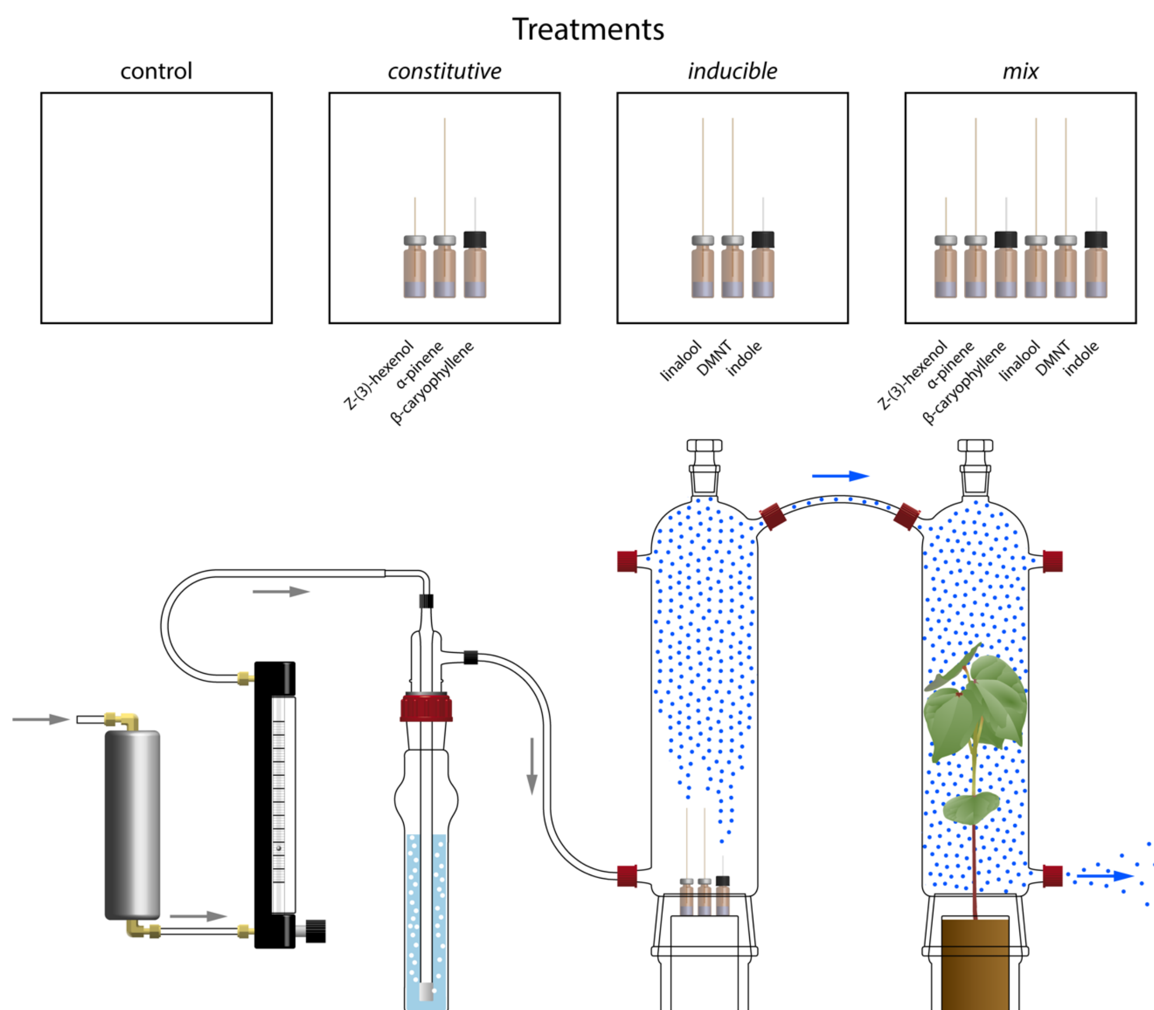

Figure 1. Set- up of the exposure system.

Gossypol and heliocides extraction and analysis

Frozen leaves were ground into a fine powder in a mortar filled with liquid nitrogen. Circa 50±5mg of powder were transferred into a frozen 1.5 mL Eppendorf tube. Then, 80 µL acetonitrile and 4-6 glass beads (1.25-1.65 mm diameter) were added to each tube. The mixture was vortexed and then homogenized for 3 minutes at 30 Hz using a beadmill (Retsch MM 300, Haan, Germany). Next, samples were centrifuged for 5 minutes at 14000 rpm. The recovered supernatant was subjected to a second centrifugation step to obtain a totally limpid solution. Finally, the supernatant was recovered and transferred into 2 mL glass vial (BGB, Germany) for HPLC analysis (Glauser *et al.*, 2013). Ultra High Performance Liquid Chromatography (UHPLC) is coupled with DAD detector set at 288 ± 2 nm (Ultimate 3000 Dionex, Thermo Fisher Scientific, MA, USA). A 5 µL aliquot of each sample was injected onto an ACQUITY UPLC® BEH C18 column (2.1 x 100mm, 1.7µm) (Waters, MA, USA). The flow rate was held

constant at 0.45 mL/min and the temperature was kept at 30°C. The mobile phase A consisted of 0.05% formic acid in MilliQ water (18 **Ω**) and the mobile phase B of 0.05% formic acid in acetonitrile (HiPerSolv, VWR Chemicals®, France). The following gradient was used: 45-90% B in 8 min, 90-100% B in 0.5 min, holding at 100% for 2.5 min followed by re-equilibration at 45% B for 3.5 min. Gossypol and heliocides (grouped H1 to H3) were identified by their retention time. Quantification was done by calculating a calibration equation obtained by linear regression from six calibration points (5 to 250 µg/mL) in gossypol equivalents. This method is the same already used in Chapter 3.

Plant hormones profiling

The level of Jasmonic Acid (JA), Jasmonic Acid-Isoleucine (JA-Ile), Salicylic acid (SA), abscisic acid (ABA) and OPDA (12-oxo-phytodienoic acid) were measured. The extraction and analysis procedures are fully described in Glauser *et al.*, (2014).

Gene expression measurement

Part of the same powder obtained from frozen leaves as described above was used for gene expression measurements. Leaf total RNA was isolated using the GeneJET Plant Purification Mini Kit (Thermo Fisher Scientific, Vilnius, Lithuania), following the manufacturer's instructions. Complete DNA removal was performed using the RNase-Free DNase Set (QIAGEN, Hilden, Germany). Each total RNA sample (500 ng) was reverse transcribed using the GoScript™ Reverse Transcription System (Promega, Madison, USA). Real time qPCR was performed on the Rotor-Gene™ 6000 (Corbett Research) using GoTaq® qPCR Master Mix (Promega, Madison, USA)). *Histone3* (GenBank accession no. AF024716) was used as a reference gene in the analyses of the relative expression of different defense-related genes. The primers used for qPCR and the respective genes are listed in Supporting Information (Tab. 4). Relative expression levels were calculated by using the ΔΔCt method (Schmittgen & Livak, 2008). This method is the same already used in chapter 3.

Additional experiment 1

The aim of this experiment was to test whether one single constitutive VOC (i.e. *(Z)*-3-hexenol, α-pinene or β-caryophyllene) is able to induce defenses in receiver plant.

To this end, plants were exposed during 48 hours to the respective treatment by means of single dispensers. Control plants, exposed to pure air, were also included in the design. At

the end of the exposure the third and the fourth leaf of each plant (including infested source plants) were harvested, frozen in liquid nitrogen and stored at -80°C until further processing. The fourth leaf was used for gossypol, heliocides and phytohormones analyses. The third leaf, which was bigger than the fourth, allowed us to perform the same set of analyses, as well as measurements of defensive gene expression. The experiment was performed in two blocks including a total of 6 biological replicates per treatment. A complete new set of dispensers was prepared for this experiment. VOCs exposures were monitored as explained in the main experiment.

Additional experiment 2

The aim of this experiment was to test whether the defense induction in plants that are exposed to the full blend of 6 VOCs (i.e. *mix*: *Z*-3-hexenol, α-pinene, β-caryophyllene, linalool, DMNT and indole) is comparable to the one that occurs in plants that are exposed to HIPVs emitted by a plant infested by 10 2nd instar S*podoptera exigua* (i.e. *HIPVs*). To this intent, plants were exposed during 48 hours to the respective treatment. Control plants, exposed to clean air, were also included in the design. At the end of the exposure the third and the fourth leaf of each plant (including four infested source plants) were harvested, frozen in liquid nitrogen and stored at -80°C until further processing. The fourth leaf was used for gossypol, heliocides and phytohormones analyses. The third leaf, which was bigger than the fourth, allowed us to perform the same set of analyses, as well as defensive genes expression measurements. Four biological replicates per treatment were tested. Dispensers were selected from the same set prepared for additional experiment 1. VOCs exposures were monitored as explained in the main experiment.

Results

At the end of the 48h exposure treatment, leaves were frozen, extracted and the extracts were analyzed to quantify the levels of specific defense metabolites. For the youngest leaf (i.e. 4th leaf), no significant differences among the treatments were found, with the results showing great variation among replicates. Gossypol and Heliocides analyses showed non-significantly increased levels in plants that were exposed to inducible VOCs compared to plants that were exposed to constitutive VOCs (Fig. 2, S.I. Tab.1). Yet, it should be noted that some of the highest levels were measured in control plants. Also surprising was that plants that had been exposed to the *mix* treatment showed a trend of lower JA levels compared to the other treatments (Fig. 3a), whereas plants that had been exposed to the constitutive treatment showed a trend of higher levels of JA-Ile compared to the other treatments (Fig. 3b). Due to limited leaf material, the relative expression of defensive genes could not be assessed with the same leaves (i.e. 4th) and we therefore used the 3rd leaves for gene expression measurements. Alongside, we also performed gossypol and heliocides analysis as well as phytohormone profiling on the 3rd leaf. No significant difference was found among the four treatments in the amounts of gossypol and heliocides produced by the third leaf (Fig. 4, S.I. Tab. 2). In conflict with the gene expression results mentioned below, a slight trend of increased gossypol levels in plants exposed to inducible VOCs was observed (Fig. 4a). No significant differences were found in hormone levels of the third leaves among the four treatments (Fig. 5, S.I. Tab. 2). A trend indicating higher levels of JA and JA-Ile for plants that were exposed to the three constitutive VOCs was observed (Fig. 5a,b). OPDA levels showed a very slight trend of increase after exposure to the three inducible VOCs (Fig. 5c). We observed a clear, but highly variable non-significant trend of increased expression of the *Cad1A* and *Cdn1C3* genes in plants exposed to the three constitutive VOCs, which are both involved in the pathway that leads to the biosynthesis of gossypol (Fig. 6, S.I. Tab. 3). This finding is in contrast with what we found in Chapter 3 for the natural volatile blends, where the results suggested that the inducible compounds may be more effective in triggering the expression of these genes. A similar trend was also observed for the plants that were exposed to the mixture with all six volatiles (i.e. *mix*). Otherwise, no significant differences among the 4 treatments were found in any of the tested genes (S.I. Tab. 3). Still, the lowest average expression of some genes was observed in *mix*-exposed plants (i.e. *GhPAL* (related to SA biosynthesis), *GhCYP82L1*, *GhCYP82L2* and *GhTPS14* (related to VOCs biosynthesis)) (Tab. 3). The dispenser release

rates were monitored during the exposure and proved extremely variable and only partially comparable to infested plant values (Tab. 4).

Fourth leaf analyses

Gossypol and heliocides

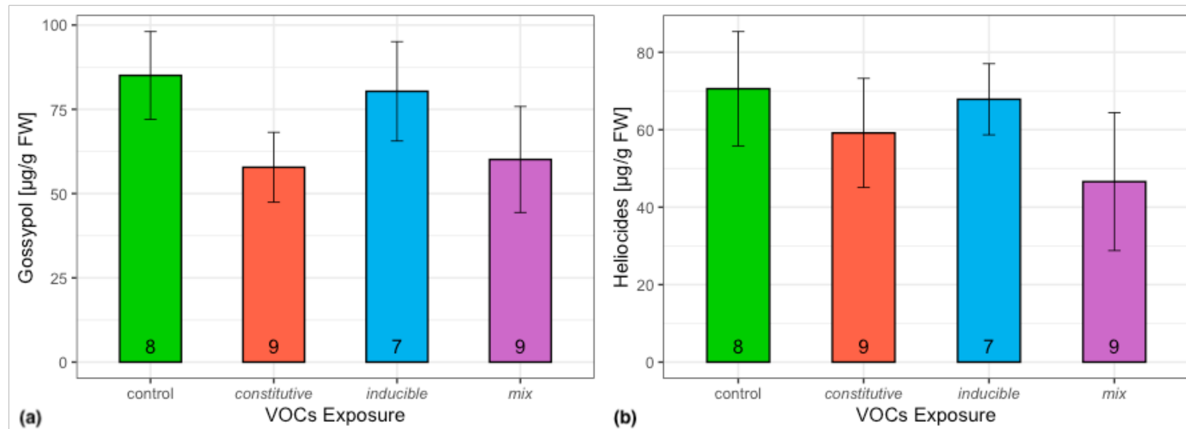

Figure 2. Gossypol (a) and Heliocides (b) levels in 4th leaf after 48h of exposure to different combinations of VOCs. Green: Control un-exposed plants; Red: *constitutive* (Z-3-hexenol, α-pinene, β-caryophyllene); Blue: *inducible* (linalool, DMNT, indole); Violet: *mix* (Z-3-hexenol, α-pinene, β-caryophyllene, linalool, DMNT, indole). No significant differences were found (ANOVA, S.I. Tab. 1). The numbers on the bottom of the bars indicate the number of single plant replicates.

Plant hormone profiling

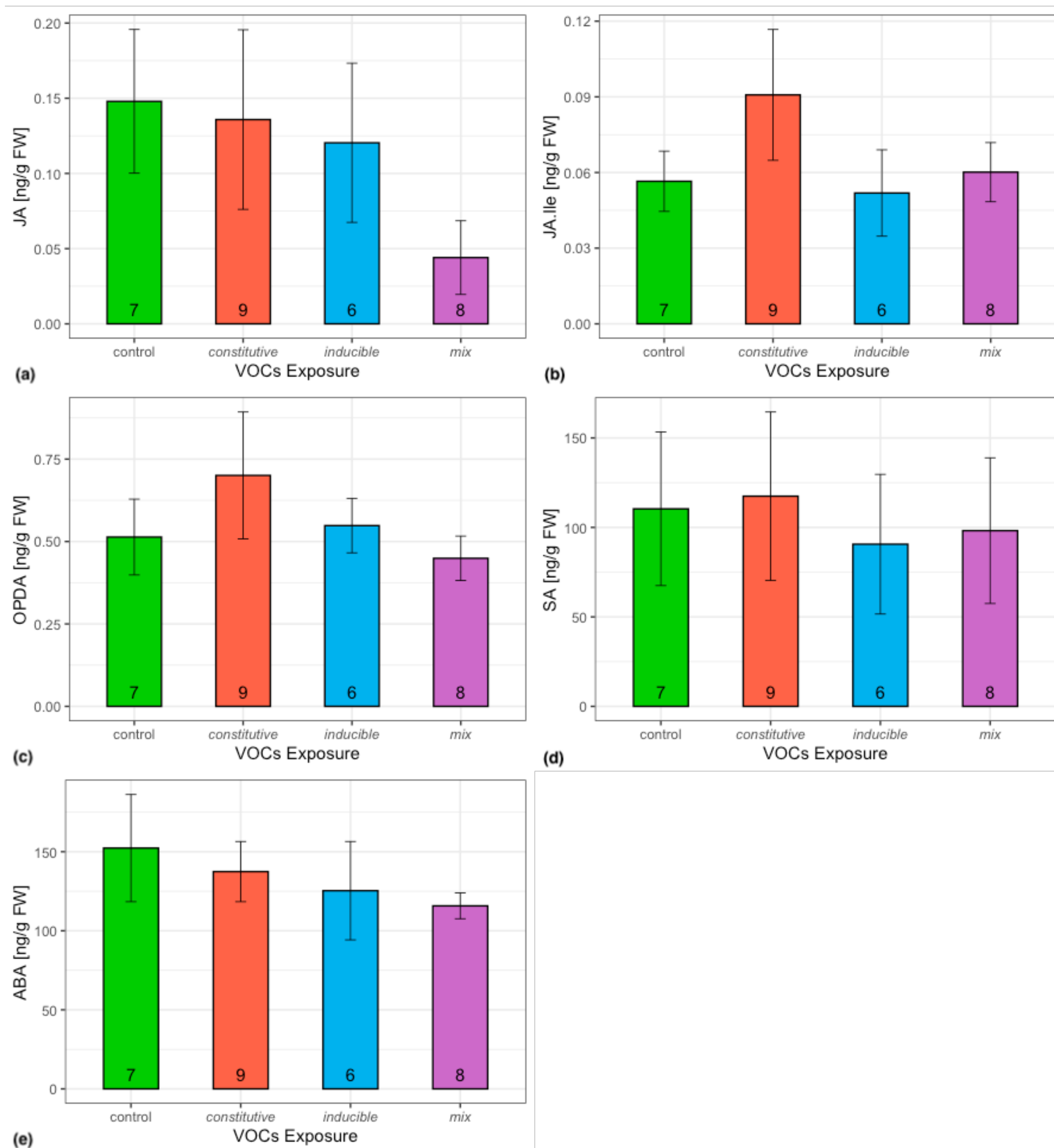

Figure 3. Different Phytohormones levels in 4th leaf after 48h of exposure to different combinations of VOCs. Green: Control un-exposed plants; Red: *constitutive* (Z-3-hexenol, α-pinene, β-caryophyllene); Blue: *inducible* (linalool, DMNT, indole); Violet: *mix* (Z-3-hexenol, α-pinene, β-caryophyllene, linalool, DMNT, indole). No significant differences were found (ANOVA, S.I. Tab. 1). The numbers on the bottom of the bars indicate the number of single plant replicates.

Third leaf analyses

Gossypol and heliocides

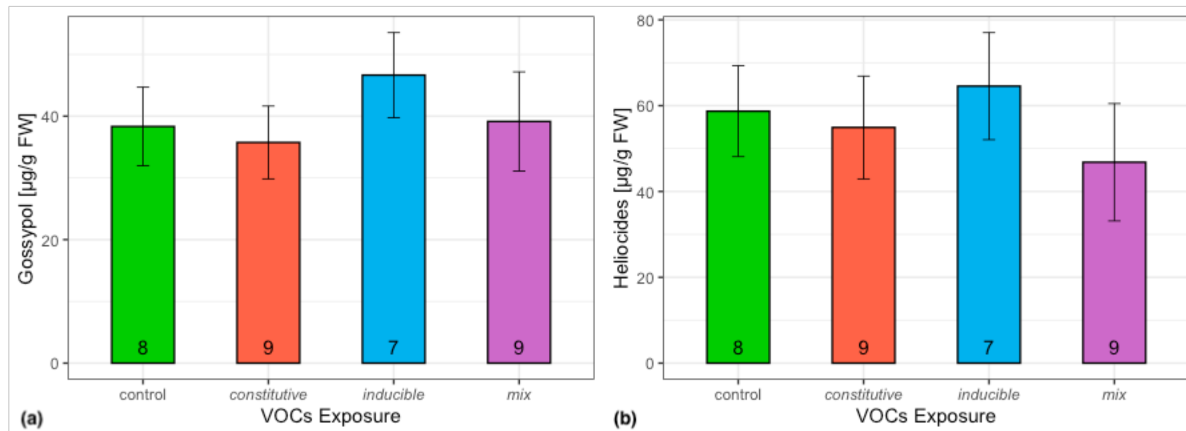

Figure 4. Gossypol (a) and heliocides (b) levels in 3rd leaf, after 48h of exposure to different combinations of VOCs. Green: Control un-exposed plants; Red: *constitutive* ((*Z)*-3-hexenol, α-pinene, β-caryophyllene); Blue: *inducible* (linalool, DMNT, indole); Violet: *mix* ((*Z)*-3-hexenol, α-pinene, β-caryophyllene, linalool, DMNT, indole). No significant differences were found (ANOVA, S.I. Tab. 2). The numbers on the bottom of the bars indicate the number of single plant replicates.

Plant hormones profiling

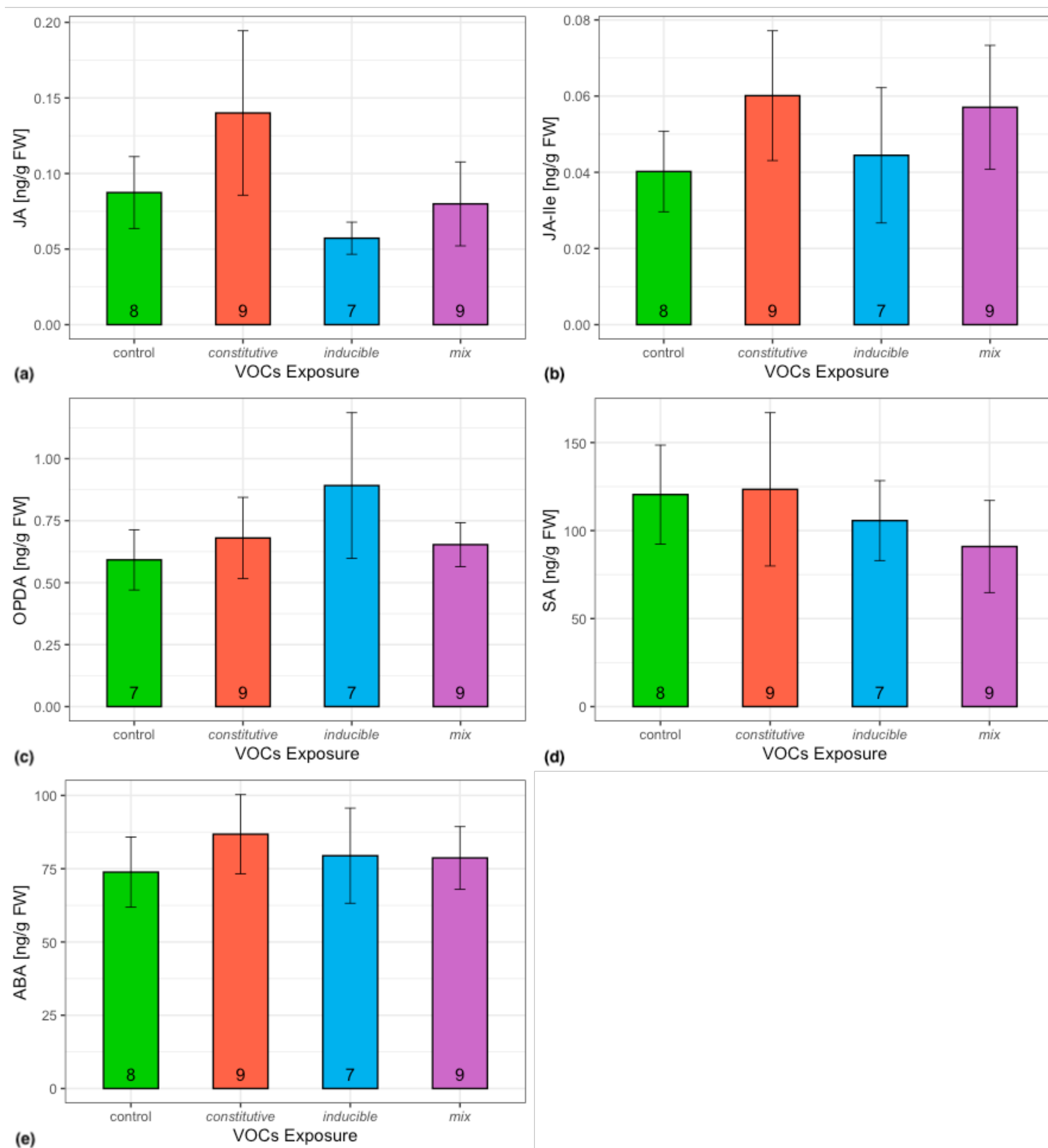

Figure 5. Phytohormones levels in 3rd leaf, after 48h of exposure to different combinations of VOCs. Green: Control un-exposed plants; Red: *constitutive* ((*Z*)-3-hexenol, α-pinene, β-caryophyllene); Blue: *inducible* (linalool, DMNT, indole); Violet: *mix* (*Z*-3-hexenol, α -pinene, β -caryophyllene, linalool, DMNT, indole). No significant differences were found (ANOVA, S.I. Tab. 2). The numbers on the bottom of the bars indicate the number of single plant replicates.

Gene expression measurements

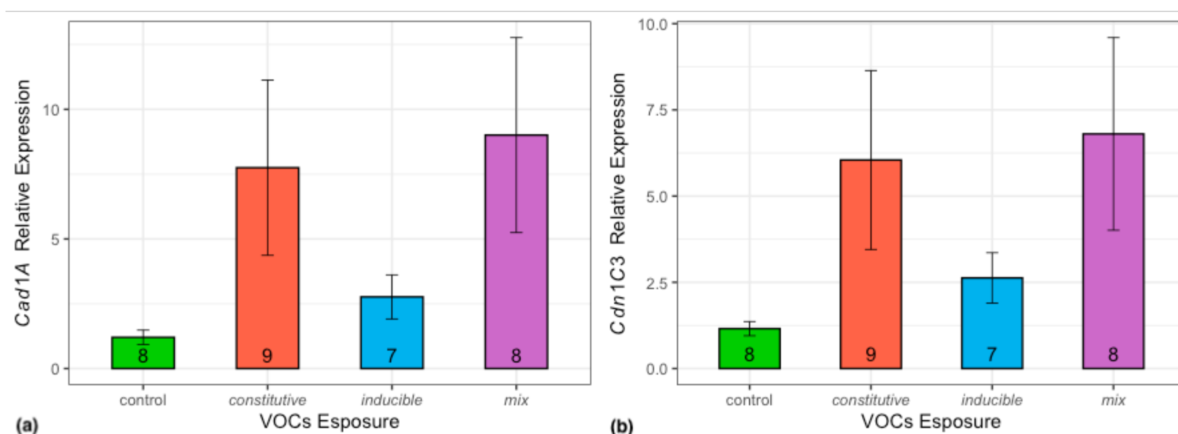

Figure 6. Relative gene expression levels in 3rd leaf after 48h of exposure to different combinations of VOCs. Left: *Cad1A*, Right: *Cdn1C3*. Green: Control un-exposed plants; Red: *constitutive* (*Z)*-3-hexenol, α-pinene, β-caryophyllene); Blue: *inducible* (linalool, DMNT, indole); Violet: *mix* ((*Z)*-3-hexenol, α -pinene, β -caryophyllene, linalool, DMNT, indole). No significant differences were found (S.I. Tab. 3). The numbers on the bottom of the bars indicate the number of single plant replicates.

Table 3. Relative expression of several defense-related genes in plants exposed to the four different VOCs treatments. (N=7-9)

Gene| Treatment	control	*constitutive*	*inducible*	*mix*
GhLOX1	1.17 ± 0.2	1.28 ± 0.29	1.53 ± 0.23	1.28 ± 0.28
GhAOS	1.38 ± 0.29	1.76 ± 0.41	2.22 ± 0.65	1 ± 0.15
GhOPR3	1.16 ± 0.18	1.26 ± 0.16	1.65 ± 0.34	1.15 ± 0.25
GhPAL	1.96 ± 0.94	2.44 ± 0.82	2.7 ± 1.07	1.15 ± 0.29
ICS1	1.12 ± 0.18	0.95 ± 0.09	1.14 ± 0.1	1.1 ± 0.16
EDS	1.19 ± 0.23	1.08 ± 0.16	1.43 ± 0.29	0.96 ± 0.22
GhCYP82L1	1.91 ± 0.61	1.72 ± 0.5	1.86 ± 0.82	0.77 ± 0.34
GhCYP82L2	1.55 ± 0.55	2.19 ± 0.83	2.17 ± 1.08	0.58 ± 0.11
GhTPS14	2.05 ± 1.14	3.57 ± 2.03	2.96 ± 1.68	0.65 ± 0.26
Cad1A	1.21 ± 0.28	7.75 ± 3.38	2.76 ± 0.85	9.01 ± 3.76
Cdn1C3	1.16 ± 0.21	6.05 ± 2.59	2.63 ± 0.73	6.8 ± 2.79
CYP706b	1.26 ± 0.33	2.15 ± 0.5	1.78 ± 0.35	2.39 ± 0.91

Table 4. Average dispensers' release rate [ng/h] during the exposure treatment. VOCs were collected twice for two hours 22 hours and 46 hours after the start of the exposure.

Block	**Position**	**Treatment**	**(*Z*)-3-hexenol**	**α-pinene**	**β-caryo phyllene**	**linalool**	**DMNT**	**indole**	**Total**
1	1	control	0	0	0	0	0	0	0
1	6	control	0	0	0	0	0	0	0
1	9	control	0	0	0	0	0	0	0
1	2	*constitutive*	8.46	10.02	36.18	0	0	0	54.66
1	5	*constitutive*	180.93	23.31	25.87	0	0	0	230.11
1	10	*constitutive*	25.76	80.18	7.23	0	0	0	113.17
1	4	*inducible*	0	0	0	68.14	9.66	130.51	208.30
1	8	*inducible*	0	0	0	71.33	53.91	73.66	198.90
1	12	*inducible*	0	0	0	45.10	41.21	128.62	214.94
1	3	*mix*	11.04	16.33	30.20	6.15	10.51	123.71	197.94
1	7	*mix*	16.66	81.77	36.88	85.37	18.31	102.68	341.66
1	11	*mix*	35.21	17.02	35.99	44.19	36.69	106.79	275.90
2	4	control	0	0	0	0	0	0	0
2	9	control	0	0	0	0	0	0	0
2	2	*constitutive*	29.69	23.57	6.27	0	0	0	59.53
2	5	*constitutive*	12.14	28.08	79.14	0	0	0	119.35
2	8	*constitutive*	38.06	49.88	29.11	0	0	0	117.06
2	6	*inducible*	0	0	0	11.98	10.85	120.70	143.52
2	1	*mix*	17.06	14.02	61.57	28.41	9.21	18.78	149.05
2	3	*mix*	36.05	58.88	64.05	16.72	44.93	18.78	239.41
2	7	*mix*	34.91	17.68	5.35	28.79	29.14	106.49	222.35
3	3	control	0	0	0	0	0	0	0
3	8	control	0	0	0	0	0	0	0
3	12	control	0	0	0	0	0	0	0
3	2	*constitutive*	17.30	167.89	98.01	0	0	0	283.20
3	6	*constitutive*	9.14	14.92	25.09	0	0	0	49.15
3	11	*constitutive*	6.94	16.61	7.73	0	0	0	31.27
3	1	*inducible*	0	0	0	34.53	59.27	80.33	174.13
3	5	*inducible*	0	0	0	42.04	16.86	90.89	149.78
3	9	*inducible*	0	0	0	27.09	108.63	94.21	229.93
3	4	*mix*	19.07	76.16	12.35	20.70	25.71	102.73	256.72
3	7	*mix*	9.22	25.19	28.27	19.96	45.28	72.52	200.44
3	10	*mix*	11.13	37.79	35.07	25.71	32.98	121.59	264.26

Additional experiment 1

None of the tested constitutive VOCs showed significantly increased levels of the extracted metabolites (Tab. 5) when compared to control. This was mainly due to high variation and low number of replicates. For JA and JA-Ile no statistical analysis was performed due to detection problems. None of the tested genes showed significant differences among the treatments except for EDS, a SA-related gene, which was significantly lower in *(Z)*-3-hexenol exposed plants compared to control (ANOVA: F=3.38, P=0.044) (Tab. 6). Interestingly, the average expression of gossypol-related genes (i.e. *Cad1A*, *Cdn1C3*, *CYP706b*) was consistently the highest in plants exposed to β-caryophyllene in spite of the highly variable dispenser emissions (Tab. 7). No correlation was found between the specific gene expression and dispenser emission rates.

Table 5. Average metabolite content (± standard error) in 3rd and 4th leaf. Gossypol and Heliocides are expressed in µg/g of fresh weight plant hormones are expressed in [ng/g fresh weight]. Number of replicates ranges from 0 to 6 depending on leaf material availability and detection. *No Detection* means that no peak could be observed in any of the replicates. When the standard error is replaced by NA, only one replicate could be detected.

	3rd Leaf			
Metabolite	control	(*Z*)-3-hexenol	α-pinene	β-caryophyllene
Gossypol	45.52 ± 7.59	32.32 ± 2.96	44.37 ± 12.87	36.72 ± 7.5
Heliocides	59.14 ± 10.89	36.88 ± 7.24	82.43 ± 32.41	47.34 ± 14.75
JA	0.3 ± NA	*No Detection*	0.41 ± NA	1.37 ± NA
JA-Ile	*No Detection*	*No Detection*	0.28 ± NA	0.3 ± NA
OPDA	0.53 ± 0.12	0.38 ± 0.07	0.36 ± 0.04	0.32 ± 0.07
ABA	108.06 ± 20.08	90.61 ± 8.78	119.06 ± 22.65	102.92 ± 32.29
SA	124.74 ± 34.27	65.94 ± 17.32	156.07 ± 38.44	96.6 ± 32.79
	4th Leaf			
Metabolite	control	(*Z*)-3-hexenol	α-pinene	β-caryophyllene
Gossypol	101.04 ± 38.54	113.47 ± 38.25	103.5 ± 31.1	89.16 ± 45.36
Heliocides	59.63 ± 13.87	40.56 ± 11.42	93.71 ± 32.75	48.03 ± 14.64
JA	*No Detection*	0.99 ± NA	2.82 ± 0.75	*No Detection*
JA-Ile	*No Detection*	0.27 ± NA	0.37 ± 0.03	*No Detection*
OPDA	0.68 ± 0.32	0.61 ± 0.23	0.3 ± 0.09	0.45 ± 0.12
ABA	132.26 ± 22.55	149.29 ± 15.95	203.77 ± 32.56	123.02 ± 18.06
SA	118.85 ± 37.62	79.79 ± 14.95	148.61 ± 41.87	55.02 ± 7.26

Table 6. Average Relative expression (± standard error) of several defense-related genes in third leaf (N=3-6).

Gene | Treatment	control	(*Z*)-3-hexenol	α-pinene	β-caryophyllene
GhLOX1	1.07 ± 0.21	1.15 ± 0.41	0.79 ± 0.15	1.46 ± 0.42
GhAOS	1.08 ± 0.24	1.13 ± 0.35	1.17 ± 0.26	0.65 ± 0.14
GhOPR3	1.06 ± 0.19	1.29 ± 0.44	1.36 ± 0.33	0.8 ± 0.23
GhPAL	1.21 ± 0.24	0.35 ± 0.16	0.64 ± 0.23	0.33 ± 0.07
ICS1	0.93 ± 0.23	0.67 ± 0.32	0.52 ± 0.22	0.98 ± 0.35
EDS	0.9 ± 0.17	0.42 ± 0.15	0.84 ± 0.19	0.57 ± 0.21
GhCYP82L1	1.28 ± 0.4	1 ± 0.3	2.22 ± 0.6	0.74 ± 0.21
GhCYP82L2	1.34 ± 0.58	0.88 ± 0.38	1.2 ± 0.25	0.88 ± 0.18
GhTPS14	1.58 ± 0.75	0.64 ± 0.24	0.8 ± 0.21	0.45 ± 0.09
Cad1A	0.99 ± 0.43	1.38 ± 0.49	2.49 ± 1.19	4.07 ± 2.09
Cdn1C3	1.19 ± 0.47	1.08 ± 0.41	1.5 ± 0.56	1.67 ± 0.71
CYP706b	1.17 ± 0.67	1.18 ± 0.17	0.92 ± 0.29	2.25 ± 0.76

Table 7. Average single dispensers' release rate [ng/h] during the exposure treatment. VOCs were collected twice for two hours 22 hours and 46 hours after the start of the exposure. On the left Block 1, on the right Block 2.

Block 1		
Position	Treatment	[ng/h]
1	(*Z*)-3-hexenol	41.64
5	(*Z*)-3-hexenol	90.22
10	(*Z*)-3-hexenol	26.01
3	α-pinene	39.13
7	α-pinene	17.62
9	α-pinene	40.57
2	β-caryophyllene	52.59
6	β-caryophyllene	55.77
11	β-caryophyllene	121.52

Block 2		
Position	Treatment	[ng/h]
4	(*Z*)-3-hexenol	15.75
8	(*Z*)-3-hexenol	36.30
12	(*Z*)-3-hexenol	92.50
2	α-pinene	26.42
6	α-pinene	9.66
10	α-pinene	23.48
3	β-caryophyllene	13.68
7	β-caryophyllene	4.06
11	β-caryophyllene	3.56

Additional experiment 2

Data on infested source plants were not included in any of the models since they experienced direct caterpillar damage, while the other treatments only experienced volatile exposure. Metabolite analysis (Tab. 8) did not show any significant differences among control, *mix* or *HIPVs* plants for any of the measured compounds in both 3rd and 4th leaf (ANOVA). For JA, JA-Ile no statistical analysis was performed due to detection problems and low number of replicates. Infested source plants were not included in the analysis, still they showed the highest levels of gossypol, JA, JA-Ile, OPDA and ABA. Gossypol level was significantly higher in the 4th leaf than in the 3rd leaf (ANOVA, $F=17.79$, $P<0.001$). Several cotton defense-related genes were tested for differences among the treatments (Tab. 9). *Cad1A* relative expression was significantly higher in *HIPVs* plants than in control plants (ANOVA: $F=4.34$, $P=0.048$). *Cdn1C3* relative expression was significantly higher in *HIPVs* plants compared to control or *mix* plants (ANOVA: $F=9.35$, $P=0.006$). Infested source plants showed the highest expression of all the gossypol-related genes (i.e. *Cad1A*, *Cdn1C3* and *CYP706b*), all the VOCs-related genes (*GhCYP82L1*, *GhCYP82L2*, *GhTPS14*) and one JA-related gene (*GhAOS*). In spite of the difference in the amount of volatiles to which *mix*- and *HIPV*-plants were exposed (Tab. 10), no significant difference could be observed between the two treatments.

Table 8. Average metabolite content (± standard error) in 3rd and 4th leaf. Gossypol and Heliocides are expressed in [µg/g fresh weight], plant hormones are expressed in [ng/g fresh weight]. Number of replicates ranges from 0 to 4 depending on leaf material availability and detection. *No Detection* means that no peak could be observed in any of the replicates. When the standard error is replaced by NA, the metabolite could only be detected in one replicate.

	3rd Leaf			
Metabolite	control	*mix*	*HIPVs*	Infested Source
Gossypol	34.61 ± 6.89	37.76 ± 4.08	26.3 ± 3.6	70.32 ± 18.28
Heliocides	37.87 ± 8	42.8 ± 10.2	29.62 ± 4.56	35.32 ± 5.77
JA	*No Detection*	*No Detection*	0.45 ± 0.03	148.47 ± 41.13
JA-Ile	*No Detection*	*No Detection*	*No Detection*	12.65 ± 2.51
OPDA	0.51 ± 0.12	0.32 ± 0.07	0.52 ± 0.13	0.92 ± 0.45
ABA	86.41 ± 5.62	74.14 ± 3.46	71.35 ± 7.56	160.3 ± 12.1
SA	185.09 ± 97.95	96.69 ± 27.1	82.94 ± 5.14	161.77 ± 71.05
	4th Leaf			
Metabolite	control	*mix*	*HIPVs*	Infested Source
Gossypol	65.81 ± 21.06	70.48 ± 14.58	51.32 ± 4.54	166.78 ± 58.49
Heliocides	39.32 ± 8.72	43.83 ± 11.92	37.2 ± 6.26	38.87 ± 5.25
JA	*No Detection*	*No Detection*	0.51 ± 0.09	38.51 ± 8.04
JA-Ile	*No Detection*	*No Detection*	0.23 ± NA	5.01 ± 0.24
OPDA	0.32 ± 0.07	0.46 ± 0.11	0.49 ± 0.23	2.96 ± 1.64
ABA	166.3 ± 25.57	126.04 ± 15.21	112.01 ± 15.06	195.03 ± 18.23
SA	111.02 ± 35.08	114.24 ± 36.44	98.45 ± 14.28	99.92 ± 19.33

Table 9. Average relative expression (± standard error) of several defense-related genes in third leaf (N=2-4).

Gene | Treatment	control	*mix*	*HIPVs*	Infested Source
GhLOX1	1.09 ± 0.25	0.68 ± 0.23	0.79 ± 0.38	0.63 ± 0.23
GhAOS	1.03 ± 0.14	0.67 ± 0.13	0.82 ± 0.3	3.87 ± 0.15
GhOPR3	1.06 ± 0.2	0.71 ± 0.15	0.77 ± 0.23	1.34 ± 0.42
GhPAL	1.48 ± 0.55	0.46 ± 0.15	1.22 ± 0.36	2.25 ± 0.65
ICS1	1.09 ± 0.28	0.89 ± 0.15	0.54 ± 0.26	0.45 ± 0.2
EDS	1.1 ± 0.25	0.56 ± 0.14	0.48 ± 0.14	0.5 ± 0.09
GhCYP82L1	2.65 ± 1.82	3.63 ± 1.88	6.76 ± 3.49	168.94 ± 14.6
GhCYP82L2	1.66 ± 0.72	2.28 ± 1	3.59 ± 0.53	24.84 ± 1.29
GhTPS14	2.08 ± 1.12	3.46 ± 1.88	1.04 ± 0.38	85.87 ± 14.73
Cad1A	1.03 ± 0.13	2.13 ± 0.17	45.7 ± 41.75	132.54 ± 71.47
Cdn1C3	1.29 ± 0.53	1.86 ± 0.28	38.91 ± 28.85	173.89 ± 110.66
CYP706b	1.11 ± 0.27	1.23 ± 0.27	7.42 ± 6.63	52.93 ± 38.21

Table 10. Single and total VOCs emission by infested source plants (HIPVs) 22h and 46h after the start of the infestation and by groups of dispensers (*mix* 1-4) [ng/h].

Compound [ng/h]	HIPVs (22 to 24)	HIPVs (46 to 48)	*mix* 1	*mix* 2	*mix* 3	*mix* 4
(*Z*)-3-hexenol	17.12	43.40	7.67	14.42	13.83	11.84
α-pinene	209.28	207.77	21.55	29.49	43.89	46.16
β-caryophyllene	111.40	162.69	45.27	31.50	38.21	34.21
linalool	8.26	83.27	10.84	16.28	15.08	22.60
DMNT	25.43	138.90	35.59	52.20	34.80	54.04
indole	40.11	307.93	86.04	103.80	87.17	102.95
Total	797.44	1818.26	206.96	247.69	232.98	271.80

Discussion

The aim of this chapter was to identify the potentially active volatile compounds responsible for the changes in defenses observed in the previous chapters. Based on the acquired knowledge, we hypothesized that truly inducible volatiles are essential compounds for the induction of enhanced defenses and therefore may be able to elicit a response in exposed plants. In our experiments we treated plants with different combination of individual dispensers each emitting one single VOC. Combinations of three constitutive volatiles, three inducible volatile or the six volatiles together were used to expose plants. No significant differences were observed in any of the tested defensive metabolites or defense-related genes, still, some trends were present. Average gossypol and heliocides levels in both 3rd and 4th leaf were higher in plants exposed to the group of inducible VOCs (i.e. linalool, DMNT and indole) compared to levels in plants exposed to the group of constitutive VOCs (i.e. (*Z*)-3-hexenol, α-pinene and β-caryophyllene) (Fig. 2 and Fig. 4). Surprisingly, the expression of two genes related to gossypol production (i.e. *Cad1A* and *Cdn1C3*) showed the opposite trend, with *constitutive*- and *mix*-exposed plants displaying higher levels compared to control and inducible exposed plants (Fig. 6). Plant hormone profiling analysis in the fourth leaf showed a trend of increased levels of JA-Ile and OPDA in *constitutive*-exposed plants compared to *inducible*-exposed plants (Fig. 3). The third leaf presented the same trend in JA and JA-Ile levels (Fig. 5). Based on the observations in gene expression and phytohormones levels, we decided to perform a first additional experiment where we exposed plants to single constitutive VOCs and compared them to control. Also in this case no significant difference could be found in any of the tested defenses. Interestingly, the average expression level of gossypol production-related genes (i.e. *Cad1A*, *Cdn1C3* and *CYP706b*) were consistently the highest in β-caryophyllene-exposed plants (S.I. 3, Tab. 6). We also conducted a second additional experiment where we aimed to compare the exposure to the six selected VOCs (i.e. *mix*) to the exposure to naturally HIPVs full blend emitted by *S. exigua*-infested plants (i.e. *HIPVs*). In this experiment infested source plants emitted huge amounts of α-pinene and β-caryophyllene compared to emissions by plants measured in chapter 3 (Tab.2) and compared to dispenser emission rates (Tab. 10). In spite of these differences, also in this case no significant difference was observed among the two treatments and control (Tab. 9). The only exception was found in two gossypol production-related genes. Indeed, *Cdn1C3* expression was significantly higher in *HIPVs* compared to *mix* or control. *Cad1A* proved to be upregulated in HIPV-exposed plants compared to controls, while the same non-significant trend was observed when *HIPVs* were compared to *mix*.

Unfortunately, this study remains inconclusive due to high variability and in some cases low number of replicates. High variability may originate from release rates varying among dispensers. Indeed, each single dispenser, in spite of previous testing and selection, had a unique emission rate, which in some cases lead to great differences within the replicates of the same treatment (S.I. 2). This problem is was never mentioned in some of the previous work where similar dispenser were used (Robert *et al.*, 2012; Hiltpold & Shriver, 2018; Hu *et al.*, 2019).

Our study is the first where plants are exposed to combinations of multiple single VOC dispensers. Differences in emission rates have therefore also led to differences in the ratio of individual compounds in the blend, which in turn may have resulted in different perception of the volatile message. Indeed, the airborne signal must be specifically received and this could occur in quality, quantity and blend ratio manner as observed with herbivore enemies (Firn & Jones, 1995; Clavijo McCormick *et al.*, 2012).

Additional or alternative explanations for the high variability and absence of significant differences among treatments may be the timing of the measurements or most likely the possibility that we might have missed the real relevant VOCs responsible for the induction of defenses in neighbor plants. Indeed, in these experiments we "only" used three constitutive and three inducible compounds in different combinations. We hypothesize that other inducible compounds, such as β-ocimene may be essential to induce the difference observed in chapter 1 and chapter 3. Some trends observed in the results of this chapter (4) suggested that also constitutive VOCs may be involved in the induction of neighbor plants. This last hypothesis is not contradicted by results in chapter 3. Indeed there, the volatile blend that induced defenses in neighbor plants was mainly composed of inducible compounds but also included constitutive compounds.

This study did not lead to the final identification of single or multiple active VOCs but provided useful information that may be used for further studies towards the ultimate goal of applying potent natural elicitors as part of an integrated pest management strategy in cotton. Further steps should include the testing of other VOCs, individually or in combination. Moreover, alternative ways to limit variation among dispenser emission rates are required.

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Supporting Information

Table 1. ANOVA table of each of the compounds extracted from fourth leaves (Fig. 2 and Fig. 3). A linear model was fitted to each response to find differences among the 4 treatments (i.e. control, *constitutive*, *inducible*, *mix*), random block effect was included as well.

Metabolite	**Df**	**F-Value**	**P-Value**	**Transformation**
Gossypol	3	1.36	0.27	log
Heliocides	3	1.25	0.31	log
JA	3	1.67	0.2	sqrt
JA-Ile	3	0.49	0.69	log
OPDA	3	0.45	0.72	log
SA	3	0.28	0.84	log
ABA	3	0.9	0.46	log

Table 2. ANOVA table of each of the compounds extracted from third leaves (Fig. 4 and Fig. 5). A linear model was fitted to each response to test for differences among the four treatments (i.e. control, *constitutive*, *inducible*, *mix*), random block effect was included as well.

Metabolite	**Df**	**F-Value**	**P-Value**	**Transformation**
Gossypol	3	0.15	0.93	log
Heliocides	3	0.57	0.64	log
JA	3	0.85	0.48	sqrt
JA-Ile	3	0.72	0.54	log
OPDA	3	0.22	0.88	log
SA	3	0.5	0.68	log
ABA	3	0.51	0.68	log

Table 3. ANOVA table of each of the gene expression in third leaves (Fig. 6 and Tab. 3). A linear model was fitted to each response to test for differences among the four treatments (i.e. control, *constitutive*, *inducible*, *mix*), random block effect was included as well.

Gene	**Df**	**F-Value**	**P-Value**	**Transformation**
GhLOX1	3	0.46	0.71	log
GhAOS	3	0.99	0.41	log
GhOPR3	3	0.79	0.51	log
GhPAL	3	0.79	0.51	log
ICS1	3	0.39	0.76	log
EDS	3	0.50	0.69	log
GhCYP82L1	3	0.75	0.53	log
GhCYP82L2	3	0.91	0.45	log
GhTPS14	3	1.06	0.38	log
Cad1A	3	1.69	0.19	log
Cdn1C3	3	1.10	0.37	log
CYP706b	3	0.38	0.77	log

Table 4. Genes information.

Gene ID	GeneBank Acc. No.	Gene Family	Function	Forward Primer Sequence	Reverse Primer Sequence	Reference
GhLOX1	AF361893	Lipoxygenase	JA biosynthesis	GCCAAGGAGAGCTTCAAGAA	TAGGGGTACTTGGCAGAACCT	Zebelo *et al*. 2016
GhAOS	KM265129	Allene oxide synthase	JA biosynthesis	ATCATGTAATCCCCGAGTTCC	CCAGCTTGATCGTTAGCTGTC	Zebelo *et al*. 2016
GhOPR3	FB505932	12-oxo-phytodienoic acid reductase	JA biosynthesis	ATGTGACGCAACCTCGTTATC	CCGCCACTACACATGAAAGTT	Zebelo *et al*. 2016
GhPAL	JN032297	phenylalanine ammonia-lyase	SA biosynthesis	CGAGGAACAAAGCATTACAT	GTGGGAGACCGTTGTTGTAG	Lv *et al*. 2017
ICS1	PPS00849	isochorismate synthase	SA biosynthesis	GTCTTCAGCCACCTAATGGAC	GCTCTGGATTCACCTCTAGCAC	Miao *et al*. 2019
EDS1	JQ766941	enhanced disease susceptibility 1	SA biosynthesis	GCAGCAACAGCTCCTCTACCT	GGCAGACCAAGACGCTACAGA	Miao *et al*. 2019
GhCYP82L1	KY247144	Cytochrome P450; CYP82L subfamily	homoterpene (DMNT and TMTT) biosynthesis	ATTCTCTGGTAACGAGTT	AACACTGATTACTGAGTC	Liu et al. 2017
GhCYP82L2	KY247145	Cytochrome P450; CYP82L subfamily	homoterpene (DMNT and TMTT) biosynthesis	ATTCTCCGGTAACGATTA	AACATTGATTATTGAGTCGA	Liu et al. 2017
GhTPS14	KX963376	Terpene Synthase	production of DMNT, TMTT, (E)-β-ocimene and linalool	GAACTGACCACCCTCACTCA	CGCCTCCACAAACCATGAAT	Huang et al. 2017
Cad1-A	Y18484	(+)-δ-cadinene synthase (CDNS; referred to as CAD); CAD1-A subfamily	Gossypol biosynthesis	ATAAGGATGAAATGCGTCC	GAAGCTTGGTAAAGTTCCA	Zebelo et al. 2017
Cdn1-C3	AF174294	(+)-δ-cadinene synthase (CDNS; referred to as CAD); CAD1-C subfamily	Gossypol biosynthesis	AACTCAAAAACGCCACCAAC	TAGTCGGAATCGAAGGGATG	Zebelo et al. 2017
CYP706B1	AF332974	Cytochrome P450 monooxygenase	Gossypol biosynthesis	GCAAGCCAATTGATTTTGGT	GCACCGGGAAAATATCAGAA	Zebelo et al. 2017
Histone (H3)	AF024716	histone (H3) ribosomal mRNA	Housekeeping Gene	GAAGCCTCATCGATACCGT	CTACCACTACCATCATGGC	Zebelo *et al*. 2016

Chapter 5: Effect of cotton topping on the production of defense-related metabolites

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Abstract

The research conducted, including the rationale: Cotton topping, which involves the manual removal of the growing point of plants just before flowering, is an ancient technique used to increase yield and reduce pest infestations. Field evidence suggests that this practice also enhances resistance against pests in neighboring un-topped plants. We conducted three field experiments (2016, 2018 and 2019) and one net house experiment (2019) in order to study the possible upregulation of plant defense metabolites related to the topping practice.

Methods: Leaves from differently treated cotton plants were harvested, and their levels of defensive compounds (gossypol, heliocides) and phytohormones were analysed. Treatments corresponded to topped plants, intact neighbor plants and intact control plants from non-topped plots.

Key Results: In the first field experiment, topped and neighbor plants presented higher levels of gossypol compared to control plants. In the second field experiment, the increased production of gossypol was observed only for topped plant. Strangely, in the third field experiment gossypol content was consistently highest in control plants, but 4 days after topping jasmonic acid (JA) and JA-isoleucine (JA-Ile) were significantly higher in topped and neighbor plants compared to control plants. Also, 8 days after topping, neighbor plants were found to contain significantly higher levels of JA and JA-Ile compared to topped plants and control plants. In the net house topped plants presented some extremely high values in phytohormones related to the JA signaling pathway.

Main Conclusion: In spite of high variability among seasons, cotton topping generally increases the content of certain defense metabolites in the plant itself, and occasionally in the neighbor plants as well.

Introduction

Cotton (*Gossypium hirsutum* L.) crops are threatened by many insect pests worldwide including leaf chewing, fruit-eating, and sap-sucking herbivores (Naranjo & Luttrell, 2008). Nowadays, in spite of the development of new genetically engineered cotton cultivars, dominant strategies are still based on repeated applications of synthetic pesticides leading to pest resistance and loss of biodiversity (Carletto *et al.*, 2010; Naranjo, 2011; Trapero *et al.*, 2016; Brévault *et al.*, 2019). With the intent of finding eco-friendly solutions for cotton pest control, plant training, which includes plant topping and pruning, was recently revisited in the light of plant defense induction (Renou *et al.*, 2011; Téréta, 2015; Llandres *et al.*, 2018). Cotton topping is an ancient cultural practice and consists of removing the top of the terminal bud of the main stem of the plant, which eventually results in prevention of apical dominance and promotes further vegetative growth (Tavernetti & Miller, 1953; Obasi & Msaakpa, 2005; Basnet, 2006; Dai & Dong, 2014). Interestingly, Renou *et al.* (2011) showed that manual cotton topping decreases bollworm infestations in cotton crops in Mali. More recently, Téréta (2015) observed that intact neighboring conspecific plants benefited from this practice, with also reduced infestations as a result. Llandres *et al.* (2018) proposed this technique as part of an integrated pest management program to reduce insecticide use and promote cotton productivity.

When damaged, cotton plants show an upregulation of phytohormone cascades and increased production of toxic secondary metabolites such as gossypol (Opitz *et al.*, 2008; Zebelo *et al.*, 2017). Moreover, damaged cotton plants emit volatile organic compounds (VOCs) that are known to repel herbivores, attract natural enemies of herbivores and improve defense in other parts of the plant itself and in neighboring conspecific plants (Röse *et al.*, 1998; Zakir *et al.*, 2013; Chapter 1).

In the context of a collaboration between CIRAD Senegal (Dr. Thierry Brévault) and the FARCE Laboratory (University of Neuchâtel), three field experiments and one net house experiment were performed in Senegal in order to study the effect of topping on production of defense-related metabolites in cotton (*G. hirsutum*, commercial variety STAM59A). For this purpose, leaves from topped plants, neighboring intact plants (close to topped plants) and control intact plants from non-topped plots were sampled. In the first two field experiments performed in 2016 and 2018, only leaf content of gossypol and heliocides was assessed. In the third field experiment and in the net house experiment, both performed in 2019, phytohormones content was also measured in addition. In the second and in the third field

experiments, cotton squares were also analyzed after 16 or 18 days post-topping, respectively. The aim of the study was to test whether defense-related metabolites are upregulated after topping in both the topped plants, as well as the neighboring plants, when compared to control plants, as a hypothesis to explain the reduced infestation levels observed in the above-mentioned studies (Renou *et al.*, 2011; Téréta, 2015).

Methods

Field experiment 1 (2016) and 2 (2018)

Both experiments were performed at the ISRA-CDH experimental station (Sangalkam, Dakar, Senegal), following the same design and procedures. The following protocol reflects the experimental procedure performed in 2016. Cotton seeds (4-5 seeds per seed hole, variety STAM59A, IER Mali) were sown in seed holes 25 cm apart within rows, with 80 cm between rows. Three weeks after sowing each group of emerged cotton plants was reduced to two plants. At the appearance of the first flower buds (circa 45 days after sowing), 25 different parcels composed by 11 lines (L1 to L11) of 10m length were defined and 5 objects (A, B, C, D, E), described in table 1, were randomly distributed in 5 blocks (Supporting Information, S.I. Fig. S1).

Table 1: Description of each of the compared objects. PSV (i.e. protection standard vulgarisé): before topping, every 14 days starting 30 days after germination, plants are treated with EMACOT 019EC (emamectin benzoate, 19 $g.l^{-1}$, Savana) and TRIUMPH 692EC (profenofos 600 $g.l^{-1}$, lambdacyhalothrin 60 $g.l^{-1}$, acetamiprid 32 $g.l^{-1}$, SPIA). NT (i.e. non traité): no pesticide application after topping. PP (i.e. protection poussée): weekly application, starting the day after topping until the opening of cotton bolls, of EMACOT 019EC and TRIUMPH 692EC on the 5 central lines (L4 to L8).

Objects	Before Topping	Topping	After Topping
A	PSV	None	NT
B	PSV	L4 to L8 (20%)	NT
C	PSV	L4 to L8 (100%)	NT
D	PSV	None	PP
E	PSV	L4 to L8 (100%)	PP

Topping was performed 10 days after the flowering date. To determine the flowering date, 10 plants per plot were marked. The flowering date of each plot was reached when at least 5 of the marked plants displayed flowers. The flowering date of the field was the average date. Topping was performed manually (Fig. 1) on plants of the 5 central lines of objects B, C and D (Tab.1).

Figure 1: Different steps of cotton topping. A) Pinching of the main stem. B) Cutting of the stem by squeezing and twisting. C) Topped cotton plant (Renou *et al.*, 2015).

In order to monitor the content of gossypol and heliocides, ten leaves were sampled on different plants from topped (L4 to L8, Object E), neighbor (L3 and L9, Object E) or control plants (Object D) 0, 2, 4, 8, 16 and 24 (only 2018) days after topping. Objects A, B and C were used for experiments not included in this collaboration. Leaves were directly frozen in liquid nitrogen and kept at -80°C until shipping to UniNe (Neuchâtel, Switzerland). Shipping was performed under dry ice in order to avoid sample thawing. Sampled leaves were always the one situated on the last node of the fruiting branch under the topping point. In 2018, 10 cotton squares (i.e. the flower bud that first appears on the plant when reproductive growth begins) per treatment were also collected 16 days after topping.

Net house experiment 1 (2019)

This experiment was performed at the ISRA-IRD Research Center (Bel-air, Dakar, Senegal) under net house conditions (i.e. field cage) to avoid insect damage. Cotton plants (variety STAM59A) were grown in 96 separated 10L soil pots in a greenhouse until flowering. Fertilizer (30g NPKM 11.9.19+2) was added to each pot. Part of the plants were topped and distributed in four different outdoor net houses placed at least 200m from each other. In each net house 6 rows of 4 plants were arranged: two net houses contained only intact plants (corresponding to control plants), while the other two net houses contained alternated rows of topped and intact neighbor plants. Leaf sampling was done 0, 2, 4, 8, 16 and 24 days after topping. Two leaves from two different plants of each treatment present in each net house were sampled, resulting in four biological replicates per day per treatment (i.e., topped, neighbor, control). Sampled plants were removed from the net houses. Leaves were harvested, frozen and shipped as described in Field experiment 1 (2016) and 2 (2018). In this experiment,

gossypol, heliocides and phytohormones were analysed as described below. No pesticides were applied on the plants.

Field experiment 3 (2019)

This field experiment was performed at the ISRA-IRD Research Center (Bel-air, Dakar, Senegal). Here, only two small plots (150 m apart) with each 10 cotton rows (10 m long) were sown (variety STAM59A). One control plot was left untouched while every other row of plants was topped in the other plot, generating at the same time the topped and intact neighbor treatments. Six leaves were sampled from different plants of each treatment 0, 2, 4, 8, 16 and 24 days after topping. Six squares per treatment were harvested 18 days after topping. Leaves and squares were frozen and shipped as described in Field experiment 1 (2016) and 2 (2018). In this experiment, gossypol, heliocides and phytohormones were analysed as described below. Plants were treated with pesticides as explained in Field Experiment 1 (2016) and 2 (2018).

Gossypol and heliocides extraction and analysis

The analysis of Gossypol and Heliocides was performed in Neuchâtel with the help of the NPAC (Neuchâtel Platform of Analytical Chemistry). For the metabolite extractions, frozen leaves were ground into a fine powder in a mortar filled with liquid nitrogen. Part of the powder (circa 50±5mg) was then transferred into a frozen 1.5 mL Eppendorf tube, the rest was kept for phytohormones profiling or backup. Next, 80 µL acetonitrile and 4-6 glass beads (1.25-1.65 mm diameter) were added to each tube. The mixture was vortexed and then homogenized for 3 minutes at 30 Hz using a beadmill (Retsch MM 300, Haan, Germany). The samples were subsequently centrifuged for 5 minutes at 14000 rpm. The recovered supernatant was subjected to a second centrifugation step to obtain a totally limpid solution. Finally, 80 µL of the supernatant were recovered and transferred into a 2 mL glass vial (BGB, Germany) for High Performance Liquid Chromatograph (HPLC) analysis (Glauser *et al.*, 2013).

Over the years two different methodologies and machines were used to run the samples (15.11.2018 transfer of method). Samples from the first field experiment were analysed via High Performance Liquid Chromatograph (HPLC) while the rest of the experiments were processed via an ultra HPLC (uHPLC).

The HPLC device was coupled to a DAD detector set at 288 ± 2 nm (HP1100, Agilent Technologies, Santa Clara, CA, USA) as described by (Glauser *et al.*, 2013). A 1.5 µL aliquot of each sample was injected onto an Extent-C18 column (2.1 x 150mm, 5µm) (Agilent

Technologies). The flow rate was held constant at 0.5 mL/min, and the temperature was kept at 30°C. The mobile phase A consisted of 0.05% formic acid in water and the mobile phase B of 0.05% formic acid in acetonitrile. The following gradient was used: 35-90% B in 20 min, 90-100% B in 1 min, holding at 100% for 3 min followed by re-equilibration at 35% B for 6 min.

Ultra High Performance Liquid Chromatography (UHPLC) was coupled with DAD detector set at 288 ± 2 nm (Ultimate 3000 Dionex, Thermo Fisher Scientific, MA, USA). A 5 µL aliquot of each sample was injected onto an ACQUITY UPLC® BEH C18 column (2.1 x 100mm, 1.7µm) (Waters, MA, USA). The flow rate was held constant at 0.45 mL/min and the temperature was kept at 30°C. The mobile phase A consisted of 0.05% formic acid in MilliQ water (18 Ω) and the mobile phase B of 0.05% formic acid in acetonitrile (HiPerSolv, VWR Chemicals®, France). The following gradient was used: 45-90% B in 8 min, 90-100% B in 0.5 min, holding at 100% for 2.5 min followed by re-equilibration at 45% B for 3.5 min.

Gossypol and heliocides (grouped H1 to H3) were identified by their retention time. Quantification was done by calculating a calibration equation obtained by linear regression from six calibration points (5 to 250 µg/mL) in gossypol equivalents.

Plant hormones profiling

Extraction and analysis procedures to determine JA (Jasmonic Acid), JA-Ile (Jasmonic Acid-Isoleucine), OPDA (12-oxo-phytodienoic acid), SA (Salicylic Acid) and ABA (Abscisic Acid) levels are fully described in Glauser *et al.* (2014). A part (circa 100±10mg) of the same leaf powder already ground for gossypol and heliocides analysis was used.

Statistical analyses

Pooled and single day analyses were performed using ANOVA and Tukey Post-hoc tests, and log or sqrt transformations were applied when necessary to meet normality assumptions on dependent variable distribution.

Results

Field experiment 1 (2016)

The results obtained during the first season (i.e. field experiment 1, 2016) were really interesting since they showed how both topped and neighbor plants had increased gossypol content after topping compared to control plants (Fig. 2 top, Fig. 3 left), while heliocides presented the same non-significant trend overall (Fig. 3 right).

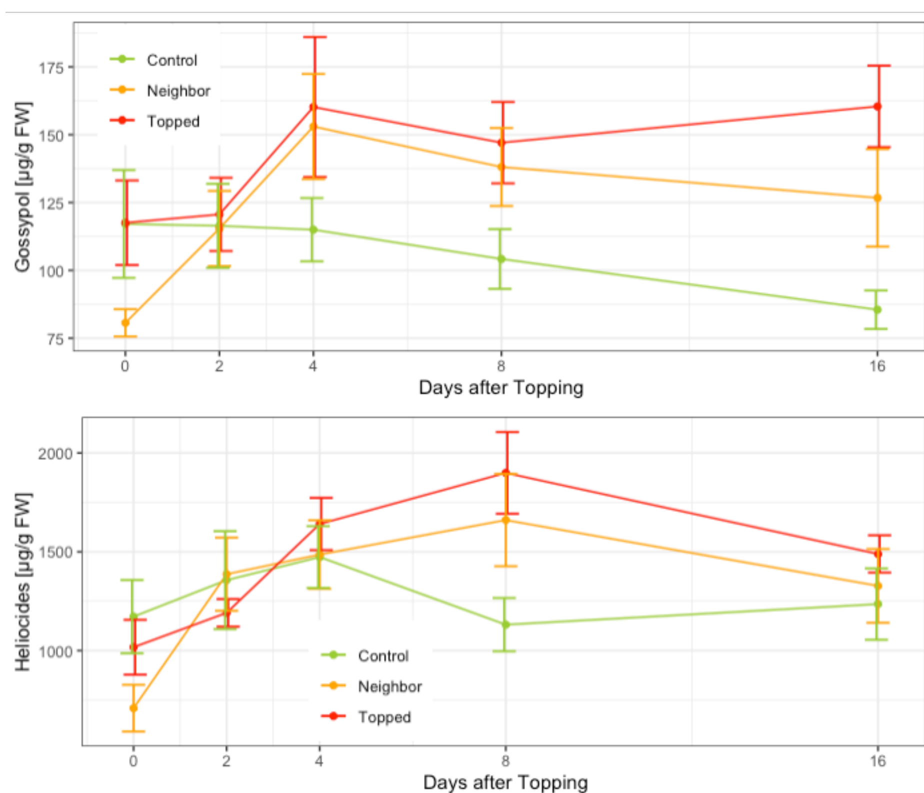

Figure 2. Dynamics of gossypol (top) and heliocides (bottom) content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 16 days after topping (N=10, each day, each treatment). No significant differences were found at any days after topping among the three treatments.

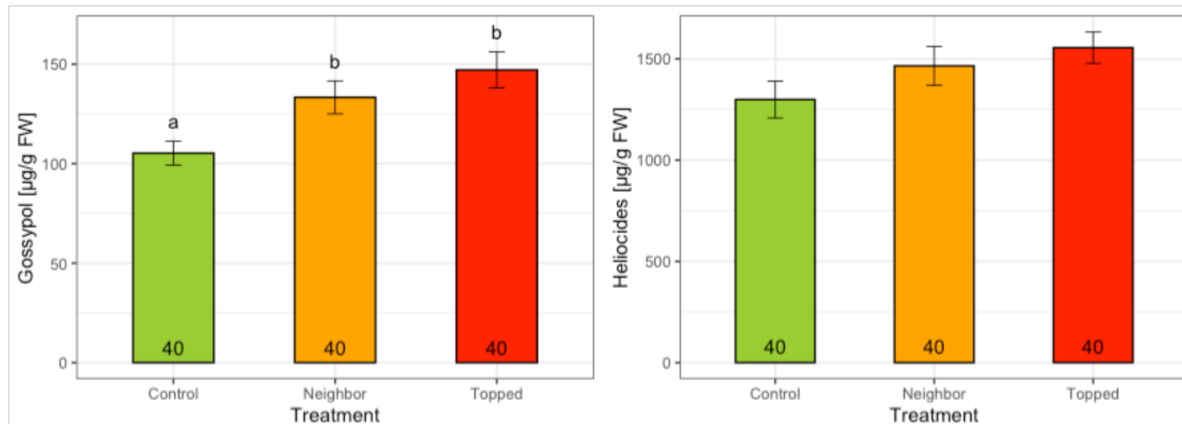

Figure 3. Gossypol (left) and heliocides (right) content in leaves from topped, non-topped neighbor, and non-topped control cotton plants. Days of harvesting were pooled together excluding day 0 (when topping was applied). Different letters indicate significant difference within the same graph (ANOVA Gossypol: F=7.73, P=0.003). Number of replicates are indicated at the base of each bar.

Field experiment 2 (2018)

Field experiment 1 (2016) results motivated us to replicate the experiment two years later. The experiment was replicated with the same design in 2018, but the gossypol induction in neighbor plants was no longer observed. Indeed, in this season, we could only record a trend of increased gossypol level in topped plants compared to neighbor and control plants (Fig. 4 left). Heliocides presented a similar trend, showing significantly higher levels in topped plants compared to neighbors (Fig. 4 right). These differences mainly originated from day 4 and day 8, where topped plants showed increased levels of both metabolites (Fig. 5).

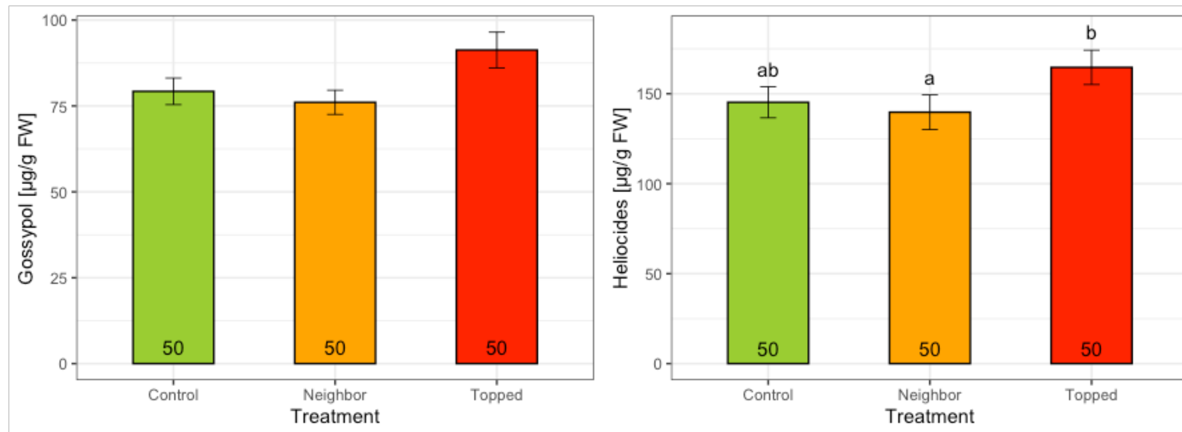

Figure 4. Gossypol (left) and heliocides (right) content in leaves from topped, non-topped neighbor, and non-topped control cotton plants. Days of harvesting were pooled together excluding day 0 (when topping was applied). Different letters indicate significant difference within the same graph (ANOVA heliocides: F=4.02, P=0.02). Number of replicates are indicated at the base of each bar.

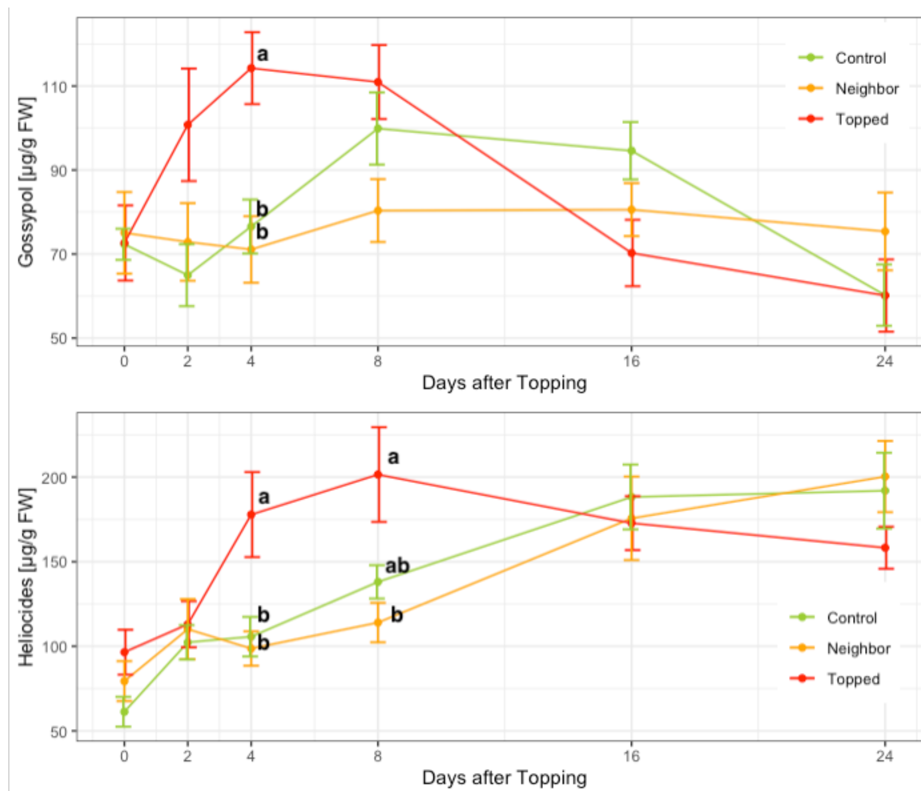

Figure 5. Dynamics of gossypol (top) and heliocides (bottom) content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 24 days after topping. N=10 for each day and each treatment, except for neighbor day 0 (N=9). Different letters indicate significant difference within the same day (ANOVA gossypol day 4: F=8.73 P=0.001; ANOVA heliocides day 4: F=7.53, P=0.003; ANOVA heliocides day 8: F=6.3, P=0.019).

In the analyses of the cotton squares, the highest concentrations were found in topped plants for both gossypol and heliocides (Fig. 6). Furthermore, heliocides levels were significantly higher in squares collected from topped plants compared to both control and neighbor plants (Fig.6 left). In comparison, gossypol levels detected in cotton squares were twice as high as those in the leaves. The differences between 2016 and 2018 experiments may be explained by drier conditions in 2018.

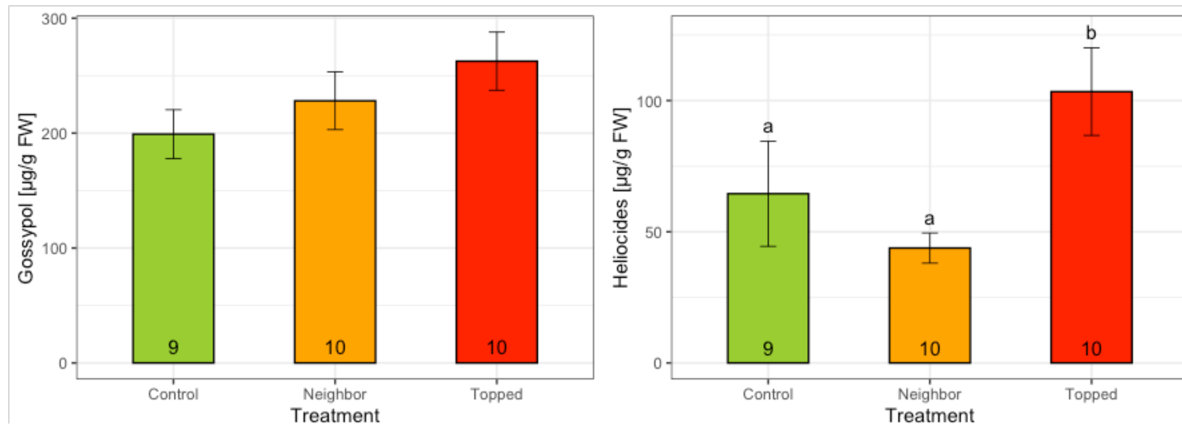

Figure 6. Gossypol (left) and heliocides (right) content in cotton square collected from topped, non-topped neighbor, and non-topped control cotton plants 16 days after topping. Different letters indicate significant difference within the same graph (ANOVA heliocides: F=7.46, P=0.003). Number of replicates are indicated at the base of each bar.

Net house experiment 1 (2019)

In order to continue studying the possible difference among the three treatments (i.e. topped, neighbor and control) a net house experiment and another field experiment with simplified design were performed in 2019. For both of these experiments, besides gossypol and heliocides, the levels of five phytohormones (i.e. JA, JA-Ile, OPDA, SA and ABA) were also determined.

Concentrations of gossypol and heliocides were the lowest in topped plants overall when compared to neighbor and control plants in the 2019 net house experiment (Fig. 7). These results were mainly explained by the difference in content observed 24 days after topping (Fig. 8).

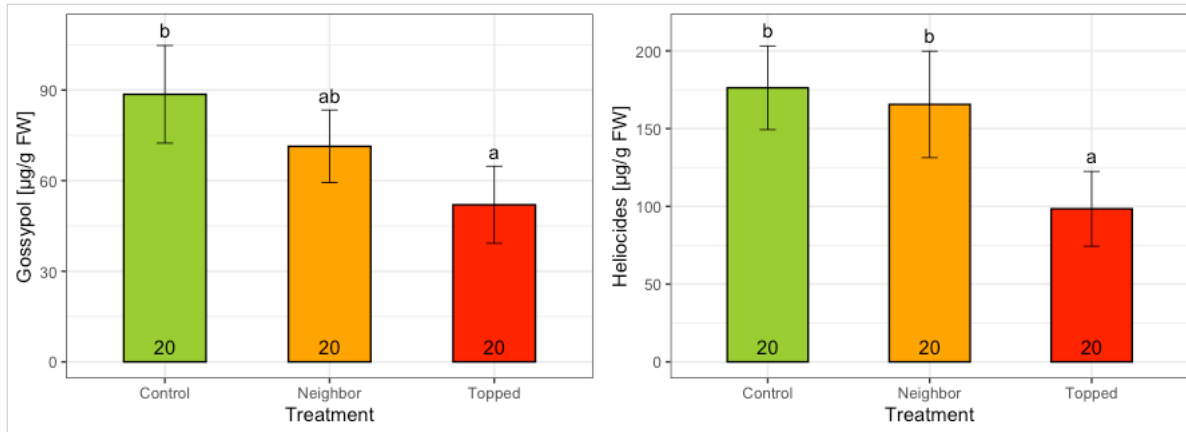

Figure 7. Gossypol (left) and heliocides (right) content in leaves from topped, non-topped neighbor, and non-topped control cotton plants. Days of harvesting were pooled together excluding day 0 (when topping was applied). Different letters indicate significant difference within the same graph (ANOVA Gossypol: F=3.78, P=0.03, ANOVA Heliocides: F=5.8, P=0.005). Number of replicates are indicated at the base of each bar.

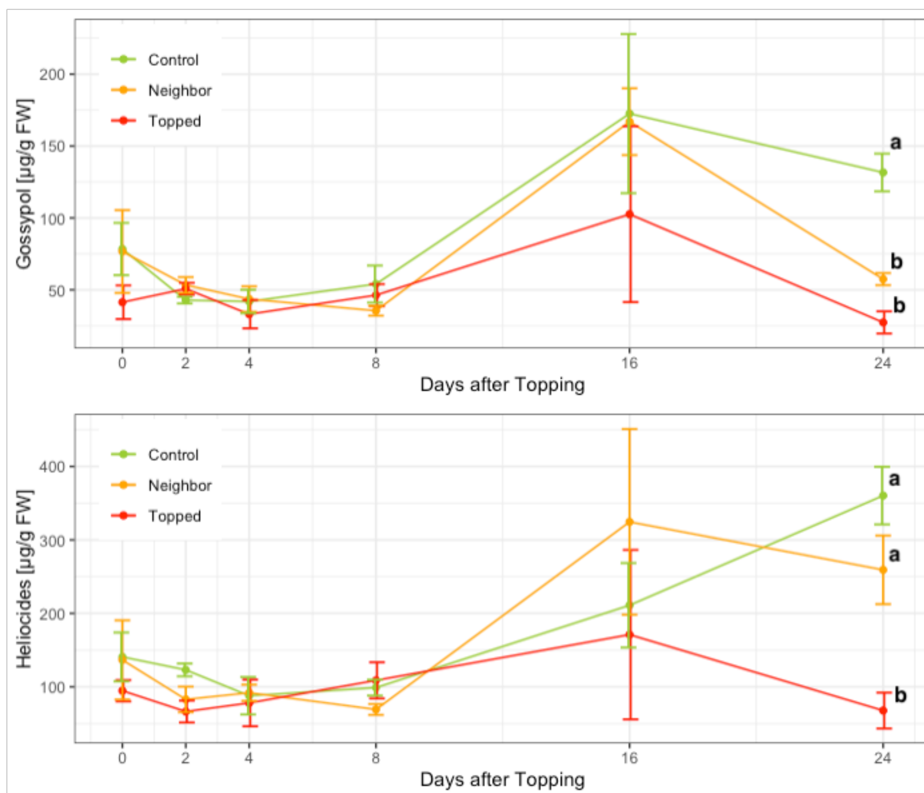

Figure 8. Dynamics of gossypol (top) and heliocides (bottom) content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 24 days after topping. N=4 each day, each treatment. Different letters indicate significant difference within the same day (ANOVA Gossypol day 24: F=34.78, P=5.828e-5; ANOVA Heliocides day 24: F=15.33, P=0.001).

We found a huge variation in phytohormone levels within the same treatment (N=4 per each day of harvesting). In spite of the great variation, trends of increased levels of JA, JA-Ile and OPDA in topped plants, mainly driven by extreme values, were observed (Fig. 9, Fig. 10, Fig. 11).

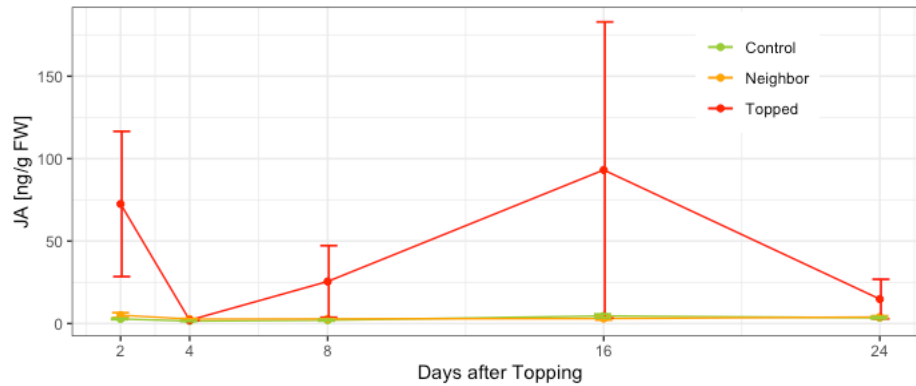

Figure 9. Dynamics of JA content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 24 days after topping. N=4 each day, each treatment. No Statistical analysis was performed due to high variation and limited number of replicates.

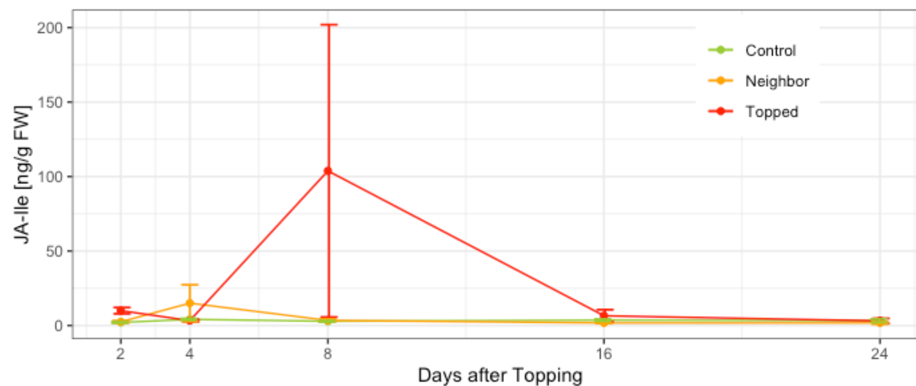

Figure 10. Dynamics of JA-Ile content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 24 days after topping. N=4 each day, each treatment. No Statistical analysis was performed due to high variation and limited number of replicates.

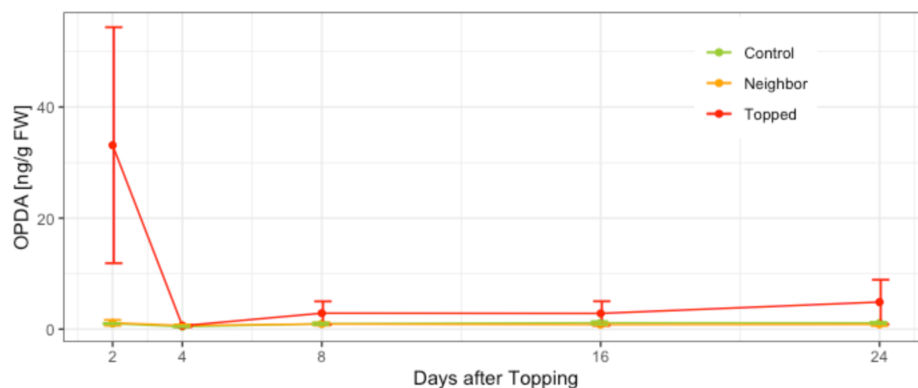

Figure 11. Dynamics of OPDA content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 24 days after topping. N=4 each day, each treatment. No Statistical analysis was performed due to high variation and limited number of replicates.

No differences were observed in SA dynamics (Fig. 12), whereas ABA level in topped plants decreased over the timepoints resulting significantly lower than in control plants 16 and 24 days after topping (Fig. 13).

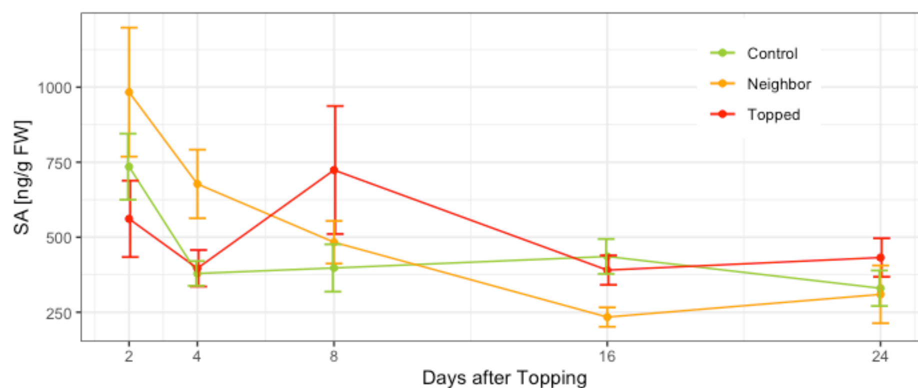

Figure 12: Dynamics of SA content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 24 days after topping. N=4 each day, each treatment. No Statistical analysis was performed due to high variation and limited number of replicates.

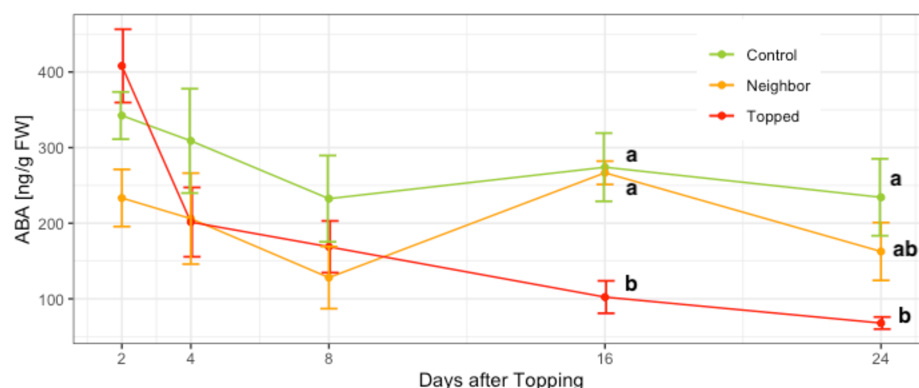

Figure 13. Dynamics of ABA content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 24 days after topping. N=4 each day, each treatment. Different letters indicate significant difference within the same day (ANOVA day 16: F=7.44, P=0.012; ANOVA day 24: F=5.08, P=0.033).

Field experiment 3 (2019)

Contrary to all other previous experiments, in the 2019 field experiment, gossypol and heliocides levels were highest in control plants (Fig. 14). These results need to be taken with precaution since the levels were already higher on the day of topping (i.e day 0, Fig. 15) suggesting that there might have been some plot-related issues. Indeed, the control plot was less fertile than the other one (Thierry Brévault personal communication).

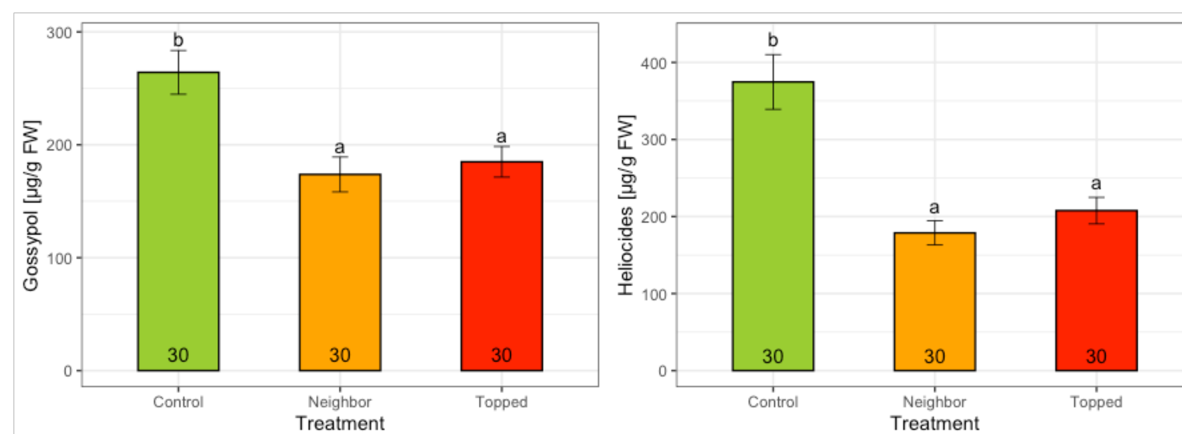

Figure 14. Gossypol (left) and heliocides (right) content in leaves from topped, non-topped neighbor, and non-topped control cotton plants. Days of harvesting were pooled together excluding day 0 (when topping was applied). Different letters indicate significant difference within the same graph (ANOVA gossypol: F=7.66, P=0.0008, ANOVA heliocides: F=19.51, P=6.348e-08). Number of replicates are indicated at the base of each bar.

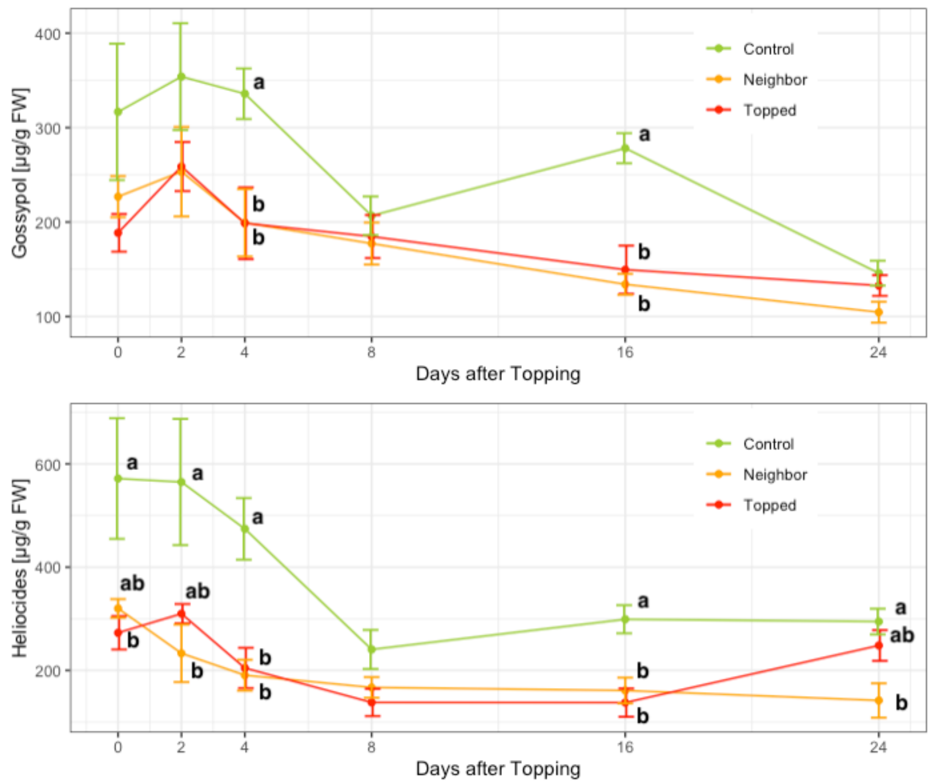

Figure 15. Dynamics of gossypol (top) and heliocides (bottom) content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 24 days after topping. N=6 each day, each treatment. Different letters indicate significant difference within the same day (ANOVA gossypol day 4: F=5.46, P=0.016; ANOVA gossypol day 16: F=18.29, P=9.49e-05; ANOVA heliocides are listed in S.I. Tab. T1). At day 24, heliocides levels in topped plants was marginally significantly higher than in neighbor plants (Tukey Posthoc: p=0.055).

Phytohormone profiling analysis showed an increase of JA and JA-Ile in topped and neighbor plants compared to controls 4 days after topping (Fig. 16, Fig. 17). In the same graphs we can observe how 8 days after topping JA and JA-Ile in neighbor plants were higher compared to topped and control plants. OPDA levels in neighbor non-topped plants similarly spiked 8 days after topping, 24 days after topping the highest level was detected in control plants (Fig. 18).

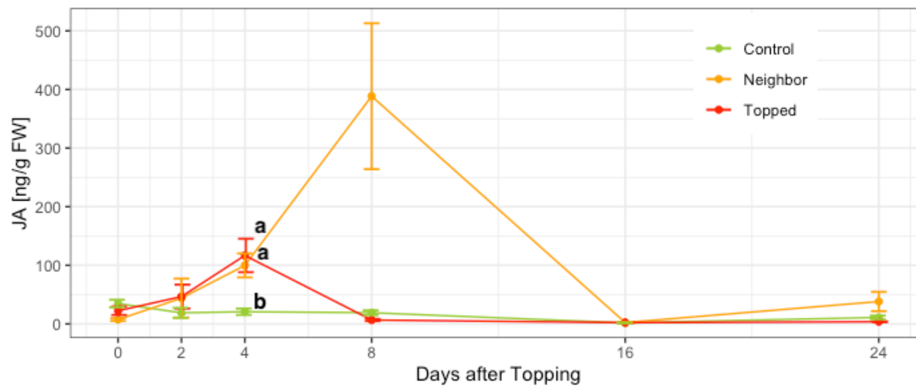

Figure 16. Dynamics of JA content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 24 days after topping. N=6 each day, each treatment. Different letters indicate significant difference within the same day (ANOVA day 4: F=6.24, P=0.01). At day 8 no linear model could be fit due to not normally distributed data.

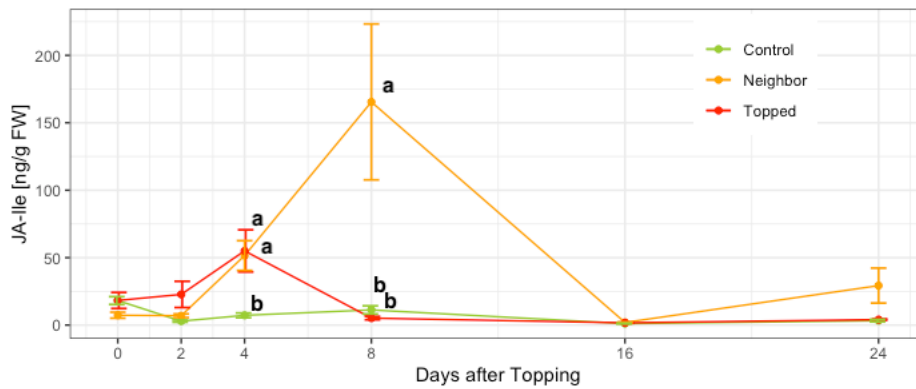

Figure 17. Dynamics of JA-Ile content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 24 days after topping. N=6 each day, each treatment. Different letters indicate significant difference within the same day (ANOVA day 4: F=5.77, P=0.014; ANOVA day 8: F=7.76, P=0.005).

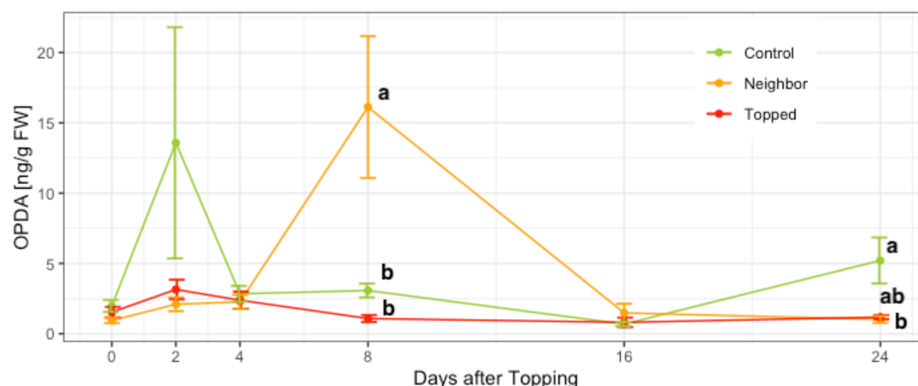

Figure 18. Dynamics of OPDA content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 24 days after topping. N=6 each day, each treatment. Different letters indicate significant difference within the same day (ANOVA day 8: F=10.6, P=0.0014; ANOVA day 24: F=5.49, P=0.016). On day 24 the difference between topped and control plants was marginally significant (Tukey Posthoc: p=0.57).

SA dynamics did not show any particular pattern (Fig. 19). In the last measured timepoint, 24 days after topping, control plants contained significantly higher ABA levels compared to the other two treatments (Fig. 20).

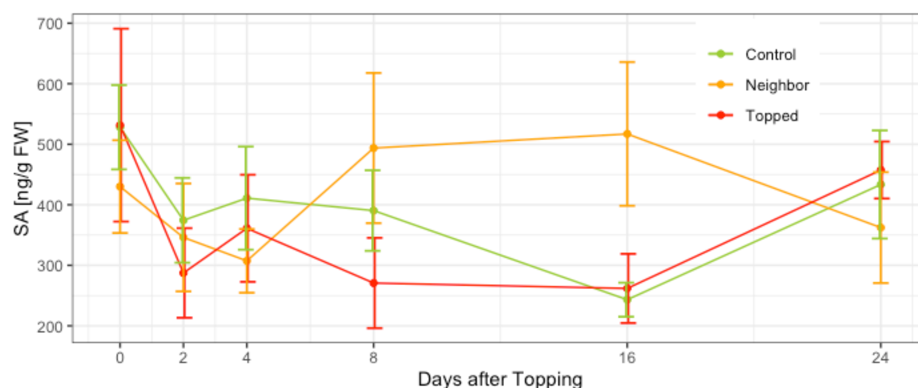

Figure 19. Dynamics of SA content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 24 days after topping. N=6 each day, each treatment. No significant difference was found at any day.

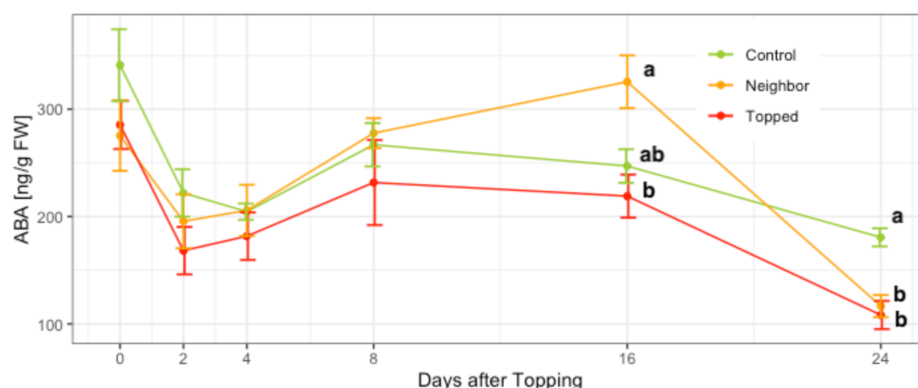

Figure 20. Dynamics of ABA content in leaves from topped, non-topped neighbor, and non-topped control cotton plants up to 24 days after topping. N=6 each day, each treatment. Different letters indicate significant difference within the same day (ANOVA day 16: F=7.27, P=0.006; ANOVA day 24: F=9.75, P=0.002). On day 16 the difference between neighbor and control plants was marginally significant (p=0.058).

Considering cotton square analysis, we could not observe any significant difference among treatments for all the tested compounds (Fig. 21). Gossypol and heliocides levels were the lowest in non-topped neighbor plants. The highest levels of JA, JA-Ile and ABA were detected in control plants.

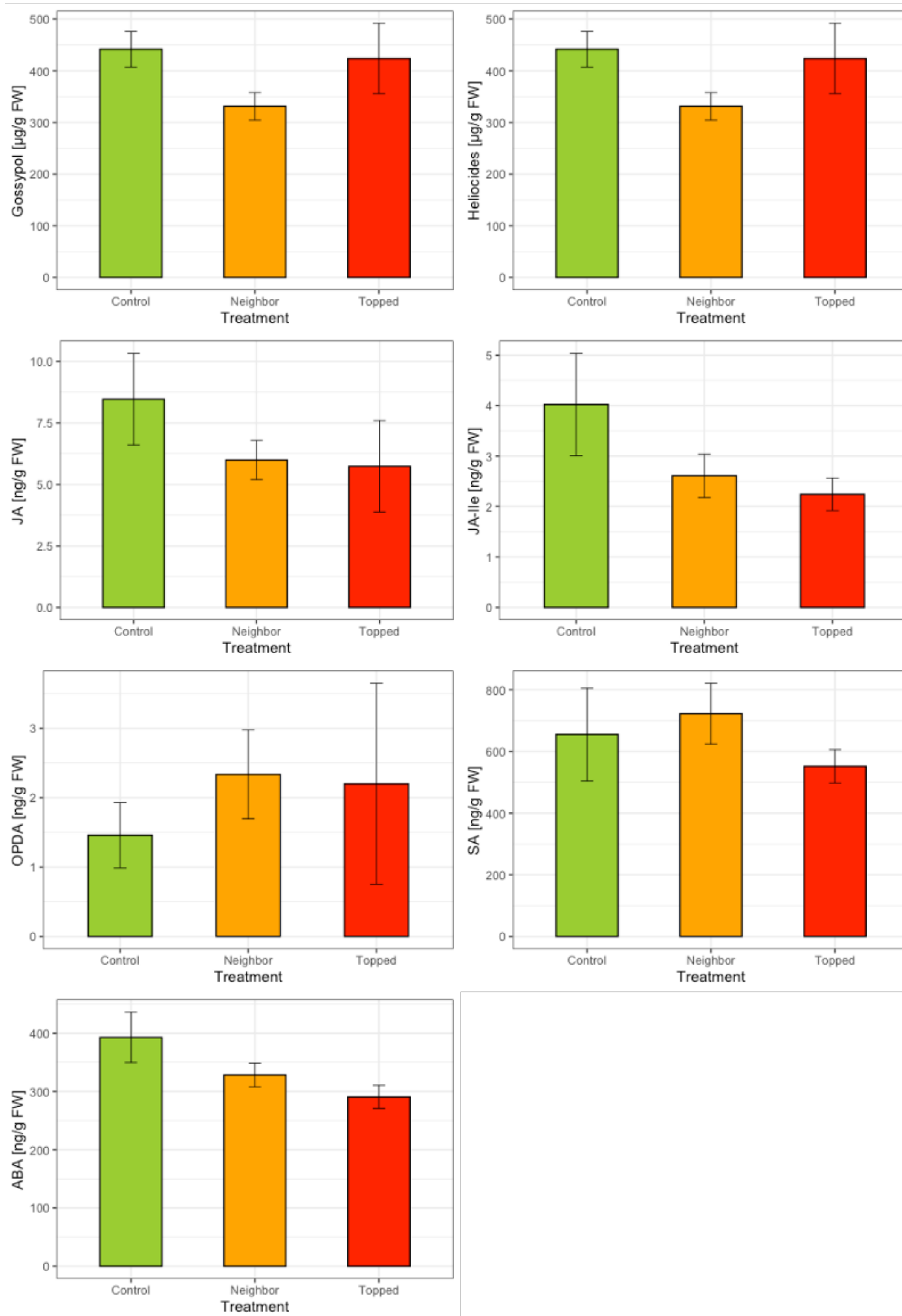

Figure 21. Levels of gossypol, heliocides and different phytohormones in cotton squares collected from topped, non-topped neighbor, and non-topped control cotton plants 18 days after topping. N=6, no significant difference among the 3 treatments was found with any compound.

Discussion

In spite of the great seasonal variability, the results obtained in Field Experiment 1 (2016) and Field Experiment 2 (2018) for gossypol and heliocides suggest that topping actually plays a role in defense activations in the plant itself. Furthermore, the first field experiment (2016) suggested that also intact neighbor plants might benefit from the topping treatment, most likely due to the VOCs emitted by the topped plants (Téréta, 2015; Llandres *et al.*, 2018).

In Field Experiment 3 (2019), gossypol and heliocide levels surprisingly were lower in topped and non-topped neighbor plants compared to control plants. Still, phytohormone analysis showed how topping resulted in increased JA and JA-Ile levels in both topped and non-topped neighbor plants 4 days after topping. Moreover 8 days after topping, non-topped neighbor plants displayed the highest detected levels of JA and JA-Ile, suggesting VOCs mediated defense induction.We need to mention that the pesticides applied before and after topping in field experiments may also have played a role in altering or boosting plants defenses independently from the applied treatment (i.e. topped, neighbor or control) and might explain the observed variability.

The net house experiment, which was performed under controlled conditions and in a pesticide-free environment, showed several different trends. As in Field Experiment 3 (2019), topped and non-topped neighbor plants displayed low levels of gossypol and heliocides compared to controls at the last measured time point. At the same time, we detected some extreme values in JA signaling pathway-related phytohormones (i.e JA, JA-Ile, OPDA) in topped plants. These results further suggest that topping plays an active role on plant defense activation. We care to mention that the detected extreme values could be possibly related to scale infestation found on some plants (Thierry Brévault personal communication). In future it would be interesting to re-conduct the experiment in a highly controlled environment. To provide a pest-free environment or a very low pest pressure only, future experiments should be performed in greenhouses or in open field during the dry season. Furthermore, it would be important to test the effects of pesticides on cotton defense metabolites, comparing pesticide and pesticide-free plots.

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Supporting Information

Parcelle n°	*101*	*102*	*103*	*104*	*105*
Object	B	D	E	A	C
	106	*107*	*108*	*109*	*110*
	E	D	A	B	C
	111	*112*	*113*	*114*	*115*
	B	E	C	D	A
	116	*117*	*118*	*119*	*120*
	C	A	D	B	E
	121	*122*	*123*	*124*	*125*
	A	E	B	D	C

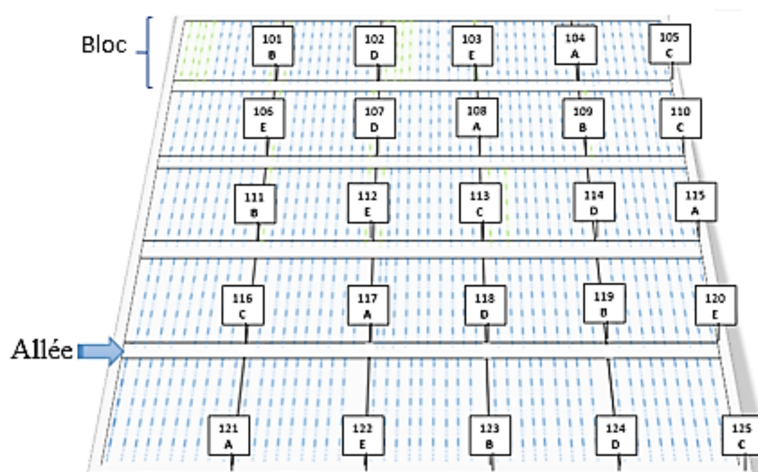

Figure S1. Arrangement of the parcels in Field Experiment 1 (2016)

Table T1. Field Experiment 3 (2019) ANOVA table Heliocides

Day	F-value	P-value
0	4.63	0.027
2	4.93	0.023
4	12.82	0.0006
16	10.77	0.001
24	7.04	0.007

Conclusions and Outlook

Conclusions

Cotton HIPVs directly induce defenses in conspecific neighbors (Chapter 1)

The aim of the first chapter of my thesis was to test the hypothesis that cotton HIPVs are able to directly induce defenses in intact conspecific neighbor plants. The hypothesis was formulated based on studies by Alain Renou and collaborators, in which they showed how topping (pruning) of cotton resulted in reduced insect infestation also in intact neighboring plants, suggesting VOC-mediated induction of cotton defenses (Renou *et al.*, 2011; Téréta, 2015; Llandres *et al.*, 2018). By means of our volatiles exposure system (Chapter 1, Fig.1), we exposed intact cotton plants during 48 hours to either a blend emitted by plants infested by *Spodoptera* caterpillars or to a control blend emitted by intact un-infested plants. We found that receiver plants that had been exposed to herbivore-induced plant volatiles (HIPVs) showed higher gossypol content and increased their volatile emissions compared to controls. Both these findings were corroborated by the increased expression of genes known to be related to both gossypol and volatiles production. Furthermore, in the choice-test we observed that caterpillars avoided HIPVs-exposed plants and fed less on them, especially on younger leaves, probably due to the volatile-mediated defense induction. Surprisingly, when the exposed plants were subjected to caterpillar attack themselves, we no longer found a difference in VOC emissions between HIPV-exposed plants and control plants. These results imply that in cotton HIPV exposure does not prime, but directly induce defenses in receiver plants. Hence, we confirmed our hypothesis that cotton HIPVs play an important role in defense activation in neighbor plants.

Based on these results, we decided to pursue two different axes of research presented in the following chapters. The first screened for strong volatiles emitters or highly inducible receivers among several wild cotton genotypes (Chapter 2), with the idea that these traits might be transferable to cultivated cotton. The second research axis aimed to identify specific compounds in the volatile blend that are responsible for the physiological changes observed in the neighboring plants (Chapter 3 and Chapter 4), again with the intent to possibly apply this knowledge for improved crop protection strategies.

HIPV-mediated defense induction in neighbor plants can be population-dependent (Chapter 2)

The aim of this study was to screen for genotypes of wild cotton that are strong emitters or highly inducible receivers. We hypothesized that wild cotton may possess better defenses compared to cultivated or feral plants. This hypothesis originated from the idea that crop plants may have partially lost their abilities to defend themselves autonomously through artificial selection in favor of specific domestication syndromes (Meyer *et al.*, 2012; Chen *et al.*, 2015a,b).

In a series of experiments, we exposed cotton plants originating from 12 different genotypes to volatiles emitted by *S. exigua*-infested plants from the same genotypes during 48 hours and took several measurements on both source and receiver plants. A total of 10 wild Mexican genotypes from 5 populations, 1 feral genotype, and 1 cultivated variety were used. We observed that two populations (referred to as HC and CO), when infested by *S. exigua*, emitted higher amounts of the typical aromatic HIPV indole compared to other genotypes. A third population (named SA) showed less caterpillar damage and slower caterpillar development. We further found that wild genotypes had a lower germination rate and grew slower compared to cultivated and feral cotton, as indicated by the attained plant height and total leaf area.

Our hypothesis on the effects of cotton domestication was therefore partially confirmed, since some wild genotypes tended to display increased defenses compared to cultivated and feral cotton. Moreover, the higher germination rates and faster growth of cultivated and feral cotton also highlighted the effects of domestication. At this point, we decided to not further extend this topic of my thesis because of the limited availability of seed of wild plants and of the extremely low germination rates of wild seeds, which both were a major impediment. Instead, in the following chapters, we focused on trying to identify specific volatile compounds responsible for the defense inductions in neighboring plants that were observed in Chapter 1.

Cotton HIPVs emitted at least one day after infestation strongly induces defenses in neighboring plants (Chapter 3)

The aim of this chapter was to follow up on the results obtained in the first chapter, trying to determine the specific roles of two sub-groups of the volatile compounds identified in the blend in inducing defense responses in intact receiver plants. We defined the two categories of volatile compounds, based on the changes over time in the composition of the blend, i.e. the

shift from mainly constitutively present volatiles to mainly inducible HIPVs upon continuous herbivore damage. We therefore exposed plants to one of these two blends during 48 hours and compared their defenses to control plants exposed to clean air. We hypothesized that the latter group, the one mainly composed of inducible volatiles enhances defenses more strongly in intact receiver plants because they are generally emitted in large amounts and represent a reliable indicator of continuous damage (Loughrin *et al.*, 1994; Paré & Tumlinson, 1997).

Our hypothesis was confirmed by some of the results. Indeed, the levels of jasmonic acid and jasmonic acid-isoleucine were the highest in plants exposed to the group mainly composed of truly inducible volatiles. Similar results were observed in the expression of several genes involved in gossypol and volatiles production. A preference test revealed that *S. exigua* caterpillars avoid leaves of plants that had been exposed to a blend mainly composed of inducible volatiles. In conclusion, these results suggest that the truly inducible compounds are the main mediators of defense induction in neighbor plants, providing resistance to caterpillars. Based on these observations we decided to expose plants to several representative authentic synthetic compounds, to possibly identify some key compounds that are essential to, singularly or in combination with others, enhance defenses in receiver plants.

Groups of or single authentic volatiles representing typical cotton HIPVs are not sufficient to induce the same responses as natural HIPV blends in receiver plants (Chapter 4)

The aim of this set of experiments was to uncover which specific HIPVs are responsible for the enhanced defenses in neighboring plants observed in the previous chapters. We hypothesized, based on the increased defenses observed in chapter 3, that some of the truly inducible volatiles may be essential to trigger these defenses. In order to test our hypothesis, we prepared dispensers of several single synthetic HIPV and we exposed intact cotton plants to different combinations of constitutive and inducible volatiles.

Unfortunately, none of the tested combinations was effective in significantly inducing defense responses in exposed plants. Contrary to our hypothesis, the expression of two genes related to gossypol production tended to be more upregulated when exposed to three constitutive volatiles or to a mix of three constitutive and three inducible VOCs, compared to exposure to three inducible volatiles only. This suggests that constitutive VOCs are also involved in the process of induction observed in neighboring plants. Based on the results we speculate that for volatile-mediated defense induction cotton plants may either require both

constitutive and inducible volatiles in specific ratios, or a complete HIPV blend. It remains also fully possible that other volatiles that were not tested, even undetected compounds, are implied in the induction.

Thus, our question and our main goal of identifying potent volatile compounds to apply on cotton in the context of IMP strategies remain open.

Cotton topping can increase the production of certain defense metabolites in the plant itself and in neighboring plants (Chapter 5)

This chapter was assembled at the very end of the thesis since it summarizes the results of field experiments performed over several seasons by Thierry Brévault and colleagues in Senegal. My contribution to this project consisted of carrying out the chemical analysis (i.e. gossypol, heliocides and phytohormones analysis) in Neuchâtel. The aim of this project was to test whether the reduced infestations observed in several previous studies in both topped and neighboring cotton plants (Renou *et al.*, 2011; Téréta, 2015; Llandres *et al.*, 2018) may be due to increased amounts of defense metabolites. Our hypothesis was that the above-mentioned defense metabolites are upregulated in leaves in topped plants and in non-topped neighboring plants when compared to control plants in un-topped plots.

Despite high seasonal variability, we could observe that topping generally resulted in an increase of some defense metabolites in a time-dependent manner in the topped plant itself and in some cases also in the neighboring plants. Indeed, in the first field experiment the amount of gossypol was higher in topped and neighboring plants compared to controls. In the second field experiment the same trend was observed in topped plants only. The third field experiment and the net house experiment did not show any clear trend in gossypol or heliocide analysis, but some extremely high JA and JA-Ile levels were detected in topped and neighbor plants.

Outlook

- We confirmed the hypothesized role of HIPVs as plant defense enhancers in cotton, which suggests that they have potential for use in IPM strategies.

- Experiments with wild Mexican cotton populations highlighted trends of improved defense or increased VOCs emissions by some genotypes. These genotypes are good candidates to use in further experiments aimed at improving the efficiency of plant defenses and plant-plant interactions in cotton. Improved methods to increase germination rates of wild cotton seeds are needed.

- Volatiles emitted by cotton plants under prolonged damage (i.e. at least one day) are potent mediator for defense induction in conspecific neighboring plants.

- Emissions of synthetic volatile compounds from dispensers can be highly variable between days or replicates. Alternative solutions to expose cotton plants to synthetic volatile blends should be considered.

- More research is required to identify potent synthetic volatiles, single compounds and/or mixtures of several compounds, able to trigger defenses in neighbor plants.

- In spite of high seasonal variability, cotton topping generally increases the content of some defense metabolites in the plant itself and in neighboring plants. Higher simultaneous replications in multiple fields and during different seasons may better unravel the full effects of topping. The possible effects of pesticide applications should be assessed.

- Field experiments including exposure to plant-derived or synthetic VOCs should be performed in order to test the efficiency of HIPV-mediated cotton pest resistance under natural conditions.

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Annex

Publications:

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RESEARCH ARTICLE

Effects of early-season insect herbivory on subsequent pathogen infection and ant abundance on wild cotton (*Gossypium hirsutum*)

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https://besjournals.onlinelibrary.wiley.com/doi/pdf/10.1111/1365-2745.13131

Bottom-up control of geographic variation in insect herbivory on wild cotton (*Gossypium hirsutum*) by plant defenses and climate

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https://bsapubs.onlinelibrary.wiley.com/doi/epdf/10.1002/ajb2.1330

Publications in Preparation:

Teresa Quijano-Medina, Ted C.J. Turlings, Paula Sosenski, **Luca Grandi**, José Carlos Cerveral, Xoaquín Moreira, and Luis Abdala-Roberts. Effects of soil salinity on the expression of direct and indirect defences in wild 2 cotton (Gossypium hirsutum) and its underlying mechanisms. (Accepted in Journal of Ecology, July 2020)

Luca Grandi, Wenfeng Ye, Armelle Vallat, Gaetan Glauser, Luis Abdala-Roberts, Thierry Brevault, Betty Benrey, Ted C.J. Turlings. Caterpillar-induced cotton volatiles activate multiple defenses in neighboring cotton plants (Tentative title) (In preparation, to be submitted in New Phytologist)

Luca Grandi, Wenfeng Ye, Armelle Vallat, Gaetan Glauser, Luis Abdala-Roberts, Thierry Brevault, Betty Benrey, Ted C.J. Turlings. Constitutive vs inducible cotton volatiles: which ones trigger defenses in neighboring cotton plants? (Tentative title) (in preparation)

Arooran Kanagendran, **Luca Grandi**, Ted C.J Turlings. Foliage volatile trapping by single and combined porous sorbents in maize: an efficiency analysis. (Tentative title) (in preparation).

Master's Thesis Supervisions

Julien Dongiovanni 2017-2018: "*The effect of Vu-In on VOCs emission and parasitoids attractiveness*".

Maeva Stoebner 2019-2020: "*A story of attraction and development, Cotesia marginiventris and cotton plants*" (tentative title)

Attended Meetings and Courses (Poster and Oral Presentations)

Annual PhD Students Meeting 2017 (Neuchâtel, Switzerland), Poster

Insect Chemical Ecology 2017 (Penn State, USA), Poster

Biogenic Volatiles – Exchange at Different Scales and Interactions with Ecosystem Processes 2017 (Copenhagen, Denmark), Oral Presentation

International Society of Chemical Ecology 34th Annual Meeting 2018 (Budapest, Hungary), Poster

Annual PhD Students Meeting 2019 (Neuchâtel, Switzerland), Oral Presentation

International Society of Chemical Ecology 35th Annual Meeting 2019 (Atlanta, USA), Oral Presentation

Extension/Vulgarisation

Swiss Television 2017 (RTS): https://avisdexperts.ch/videos/view/6877

Swiss Television 2018 (RTS): https://avisdexperts.ch/videos/view/8272

Fête des Sciences, Ecole Moser S.A 2018 (Nyon, Switzerland) Oral Presentation

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Research Skills

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Secondary Metabolites

Basic Molecular Biology
DNA/RNA Extractions & qPCR

Computational Skills
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Sterile Work
Bacteria Culturing
Media Preparation
Plant Virus Inoculation (ELISA)

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Italian:	Native
English:	Fluent
French:	Fluent
German:	Fluent
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Swiss Army

Sergeant (Group Leader)
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Profile

I am a result-oriented person who communicates well with other people. In the working environment, I am punctual, organized, and I perform tasks efficiently while paying attention to details. In my free time, I enjoy practicing team sports and spending time in nature with friends and family.

Education

- **PhD in Biology**
  University of Neuchâtel | 2016 – 2020
  Fundamental & Applied Research in Chemical Ecology Lab. (Prof. T.Turlings)
- **MSc in Biology**
  ETH Zurich | 2013– 2015
  Biocommunication & Entomology Lab. (Prof. C. De Moraes)
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  ETH Zurich | 2010 - 2013

Working Experience

- **Supervision & Teaching (MSc & BSc Students)**
  FARCE Lab., University of Neuchâtel | 2016 - 2020
- **Events Organisation & Tutoring**
  University of Neuchâtel | 2016 – 2020
  VeBiS, ETH Zurich | 2011-2013
- **Research Assistant**
  Biocommunication & Entomology Lab., ETH Zurich | 2013 - 2015
- **Department President**
  VeBiS, ETH Zurich | 2012 - 2013

Selected Presentations & Paper

- **International Society of Chemical Ecology Meeting, Atlanta (USA)**
  Invited Speaker | 2019
- **PhD Annual Meeting University of Neuchâtel, Neuchâtel (CH)**
  Best Talk Award | 2019
- **Publication in Journal of Ecology**
  Abdala-Roberts, L. & Pérez Niño, B. & Moreira, X. & Parra-Tabla, V. & **Grandi, L.** & Glauser, G. & Benrey, B. & Turlings, T. “Effects of early-season insect herbivory on subsequent pathogen infection and ant abundance on wild cotton (*Gossypium hirsutum*)” | 2019

Student Jobs

- Promotion & Logistic (Spool, ZH)
- Bartender (St. Moritz, GR)
- Crew Member (Mc Donald‘s, TI)
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