CARLA CRISTINA MARQUES ARCE

INDUCED DEFENSES AND THEIR EFFECT ON INTERACTIONS AMONG HERBIVORES ON SOLANACEOUS PLANTS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

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"Desconfie do destino e acredite em você. Gaste mais horas realizando
que sonhando, fazendo que planejando, vivendo que esperando...
Porque, embora quem quase morre esteja vivo, quem quase vive, já morreu..."
Sarah Westphal

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SUMÁRIO

RESUMO

ARCE, Carla Cristina Marques, D. Sc., Universidade Federal de Viçosa, Agosto de 2014. **Induced defenses and their effect on interactions among herbivores on Solanaceous plants**. Orientador: Eraldo Rodrigues de Lima. Coorientadores: Arnoldus Rudolf Maria Janssen e Flávia Maria da Silva Carmo.

Plantas tem desenvolvido diversas estratégias afim de se defender contra herbivoria. Depois de atacadas plantas induzem defesa, mas por outro lado herbívoros podem evitar plantas com altos níveis de defesa induzida. Além disso, muitos herbívoros podem interagir uns com os outros através da defesa induzida na planta hospedeira. Esta tese teve como foco a indução de defesa local e sistêmica de plantas da família Solanaceae. O principal objetivo foi elucidar os mecanismos e relevâncias ecológicas sobre interações entre herbívoros acima e abaixo do solo mediadas pela defesa da planta hospedeira. Os resultados sugerem que a ausência de uma enzima específica do metabolismo de açúcares alterou os metabólitos induzidos pela planta depois de herbivoria foliar, mas nenhum efeito no inseto foi encontrado. Quando um herbívoro foliar se estabelece primeiro na planta hospedeira, um herbívoro subsequente na raiz foi positivamente afetado e também o fitness da planta hospedeira. De maneira oposta, o herbívoro de raiz alterou a seleção da planta hospedeira do herbívoro foliar que compartilhava a planta hospedeira. Este preferiu ovipositar em plantas sem o dano na raiz e esta preferência impactou negativamente o desenvolvimento da prole. Em geral, esta tese adicionou importantes evidências sobre os mecanismos e fatores ecológicos envolvidos em defesa induzida de plantas modulando interações entre herbívoros.

ABSTRACT

ARCE, Carla Cristina Marques, D. Sc., Universidade Federal de Viçosa, August, 2014. **Induced defenses and their effect on interactions among herbivores on Solanaceous plants**. Adviser: Eraldo Rodrigues da Silva. Co-advisers: Arnoldus Rudolf Maria Janssen and Flávia Maria da Silva Carmo.

Plants have evolved a range of strategies to defend themselves against herbivory. Including the induced defense after a herbivore attack, on the other hand, herbivores can avoid plants with higher induced defense. Moreover, many herbivores can interact with each other mediated by induced plant defenses, despite being on different parts of the host plant. In this thesis I have focused on local and systemic induction of defense in Solanaceae plants. The main aim was to elucidate mechanisms and ecological relevance around interactions between above- and below-ground herbivores mediated by plant defense. The results showed that the absence of a specific enzyme in sugar metabolism changed the induced plant metabolites under herbivory, but no strong effect on herbivore was observed. When a foliar herbivore establishes first on the host plant, a subsequent herbivore in the root was positively affected and the host plant fitness was also impacted. In the opposite way, a root feeder changed the host plant selection of the leaf herbivore that was sharing the same plant. The leaf herbivore preferred to oviposit in plants without root damage and their preference negatively impacted the offspring development. In general, this thesis add important evidences about the mechanisms and ecological involved on induced plant defense modulating the interactions between herbivores.

Chapter 1

General Introduction

Plants and insects are associated for millions of years and their coexistence demonstrates intricate interdependence and interactions. Plants need insect pollinators for their reproduction and phytophagous insects need plants for their development and protection. In the context of evolutionary arms races with their enemies, plants have evolved various strategies to defend themselves against herbivores. The constitutive defenses include any continuously present plant trait (e.g. trichomes, toxins and digestibility reducers) that improves the resistance of a host plant to insect attack (Karban & Baldwin, 1997; Kessler & Baldwin, 2002). Inducible defenses, on the other hand, are traits that become more abundant or active when the plant is under attack (Karban & Myers, 1989). Two types of inducible defenses have been observed: direct and indirect defenses. Direct defenses are characterized by their immediate negative impact on herbivores, while indirect defenses act through the attraction or maintenance of natural enemies of the herbivores (Kessler & Baldwin, 2001, 2002; Heil, 2008). Induced defenses may have several advantages such as reducing biosynthetic costs of defense or avoiding that other organisms can exploit the defense for their own benefit (Karban & Baldwin, 1997; Cipollini et al., 2003; Zangerl, 2003).

Plant tissues are not attacked uniformly nor are they uniformly responsive to insect herbivores, in part because they are modular organisms made up of source and sink tissues. While sink tissues (e.g. young leaves, flowers, and roots) are usually the major determinants of carbon partitioning in plants, defense induction can also reconfigure the primary metabolism to provide additional substrates and energy required to produce secondary metabolites (Schwachtje & Baldwin, 2008). This results in complex patterns of defense that may vary spatially and temporally (Karban & Baldwin, 1997).

Herbivore-induced changes do not only occur locally, but involve non-attacked tissues as well (Schwachtje & Baldwin, 2008; Erb et al., 2009a). Systemic effects following herbivory can have fitness consequences for temporally or spatially separated organisms (Sticher et al., 1997; Poelman et al., 2008; Erb et al., 2009b). Both primary and secondary metabolic profiles of shoots can be altered upon root herbivory and *vice versa* (Gange & Brown, 1989; Bezemer et al., 2003; Hol et al., 2004). Root herbivory causes systemic induction of plant defense compounds in the foliage and *vice versa*. Root feeding by wireworms can increase the amount of terpenoids in 50% in leaves (Bezemer et al., 2003). Several other studies have a reported that plant defense compounds increase in the foliage following root herbivory (e.g. Bezemer et al., 2004; Soler et al., 2005; van Dam et al., 2005). But a decrease of defensive compounds can occur as well (e.g. Bezemer & Van Dam, 2005; van Dam et al., 2005). Currently, more studies examine the impact of root-feeders on above-ground herbivores than the contrary, possibly because the performance of root-feeders is more difficult to observe and quantify (Johnson et al., 2007). Soler et al. (2007) reported a significant increase in root indole glucosinolates following foliar herbivory. Other studies also show that foliar herbivory can lead to an increase in plant defense compounds in roots (Ludwig-Muller et al., 1997; van Dam et al., 2005). Besides foliar nutritional resources are allocated in roots (Schwachtje et al., 2006). According to Kaplan et al. (2008), an increase in fecundity of nematodes was associated with an increase in roots of photoassimilates elicited by caterpillar feeding. The same author found no evidence that caterpillar leaf herbivory alters the secondary chemistry of tobacco roots (Kaplan et al., 2008).

In this thesis I have focused on local and systemic induction of defense in Solanaceae plants. The main aim was to elucidate mechanisms and ecological relevance around interactions between above- and below-ground herbivores mediated by plant defense.

The first chapter (this chapter) is the General Introduction that provides to readers an overview of the central topic of the thesis and the specific questions addressed in the following chapters. In chapter 2, the central question is: What is the role of cell wall invertase inhibitor in plant-insect interaction? A range of regulatory mechanisms supports the idea that invertases play a crucial role in plant metabolism. It is also reported that this invertase expression is related to wounding and pathogen infections. Therefore none information about this invertase inhibitor involved in plant defense against insects have been reported. This chapter provides through the plant mechanism and the ecological impact in the insect, the first step to elucidate the role of cell wall invertase in plant defense. To accomplish this goal, we used the *Nicotiana attenuata* plants and its specialist herbivore *Manduca sexta*; the studied system has been reported as an ecological model for plant-insect interaction. Besides the fact that plant-insect interactions are complex with plants being simultaneously attacked by more than one herbivore; this multiple attack can occur in the same parts of the plant or spatially separated (e.g. leaves and roots). Sharing of the same host plant by many herbivores can mediate interactions among them, through plant defense. Thus, in subsequent chapters I evaluated the interactions mediated by host plants among above- and bellow-ground herbivores. In chapter 3, the central question is: What is the impact of a foliar herbivory in the root nematodes and their consequences to the plant with regard to costs associated in defense? Studies about above- and below-ground interactions have been well documented in the last years, however, the effect of above-ground on below-ground herbivores has been poor investigated. For this, we used the *N. attenuata* plants, its specialist above-ground herbivore *M. sexta* and the generalist root-knot nematode *Meloidogyne incognita*. In chapter 4, the central question is: How is the influence of a below-ground herbivore on the preference-performance of a foliar herbivore? Here, we used the cultivated tomato plants *Solanum lycopersicon*, its specialist above-ground herbivore *Tuta absoluta* and the generalist root-knot nematode *Meloidogyne incognita*. Chapter 5 is the General Conclusion of all results obtained from this thesis. In the following subsection I will briefly describe the model systems used in the experiments.

The systems

Nicotiana attenuata is a tobacco species native to southwestern United States. Because *N. attenuata* is highly plastic in nature, it should have facilitated its adaptation to natural habitats. Habitat selection of *N. attenuata* is largely determined by its peculiar germination behavior. Seeds from a long-lived seed bank germinate in post-fire environments by responding to germination stimulants in wood-smoke (Baldwin et al., 1994). In post-fire burns with high nitrogen (N) content in the soil, these plants act as pioneering species. With highly synchronized germination behavior and living in a habitat with fast rising temperatures and resources being lastly depleted, *N. attenuata* plants have been selected for rapid growth. The representation of a genotype of *N. attenauta* in the seed banks is determined by how successfully a particular genotype alters its phenotype to respond to these highly variable biotic selection regimes, translating vegetative growth into reproductive output, i.e. seed (Baldwin, 2001). Therefore, *N. attenuata* caters to the needs of an ideal model system for understanding the genetic basis of regulation of phenotypic plasticity. Herbivores from more than 20 different taxa attack these plants, and specific herbivore populations vary from year to year because they also recolonize the habitat after fire. An insect that is frequently found attacking *N. attenuata* plants is the tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae), the larvae are polyphagous and become oligophagous when feeding on Solanaceous plants. They remain polyphagous when reared on artificial diet or non-host plants (Yamamoto, 1974). *M. sexta* is used extensively in research, particularly for physiological and biochemical studies (Kanost et al., 2004). Its large size (± 10 cm), makes this insect easy to manipulate physically.

Tomato, *Solanum lypersicon* L. (Solanaceae), is one of the most important vegetable crops grown worldwide and can be used as a host by approximately 200 species of herbivores. Tomato plants have been used as a model for studies involving gene expression (Fung et al., 2006; Kok et al., 2007), induced defenses against bacteria (Cavalcanti et al., 2006), nematodes (Molinari & Loffredo, 2006) and volatiles released after injury (Farag & Paré, 2002). Among the insects associated with this culture, the specialist tomato leafminer *Tuta absoluta* M. (Lepidoptera: Gelechiidae) which attacks leaves, flowers and fruits, which may cause even death of the plant and nowadays is considered a key pest of this crop in Brazil. The first occurrence of *T. absoluta* in Brazil dates from 1979, at the coast of Paraná state (Muszinski et al., 1982), and at the end of the 80s this pest was reported in all fields of tomato production in this country (France, 1993). Under laboratory conditions, the entire life cycle of *T. absoluta* ranges 26-35 days, their miner larvae feed initially on the leaf mesophyll, building transparent galleries.

A generalist organism that attacks many plants and exploits a wide variety of ecological niches are the nematodes. According to Semblat et al. (2000), the root-knot nematodes belonging to the genus *Meloidogyne* (Treud) are sedentary endoparasites that cause extensive damage to a wide range of economically plants, such as Solanaceae plants (e.g. *Nicotiana attenuata* and tomato plants). Infected plants show reduced growth, with small, yellowish leaves, which wilt prematurely. The *M.*

incognita and *M. javanica* (Chitwood) species are among the most harmful species of Brazilian agriculture (Peixoto et al., 1999).

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Chapter 2

The effect of silencing a *Nicotiana attenuata* proteinaceous invertase inhibitor on food utilization by specialist herbivore *Manduca sexta*

Abstract

Plant invertases are sucrolytic enzymes essential for plant metabolism and development whose activity is regulated post-translationally by proteinaceous inhibitor. Changes in plant invertase activity have been well documented in response to abiotic and biotic stresses, including pathogens and insects. However, the role of proteinaceous inhibitors in regulating these changes within the context of plant defense is unknown. Here we show that a putative invertase inhibitor (NaCWII) is up-regulated 5 hours following attack by the specialists herbivore, *Manduca sexta.* To understand the potential defensive role of NaCWII, we silenced its expression by *Agrobacterium* transformation and measured *M. sexta* performance and utilization of irCWII and wild-type (EV) plants. Larval performance was correlated to changes in plant primary and secondary metabolism following simulated herbivory. Although we found that irCWII displayed a greater depletion of carbohydrates and secondary defense metabolites relative to their EV controls, we found that these changes did not impact *M. sexta* performance despite changes in digestibility of the leaf tissue. Despite the lack of evidence for a defensive role for NaCWII this work highlights the strength of using plant transformation to uncover novel roles for herbivore-induced genes of interest, and provides the foundation for future studies to understand NaCWII's role in post-translational regulation of cell-wall invertase during the process of plant defense.

Keywords: Cell wall invertase inhibitor, *Nicotiana attenuata*, tradeoff growth-defense, *Manduca sexta*

Introduction

Plant response to attack by insects lead to a local need for resources to support defense by inducing a profound and dynamic reconfiguration of both primary and secondary metabolism (Schwachtje & Baldwin, 2008). This induction of defense involves a massive redistribution of energy towards defense response. Carbon is in high demand when a plant tissue is requested upon to defend itself (this incurs a metabolic cost in terms of biosynthesis, storage, and fitness impacts). Yet, local photosynthetic rates are known to decrease upon herbivory (Creelman & Mullet, 1997; Hristova & Popova, 2002; Izaguirre et al., 2003; Schwachtje & Baldwin, 2008; Gómez et al., 2010).

Plants may not be adding to their carbon pool via photosynthesis; however they may rely on other means to amass/store soluble sugars and starch to support secondary metabolism generating plant defenses, as well as support growth (Chapin et al., 1990). For example, re-allocating resources from other tissues/inducing local sink strength (reviewed by Schultz et al., 2013), or manipulating invertase activity to ‘protect’ sucrose (via regulatory mechanisms, including protein inhibitors). Cell wall invertase (CWI), an enzyme ionically that bound to plant cell walls, facilitates phloem unloading at sink tissues by cleaving sucrose into fructose and glucose (Sturm & Tang, 1999). In addition to its pivotal role in plant development (Roitsch & González, 2004), invertases have also been implicated in response to stress via controlling carbon allocation and sugar signalling (Koch, 2004). For these reasons, the activity of CWI needs to be tightly regulated at transcriptional and post-transcriptional levels (Webe et al., 1996; Rausch & Greiner, 2004; Essmann et al., 2008).

Studies have focused primarily on transcriptional induction of invertase genes or direct changes in enzymatic activity in extracts. However, apart from transcriptional and translational regulation, cell wall invertase activity are known to be also highly regulated at the post-translational level through interaction with their inhibitors, a group of small peptides with molecular masses between 15-23kDa (Ruan et al., 2010).

These regulatory mechanisms are known to be affected by mechanical damage, grazing, pathogen infection, galling, all classes of plant hormones, and the development of floral organs, fruits, and seeds, among other tissue types (Roitsch & González, 2004). There has been significant progress in understand transcriptional regulation of invertases, yet their regulation by proteinaceous inhibitors is less understood. Those studies which have examined the role of invertase inhibitors have focused on their role in plant development (Jin et al., 2009) or during plant-pathogen interactions (Bonfig et al., 2010). Jin et al. (2009) found that silencing the tomato invertase inhibitor (INVINH1) increases invertase activity in developing fruits, increases seed size, and delays leaf senescence. Bonfig et al. (2010) demonstrates a role for proteinaceous invertase inhibitors in post-translational regulation of invertase activity during pathogen infection. They found that the expression and activity of invertase inhibitors were suppressed in *Arabidopsis thaliana* during *Pseudomonas syringae* infection, which released cell-wall invertase from post-translational inhibition and allowed a successful induction of plant defense. Infiltration of *A. thaliana* leaves with acarbose (a chemical invertase inhibitor) increased plant susceptibility to this pathogen, suggesting a role for extracellular invertase in successful to plant defense.

Although the presence of proteinaceous invertase inhibitors in plants has been reported for many species (Pressey, 1994; Krausgrill et al., 1998; Bate et al., 2004; Reca et al., 2008; Bonfig et al., 2010), and their role in controlling sucrose use in plants and developmental processes has been well-established (Rausch & Greiner, 2004; Jin et al., 2009), their involvement in regulating growth and metabolism within the context of plant defense systems against insect herbivores has never been addressed. Here, we explore the function of INVINH1 in the interactions between *Nicotiana attenuata* and its specialist herbivore, *Manduca sexta* by cloning its respective homolog in *N. attenuata* (NaCWII) and silencing transcript levels using an *Agrobacterium*-mediated transformation method. Subsequent changes in plant metabolism and its impact on *M. sexta* larvae incurred following simulated herbivore attack were examined to understand the role of NaCWII in regulating the defense against insect herbivore in *N. attenuata*.

Materials & Methods

Plant growth conditions

Seeds of the 31^{st} generation of an inbred line of *Nicotiana attenuata* Torr. Ex Watts originally collected from Utah, USA, were used as the wild-type genotype in all experiments. Seeds were first surface sterilized and incubated with 1:50 (v/v) diluted liquid smoke (House of Herbs, Passaic, NY, USA) and 0.1M gibberellic acid (GA3), and germinated on plates containing Gamborg's B5 medium as described in Krugel et al. (2002). Ten days after germination, seedlings were transferred to Teku pots (Poppelmann GmbH & Co. KG, Lohne, Germany) for an additional 10-12 days, followed by a final transplanting to 1L pots filled with washed sand. Plants were grown under glasshouse conditions at 45-55% relative humidity, 24-26°C, and 16:8 light:dark and 14:14:14 of N:P:K twice per week. (Krugel et al., 2002).

Identification of a CWII homolog in *N. attenuata* and plant transformation

The amino acid sequence of an invertase inhibitor in tomato INVINH1 *Solanum lycopersicum* (Jin et al., 2009) was used to search *Nicotiana* RefSeq databases by basic local alignment (BLAST). Full-length amino acid sequences of *Nicotiana spp.* were aligned using BioEdit version 7.0.9.0 (Hall, 1999). Accession numbers of tobacco and tomato can be found in the GenBank/EMBL database under: NT VINVINH, AY594170; NT CWINVINH, Y12805; LE INVINH1, AJ010943. Specific primers (forward 5‘-ACATGCAAGAACACACCAAATTACCAA and reverse 5‘-TCCCCTGTTTCACTCCGTTTGTCC) were designed to via PCR amplify the respective homolog in *N. attenuata* (NaCWII). PCR product was obtained using *N. attenuata* leaf cDNA as template and a 293-bp fragment was cloned into the pRESC8 transformation vector in an inverted repeat orientation driven by the CaMV 35S promoter (Gase et al., 2011). This vector was transformed into wild-type *N. attenuata* plants, using *Agrobacterium*-mediated transformation methods described by

Krugel et al. (2002). Independent transgenic lines were selected for homozygosity by hygromycin resistance (Gase et al., 2011). Real-time qPCR was used to confirm silencing efficiency and to select two single-insert-containing transgenic lines, irCWII439-5 and irCWII432-3, which were used in further experiments as irCWII-1 and irCWII-2, respectively. For all experiments, WT plants transformed with an empty vector construct (EV, line A-03-9-1) were used as control.

Regulation of a *N. attenuata* CWII by wounding and simulated herbivory

A time course experiment was conducted to establish expression dynamics of NaCWII following simulated herbivory. Rosettes of EV, irCWII-1 and irCWII-2 plants(n=27) were treated by rolling a serrated fabric pattern wheel three times on each side of the midvein of three middle-aged leaves per plant, and by immediately applying 20 μL of water (W+W) or *M. sexta* oral secretions (W+OS) diluted 1:5 in distilled water to the fresh puncture wounds of each leaf. Control leaves remained untreated. Leaves were harvested 1, 3 and 6 h after treatments and immediately frozen in liquid nitrogen for subsequent analysis. Total RNA was extracted from ground tissue by TRIZOL method, followed by DNase-I treatment (Fermentas) according to the manufacturer's instructions. Five micrograms of total RNA were reverse-transcribed using oligo(dT)18 and the SuperScript-II Reverse Transcriptase kit (Invitrogen). Resulting cDNA was used for quantitative analysis with SYBR Green I following the manufacturer's protocol, and the $\triangle$Ct method was used for transcript evaluation (Bubner & Baldwin, 2004). ACTIN was used as an endogenous reference.

Regulation of cell-wall and soluble invertase activity by NaCWII after simulated herbivory

To investigate whether *M. sexta* attack alter invertase activity in *N. attenuata* we measured cell-wall and soluble invertase activities in leaf extracts of EV, irCWII-1 and irCWII-2 plants following repeated W+OS treatments. Every 48 h, two leaves per plant were treated with 20 μL per leaf of 1:5 diluted *M. sexta* oral secretions over a total of 6 days, resulting in six treated leaves per plant. Leaves (n=30) were harvested 6 days after the first treatment at mid-day. Cell-wall and soluble invertase activities were determined according to Ferrieri et al. (2013).

Primary and secondary metabolite profiles of NaCWII-silenced plants

An additional time course experiment was performed to assess the impact of silencing NaCWII on herbivore-induced reconfiguration of primary metabolism (Schwachtje & Baldwin, 2008). Herbivory was simulated every other day for 8 days using W+OS treatments on two leaves per plant (4 treatments, 8 damaged leaves per plant in total), as described above. Leaves were harvested at 2:00 AM, 6:00 AM, 2:00 PM and 10:00 PM following the last day of treatment. Soluble sugars (glucose, fructose and sucrose) and starch levels were extracted from 100 mg of frozen leaf material using an 80% (v/v) ethanol extraction procedure according to Machado et al. (2013). Sucrose, glucose and fructose were quantified enzymatically in supernatant pooled during the extraction steps as described by Velterop & Vos (2001), and remaining pellets were used for starch determination according to Smith & Zeeman (2006).

Secondary metabolites were extracted from 100 mg of frozen leaf material using a 40% methanol extraction procedure (Gaquerel et al., 2010). Separation of leaf extracts was performed using a RSLC system (Dionex) according to Kim et al. (2011). Briefly, 4 μL of extract were injected onto a C18 column (Acclaim, 2.2 μm particle size, 150 mm × 2.1 mm diameter, Dionex Corporation, Sunnyvale, USA). The mobile phase consisted of solvent A (0.1% (v/v) acetonitrile and 0.05% (v/v) formic acid in deionized water) and solvent B (0.05% (v/v) formic acid in acetonitrile) in the following gradient: 0-0.5 min 10% B, 0.5-6.5 min linear gradient 80% B, 6.5-10 min 80% B, and re-equilibration at 10% B for 3 min with a flow rate of 300 μL/min. An ESI-TOF mass spectrometer (Bruker Daltonic, Bremen, Germany) was used to determine the molecular mass of ionized molecular fragments and the amounts of the eluted analytes. The capillary voltage was 4500V, and dry gas (200°C) flow rate was 8 L/min. Detected ion range was from m/z 200 to 1400 at a repetition rate of 1 Hz. The mass calibration was achieved using a sodium formate solution (10 mM sodium hydroxide and 0.2% formic acid in isopropanol/water 1:1, v/v).

The effect of NaCWII on *M. sexta* performance and behavior

To test whether the *N. attenuata* cell wall invertase inhibitor affects the activity of *M. sexta* mid-gut invertase, insect nutritional indices (Waldbauer, 1968) were assessed for larvae feeding on EV, irCWII-1 and irCWII-2 tissue and mid-gut tissues were harvested for invertase activity assays. *Manduca sexta* larvae (n=150) were reared to 4^{th} instar on irCWII-1, irCWII-2 or EV plants under normal glasshouse conditions after which they were transferred to individual Petri dishes (3.5 × 4.5 cm; DG-Distler-Gastro GmbH, Erfurt, Germany) placed in a climate chamber (70±5%RH, 28±2°C, 16:8h light:dark) for 24 h. Larvae were weighed before and after this starvation period. During the following 2 days, larvae were fed *ad libitum* in a diet of irCWII-1, irCWII-2 or EV leaves previously treated with 15 μL of *M. sexta* oral secretion (W+OS). Initial leaf fresh masses were recorded at each feeding time. At the end of the experiment, remaining plant tissue, larvae, and frass were dried and weighed. The following nutritional indexes were calculated according Waldbauer (1968): CI = [C/(G*T)] measures the amount of leaf mass consumed; AD = [(C-F)/C]*100 approximated digestibility; ECI = (G/C)*100 measures the efficiency of digestion of ingested food; ECD = [G/(C-F)]*100 measures the efficiency with which digested food is converted to body mass. C = ingested food (g), G = insect mass gain, T = duration of feeding period (days) and F = frass produced (mg).

A total of 75 larvae were used for mid-gut invertase activity measurements. To extract larval mid-gut invertase, larvae were starved for 4 h and placed on ice to facilitate mid-gut removal. The 3^{rd} and 5^{th} segments from the head were cut and mid-gut was removed using a fine forceps. Mid-guts were then washed in distilled water, dried, weighed, and placed in eppendorf tubes containing 100 μL of 50% (v/v) MeOH. Samples were centrifuged for 20 min at 4°C and 13000 rpm and supernatants were used to quantify soluble invertase activity levels according Ferrieri et al. (2013).

Larval performance was also assessed under glasshouse conditions by placing freshly hatched *M. sexta* neonates directly on fully developed leaves of rosette stage plants (1 neonate/plant n = 150) and allowing them to feed freely for 10 days. Larvae were weighed after 6, 8 and 10 days of feeding using a microbalance (Sartorius TE214S; Data Weighing Systems Inc., Elk Grove, IL, USA).

Additional behavioral measurements were recorded during the course of the experiment to further evaluate larval performance. We assessed feeding patterns of *M. sexta* larvae (n = 50) by quantifying area of leaf material eaten (cm^2) via digital image analysis of damaged leaves each day at 6:00 AM and 10:00 PM (day and night). Pictures were taken during first seven days of the larvae on plants.

Nicotiana attenuata plants with reduced direct defenses (e.g. protease inhibitors) have been shown to increase *M. sexta* larvae aggressiveness and subsequent fitness as this behavior acts to protect larvae against attack by Geocoris spp. predators (Schuman et al., 2012). Given this previous observation, we measured larval aggressiveness 6 days following feeding according to protocols described by (Schuman et al., 2012) fed on EV, irCWII-1 and irCWII-2 plants. We first poked larvae (n = 75) below the horn five times, 3 s apart, with the end of a toothpick and counted how often they attacked the toothpick. Here, a successful attack was defined by whether the larva could whip its head around toward the toothpick and make direct contact. Larvae were then lifted with a forceps for 10 s during which we counted how often they attempted to attack, or succeeded in attacking (made direct contact with) the forceps.

Water content and leaf area of NaCWII-silenced plants

To evaluate whether silencing NaCWII alters leaf water content, we collected one undamaged rosette leaf from EV, ir-CWII-1 and ir-CWII-2 plants (n = 75). The leaves were weighed using a microbalance (Sartorius TE214S; Data Weighing Systems Inc., Elk Grove, IL, USA), scanned at 300dpi using an Epson Expression scanner (Seiko Epson Corp; Nagano, Japan) and areas were calculated in ImageJ (Fiji).

Jasmonate levels of NaCWII-silenced plants

To test whether functional NaCWII is required for the herbivore-induced JA burst and other phytohormones, we quantified levels of jasmonic acid (JA), ((+)-7-

iso-jasmonoyl-L-isoleucine) (JA-Ile), abscisic acid (ABA) and salicylic acid (SA) 1 h following W+OS simulated herbivory on EV, irCWII-1 and irCWII-2 plants (n=30). Phytohormone extraction and quantification was performed according to Machado et al. (2013).

Statistical analysis

All data were analyzed using Generalized Linear Models (GLM) under normal distribution. When necessary, model simplification was performed through Pairwise GLM contrast analyses with F tests, combining treatment levels when it did not cause significant ($P < 0.05$) changes in the model, as recommended by Crawley (2007). All analyses were performed in R (R Development Core Team, 2012), followed by residual analysis to verify the suitability of error distribution and model fitting. The model constructed were described in (Tab. 2.1)

Table 2.1: Constructed models for each figures. **y-var** = response variable and **x-var** = explanatory variable.

	Models	
Figures	*y-var*	*x-var*
1 (A)	Expression of the CWII in local leaves	Herbivory and time points
1 (B)	Expression of the CWII in systemic leaves	Herbivory and time points
1 (C)	Expression of the CWII in roots	Herbivory and time points
3	CWII Relative transcript abundance	Plant genotypes, herbivory and time points
4 (A)	Cell wall invertase activity	Plant genotypes and herbivroy
4 (B)	Soluble invertase activity	Plant genotypes and herbivroy
5 (A)	Glucose + fructose	Plant genotypes, herbivory and time points
5 (B)	Sucrose	Plant genotypes, herbivory and time points
5 (C)	Starch	Plant genotypes, herbivory and time points
6 (A)	Nicotine	Plant genotypes, herbivory and time points
6 (B)	Caffeoylputrescine	Plant genotypes, herbivory and time points
6 (C)	Dicaffeoylpermidine	Plant genotypes, herbivory and time points
6 (D)	Rutin	Plant genotypes, herbivory and time points
7	Larval mass	Plant genotypes
8 (A)	Cell wall invertase activity in the mid gut	Plant genotypes
8 (B)	Soluble invertase activity in the mid gut	Plant genotypes
9	Leaf area comsumed	Plant genotypes and time
10	Succesful attacks (poke and lift)	Plant genotypes
11 (A)	Water content	Plant genotypes
11 (B)	Fresh weight /area of leaves	Plant genotypes
12 (A)	Jasmonic acid (JA)	Plant genotypes and herbivory
12 (B)	Jasmonic acid L-isoleucine (JA-ile)	Plant genotypes and herbivory
12 (C)	Abscisic acid (ABA)	Plant genotypes and herbivory
12 (D)	Salicylic acid (SA)	Plant genotypes and herbivory
Table 1	CI	Plant genotypes
Table 1	AD	Plant genotypes
Table 1	ECI	Plant genotypes
Table 1	ECD	Plant genotypes
Table 1	Frass	Plant genotypes
Table 1	Larval mass	Plant genotypes

Results

Cloning of NaCWII, a cDNA encoding a putative invertase inhibitor in *N. attenuata*

We utilized a previously conducted microarray to evaluate the expression patterns of a putative NaCWII. In this initial analysis, we discovered that the expression of NaCWII appears to be up-regulated at 6 hours following simulated herbivory (W+OS). This up-regulation is specific to local leaves (Fig. 2.1A), but also occurs in systemic leaves after 12hr (Fig. 2.1B). No changes following simulated herbivory were observed in roots (Fig. 2.1C). As the first step toward elucidating the role of cell wall invertase inhibitor in *N. attenuata*, we found one putative *N. attenuata* invertase inhibitor gene sequence using internal *Nicotiana spp.* BLAST server. Full-length cDNA was then cloned from *N. attenuata* leaves and named NaCWII. Sequence alignment of CWII with invertase inhibitors from tomato and tobacco showed the conserved four Cys residues (Fig. 2.2), a hallmark of all known plant invertase inhibitors (Rausch & Greiner, 2004).

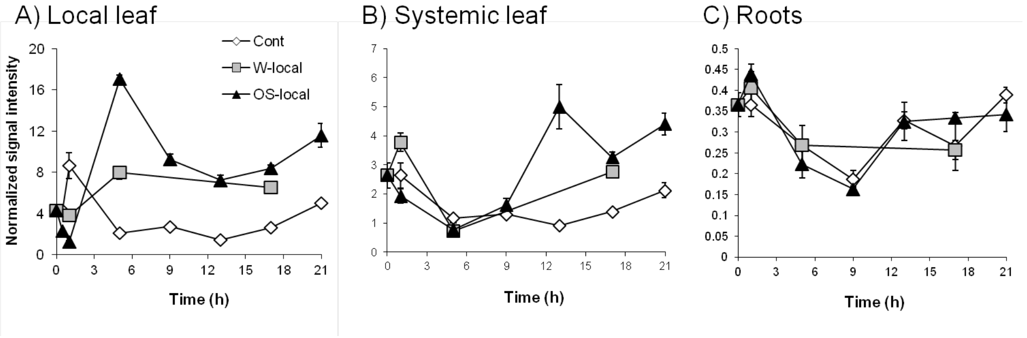

Figure 2.1: A putative *N. attenuata* invertase inhibitor is up-regulated in local and systemic leaves leaves, but not roots, following simulated herbivory. EV rosette stage leaves were treated by wounding (W-local), wounding and oral secretion of *M. sexta* larvae (OS-local) and untreated leaves (control). Expression patterns were extracted from a previously conducted microarray. Spotting was performed on a VersArray Chip Writer Compact System. Sample preparation, probe-target hybridization, and the collection of raw microarray data was performed in house using a GMS 418 Array Scanner.

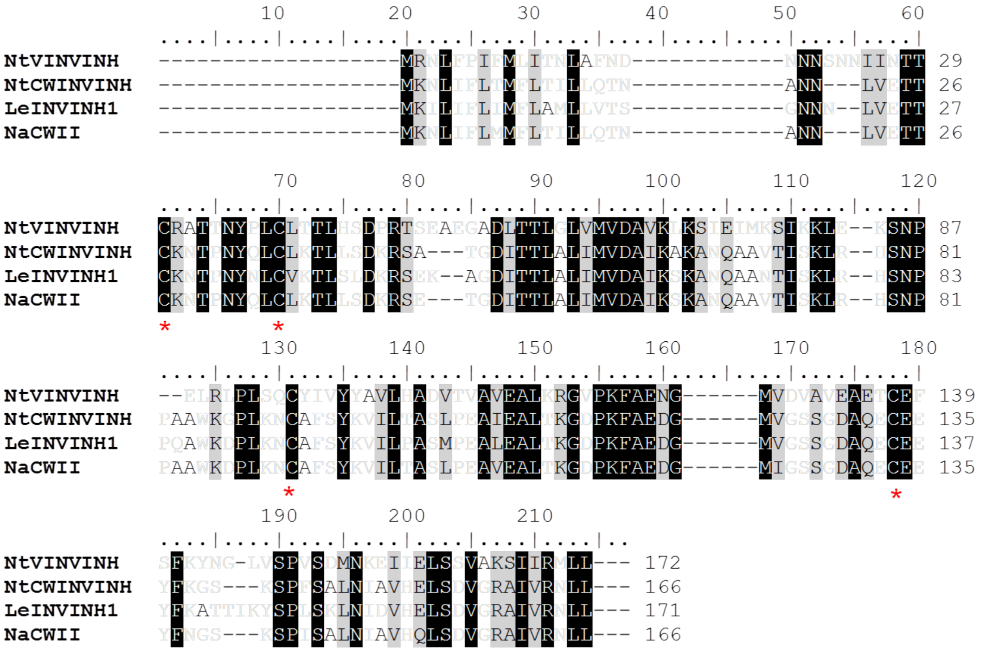

Figure 2.2: *N. attenuata* invertase inhibitor (NaCWII) shares high homology with known plant invertase inhibitors. Alignment of NaCWII with amino acid sequences of tobacco (NT CWINVIN and NT VINVINH) and tomato (LeINVINH1) invertase inhibitors. Asterisks denote four conserved Cys residues characteristic of plant invertase inhibitors. The gray and black vertical shadings represent regions exhibiting medium and high degrees of amino acid identities, respectively.

NaCWII is up-regulated upon simulated herbivory

From our initial time course experiment, we found a strong, OS-specific up-regulation of NaCWII (16-fold increase relative to control) 6h following simulated herbivory on EV plants (Fig. 2.3A). In contrast to EV plants, NaCWII expression in two independently transformed irCWII lines remained at control levels following both W+W (Fig. 2.3B) and W+OS (Fig. 2.3C) treatments.

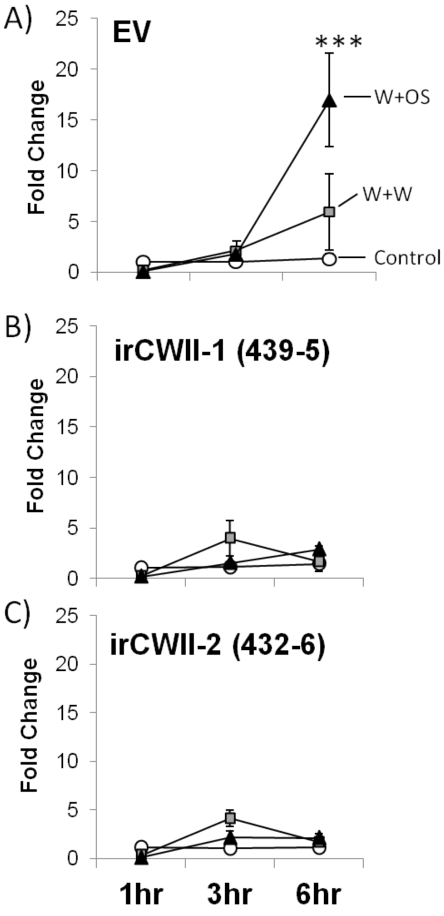

Figure 2.3: Silencing NaCWII suppresses herbivore-induced up-regulation of NaCWII. Relative transcript abundance of NaCWII in EV (A) and two independently transformed irCWII lines, irCWII-1 (B) and irCWII-2 (C). Analysis of mRNA was quantified by RT-qPCR in unelicited leaves, and at different times following wounding and treatment with 20 μL of water (W+W) or 1:5 water-diluted *M. sexta* oral secretions (W+OS). NaCWII mRNA levels are expressed as the ratio of abundance relative to an endogenous reference gene (ACTIN). Mean ±SE (n=3) are shown. Asterisks indicate significant effect of W+OS relative to control. $***$ $P < 0.001$

Simulated herbivory decreases plant cell-wall invertase activity in a NaCWII-dependent manner

To investigate whether the activity of plant invertase is regulated by NaCWII, we quantified cell-wall and soluble invertases activities in control and W+OS-treated EV and irCWII plants. We found that multiple W+OS treatments significantly suppressed cell-wall invertase activity in EV, but not irCWII plants (Fig. 2.4A). Although soluble invertase was constitutively higher in irCWII lines (Fig. 2.4B), activity levels were unaltered by W+OS treatment, regardless of plant genotype.

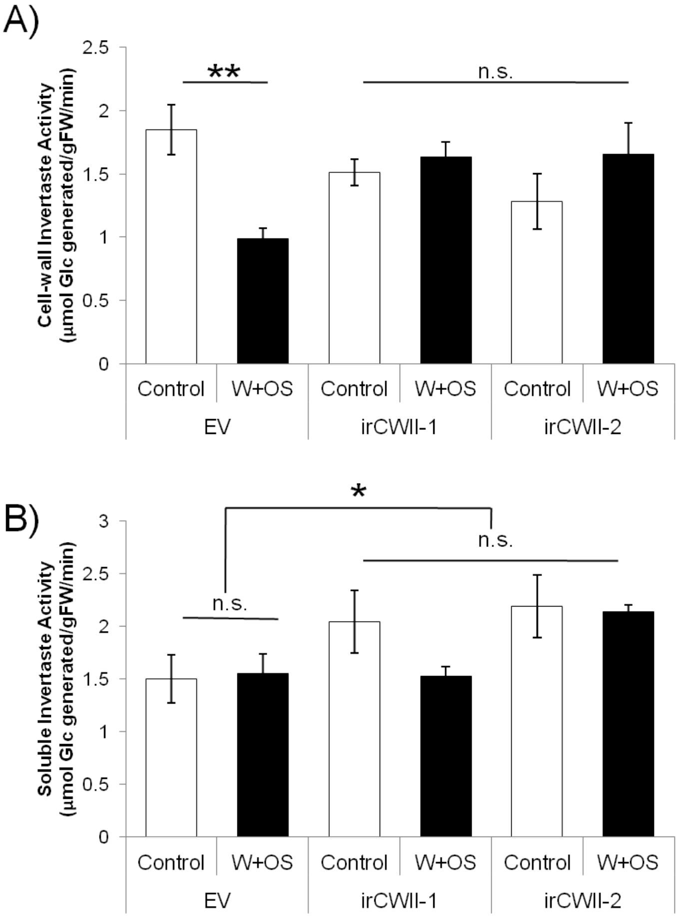

Figure 2.4: Simulated herbivory decreases cell-wall invertase activity in a NaCWII-dependent manner. Cell-wall (A) and soluble (B) invertase activities in EV and two NaCWII-silenced lines (irCWII-1 and irCWII-2). Two rosette leaves were treated every 48 h for 6 days with 1:5 water-diluted OS (W+OS, black) from *M. sexta* larvae and untreated as control (white). Bars represent average ± SE (n = 5). Asterisks indicate significant differences between treatments within each genotype and differences among genotypes ($**$ $P < 0.01$, $*$ $P < 0.05$), n.s., not significant.

NaCWII regulates *N. attenuata* primary and secondary metabolism following simulated herbivory

To examine whether NaCWII affects long-term dynamics of plant primary and secondary metabolism following simulated herbivory, we measured foliar concentrations of soluble sugars, starch, nicotine, caffeoylputrescine, dicaffeoylspermidine, and rutin in EV and irCWII genotypes in a time course following repeated damage.

In general pattern, CWII-silenced plants had more depletion of soluble sugars. While starch levels did not differ significantly between the genotypes at night (2:00 AM and 6:00 AM); however during the day (2:00 PM and 10:00 PM), EV plants accumulated more starch relative to irCWII plant. As we expected based on previously published studies (Machado et al., 2013), soluble sugars (Fig. 2.5A and B) and starch (Fig. 2.5C) were significantly depleted in leaves uponsimulated herbivroy (W+OS). This depletion was found to be driven primarily by a reduction in glucose and fructose (Fig. 2.5A), rather than sucrose (Fig. 2.5B), which occurred more dramatically in CWII-silenced plants at 6:00AM and 2:00PM relative to EV (Fig. 2.5A).

Six days after W+OS treatments, concentrations of nicotine, caffeoylputrescine, and dicaffeoylspermidine were increased upon W+OS application (Fig. 2.6A-C) in both genotypes. EV plants, in particular, contained higher levels of dicaffeoylspermidine following W+OS relative to irCWII plants (Fig. 2.6C). Foliar rutin concentration was reduced following W+OS, which occurred to a greater extent in EV plants (Fig. 2.6D).

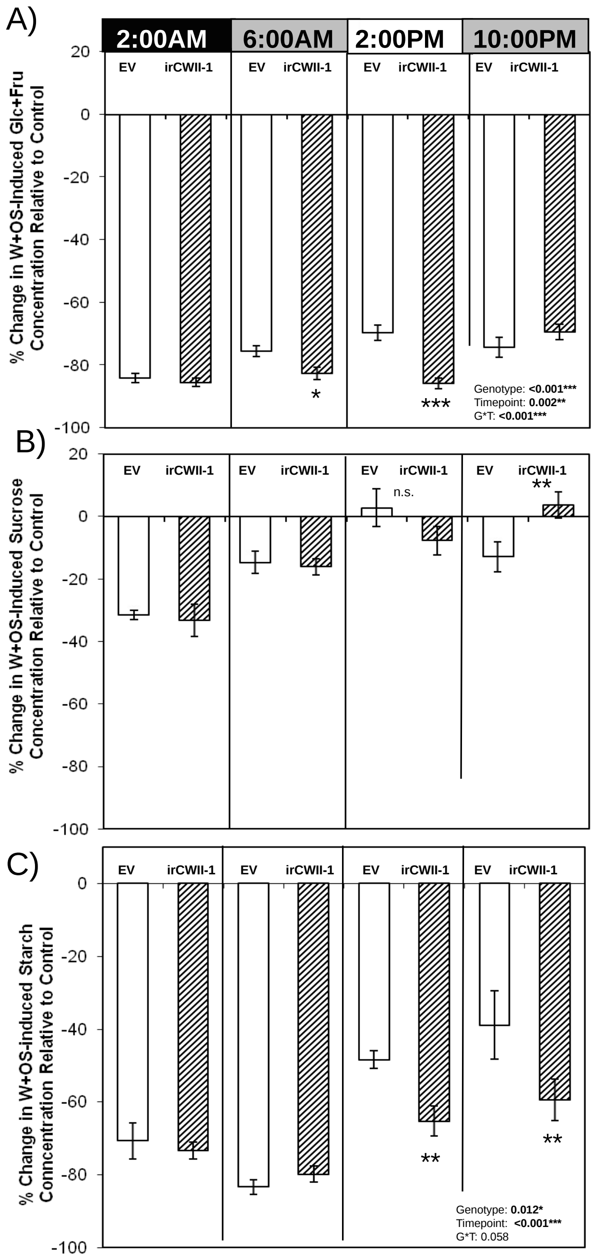

Figure 2.5: Silencing NaCWII increases herbivore-induced carbohydrate depletion in leaves. Combined glucose and fructose (A), sucrose (B) and starch (C) concentrations were measured in a time course following 4 rounds of simulated herbivory (W+OS). Bars represents mean concentration in W+OS tissue relative to mean control ±SE (n = 4). Asterisks indicate significant differences between EV (white) and NaCWII-silenced plants (irCWII-1, hatched within each timepoint. $* * *$ P $<$ 0.001, $**$ P $<$ 0.01, $*$ P $<$ 0.05, n.s., not significant.

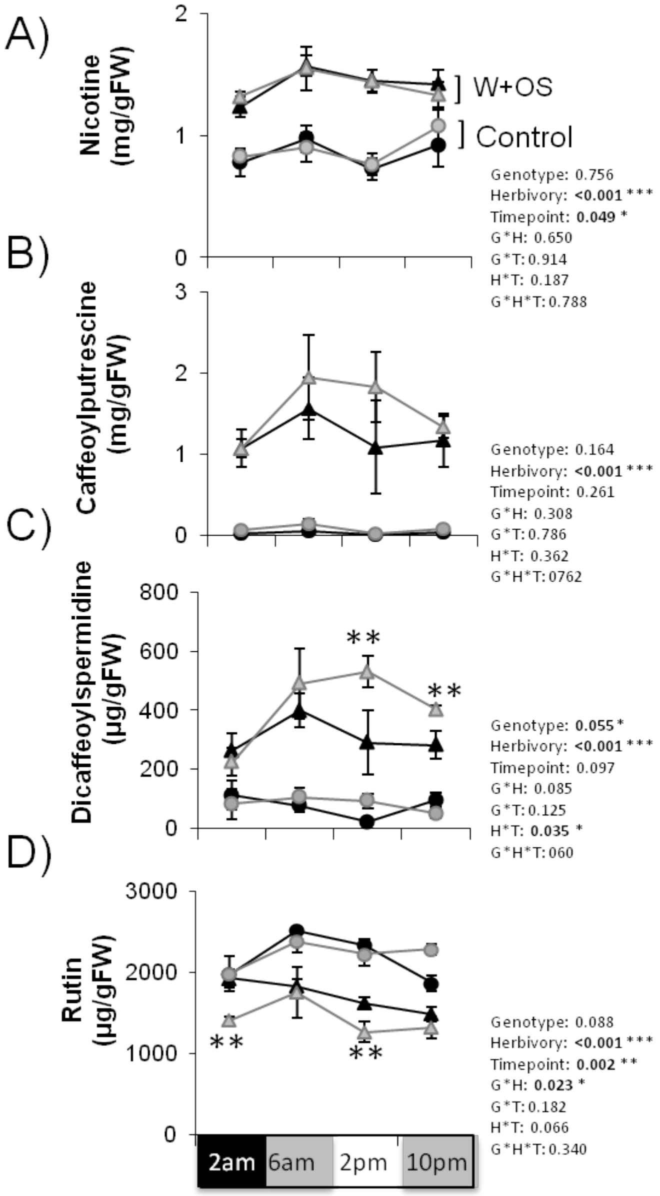

Figure 2.6: Silencing NaCWII attenuates the production of herbviore-induced secondary metabolites in leaves. Circles: untreated controls. Triangles: simulated herbivory (W+OS). Asterisks indicate significant differences between EV (grey) and irCWII (black) genotypes within herbivory treatment and timepoint ($**$ $P < 0.05$).

NaCWII regulates *N. attenuata*, not *M. sexta*, invertase activity and behavior

We took a two-fold approach to assess whether NaCWII directly influences the growth and performance of *M. sexta* larvae. First, we assessed the utilization of leaf material by *M. sexta* larvae using a Waldbauer and glasshouse performance assays. Second, we measured the activity of invertases residing in mid-gut of larvae fed EV or irCWII tissue. Results from the Waldbauer assay show that *M. sexta* feeding on elicited EV and irCWII tissues both consume (CI), and effectively convert ingested material (ECI), to the same degree (Tab. 4.1). Larvae feeding on elicited irCWII leaves displayed a greater ability to digest this leaf material (AD); however, the conversion of digested material to larval biomass occurred at a reduced capacity compared to larvae fed elicited EV leaves (ECD). Despite these differences, all larvae produced equivalent amounts of frass and reached similar body masses by the end of the experiment (Tab. 4.1). Consistent with these findings, *M. sexta* larvae mass gain was not influenced by host plant genotype in the glasshouse performance assay (Fig. 2.7). We found no difference in the activities of insoluble (Fig. 2.8A) or soluble (Fig. 2.8B) invertases extracted from the mid-gut of larvae fed EV or irCWII tissue. Another approach was the behavior measurements; in agreement with the Waldbauer assay *M. sexta* during first seven days consumed the same amount of leaf tissue when fed in EV nd irCWII plants (Fig. 2.9). But not surprising, larvae ingested more leaf tissue during the day than at night (Fig. 2.9). Concerning to aggressiveness larvae reared on EV, irCWII-1 and irCWII-2 plants had the same response pattern when poked and lifted (Fig. 2.10).

Table 2.2: Silencing NaCWII increases leaf digestibility to *M. sexta* larvae. Nutritional indices calculated from *M. sexta* feeding on elicited EV, and NaCWII-silenced plants according to Waldbauer(1968). Mean consumption index (gFW plant tissue/gFW larvae/day), approximate digestibility (%), efficiency of conversion of ingested food (%), efficiency of conversion of digested food (%), and 2-day frass production (g) and mass gain of larvae (mg) are shown ± SD. C = ingested food (g), G = insect mass gain, T = duration of feeding period (days), F = frass produced (mg). Different letters indicate significant differences among plant genotypes. ($***$ P < 0.05).

Nutritional Index	**Formula**	**EV**	**irCWII-1**	**irCWII-2**	***F value***	***P value***
Consumption index (CI)	[C/(G*T)]	2.13 4 ± 0.07a	2.29 ± 0.06a	2.24 ± 0.06a	$_{[2,67]}$ 1.328	0.271
Approximate digestibility (AD)	[(C-F)/C]*100	74 ± 0.9a	79 ± 0.9b	77 ± 1.2b	$_{[1,68]}$ 9.897	0.002 ***
Efficiency of conversion of ingested food (ECI)	(G/C)*100	24 ± 0.8a	22 ± 0.6a	23 ± 0.6a	$_{[2,67]}$ 1.633	0.203
Efficiency of conversion of digestive food (ECD)	[G/(C-F)]*100	33 ± 1.4a	28 ± 0.9b	30 ± 1.1b	$_{[1,68]}$ 5.821	0.01 ***
Frass		0.274 ± 0.01a	0.276 ± 0.01a	0.278 ± 0.02a	$_{[2,66]}$ 0.092	0.912
Larval mass		1246 ± 59.2a	1268 ± 61a	1313 ± 81a	$_{[2,66]}$ 2.035	0.138

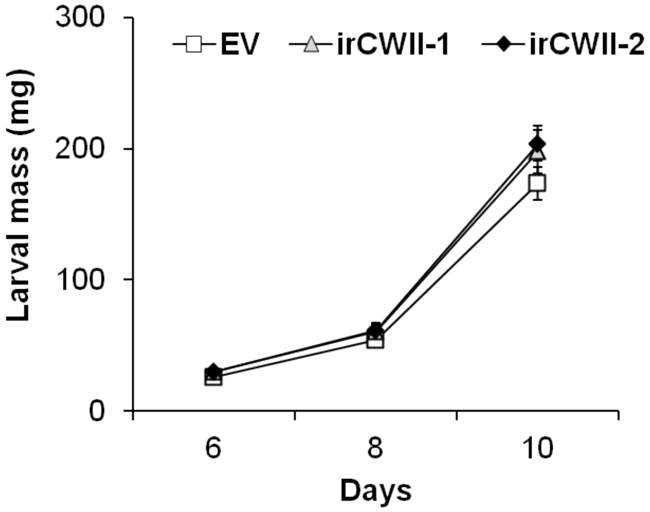

Figure 2.7: Silencing NaCWII does not affect *M. sexta* growth. The performance of the larvae was observed on EV and silencing irCWII-1 and irCWII-2 plants under glasshouse conditions. Average (±SE) of larval mass after 6, 8, and 10 days of feeding on 25 replicates of each line. ($P > 0.05$).

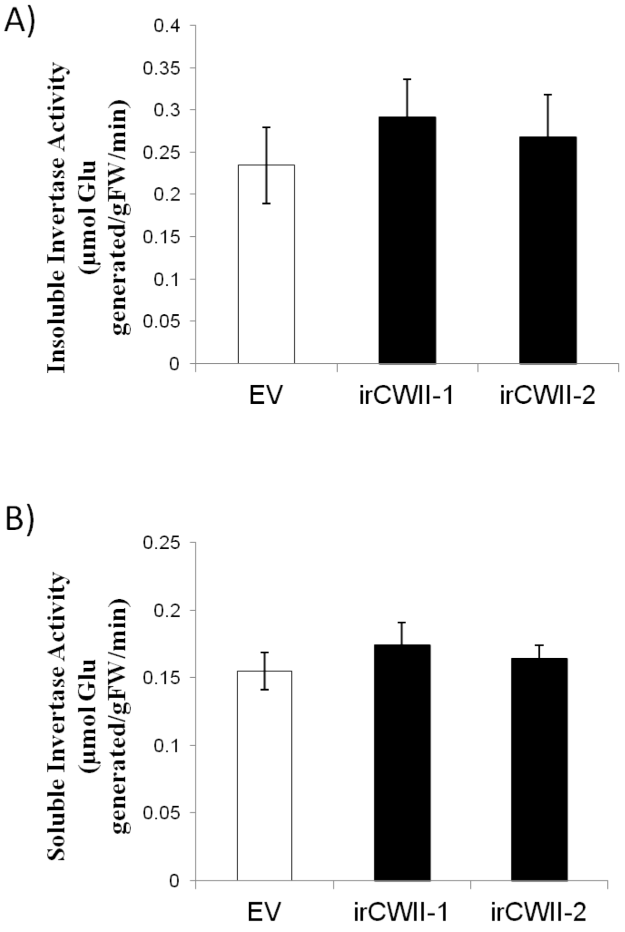

Figure 2.8: Silencing the *N. attenuata* invertase inhibitor does not affect the activity of insoluble (A) and soluble (B) invertases present in the mid-gut of *M. sexta* larvae. Invertases were extracted from the mid-gut of 4th instar feeding on leaves of EV, irCWII-1 and irCWII-2 plants. Each bar represents average ±SE (n = 25). ($P > 0.05$).

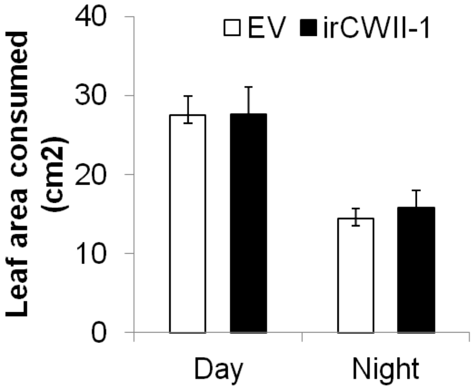

Figure 2.9: *M. sexta* exhibits similar feeding patterns on EV (white) and irCWII-1 (black) genotypes. Leaf area eaten by larvae during seven days was quantified during the day and at night. Each bar represents average leaf area consumed ±SE (n = 25). (P > 0.05).

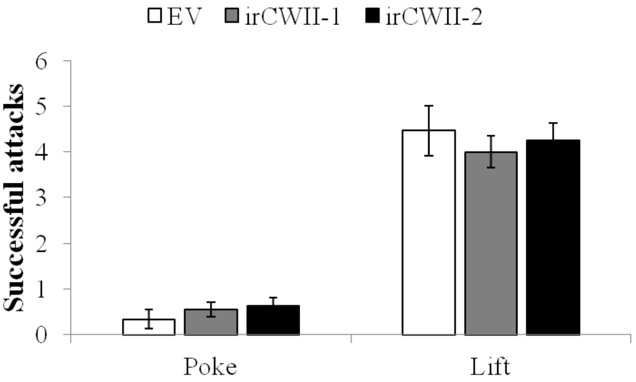

Figure 2.10: Silencing NaCWII does not affect the aggressiveness of 6 day-old *M. sexta*. Larvae on EV (white), NaCWII-silenced lines (black) were poked and lifted to mimic predation. Each bar represents average number of successful attacks by larvae ±SE (n = 25). (P > 0.05).

Water content and leaf area of NaCWII-silenced plants does not change

As we expected the water content in undamaged leaves was around 90% in EV and both irCWII plants (FigS. 2.11A) and also the amount of fresh tissue (g) *per* area was the same for all lines evaluated (FigS. 2.11B). These results strengthen our findings on *M. sexta* Waldbauer assay, showing that larvae had eaten the same quantity of leaf tissue.

Simulated herbivory induced JA and JA-Ile in leaves of EV and irCWII plants, but not change ABA and SA

Both jasmonic acid (JA) and (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) were up-regulated within 1 h following W+OS treatments to EV and irCWII leaves (FigS. 2.12A and B). However, abscisic acid (ABA) and salicylic acid (SA) 1 h after W+OS simulated herbivory were no induced in EV and neither irCWIIs lines(FigS. 2.12C and D).

Supplemental Figures

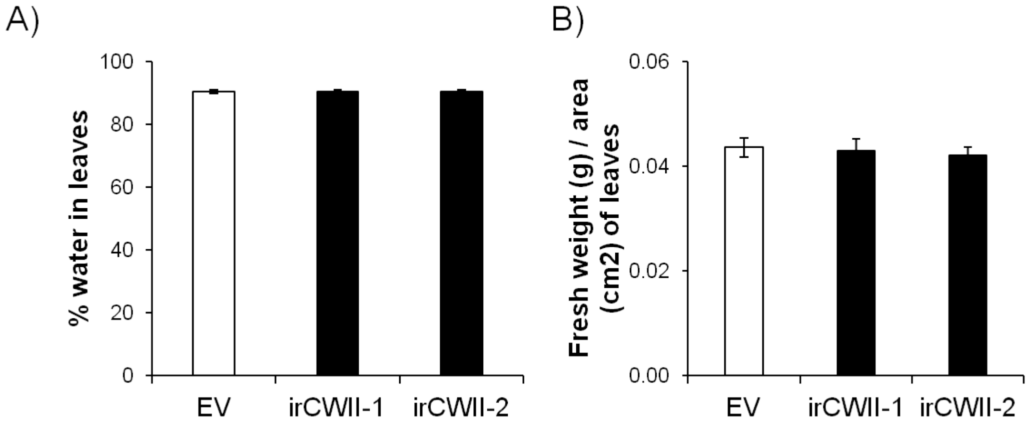

Figure 2.11: Silencing NaCWII does not affect leaf water content (A) or mass per area (B). Undamaged leaves from *N. attenuata* plants (EV, irCWII-1 and irCWII-2) in the rosette stage were weighed, scanned and dried. Bars represent average ±SE (n = 25), ($P > 0.05$

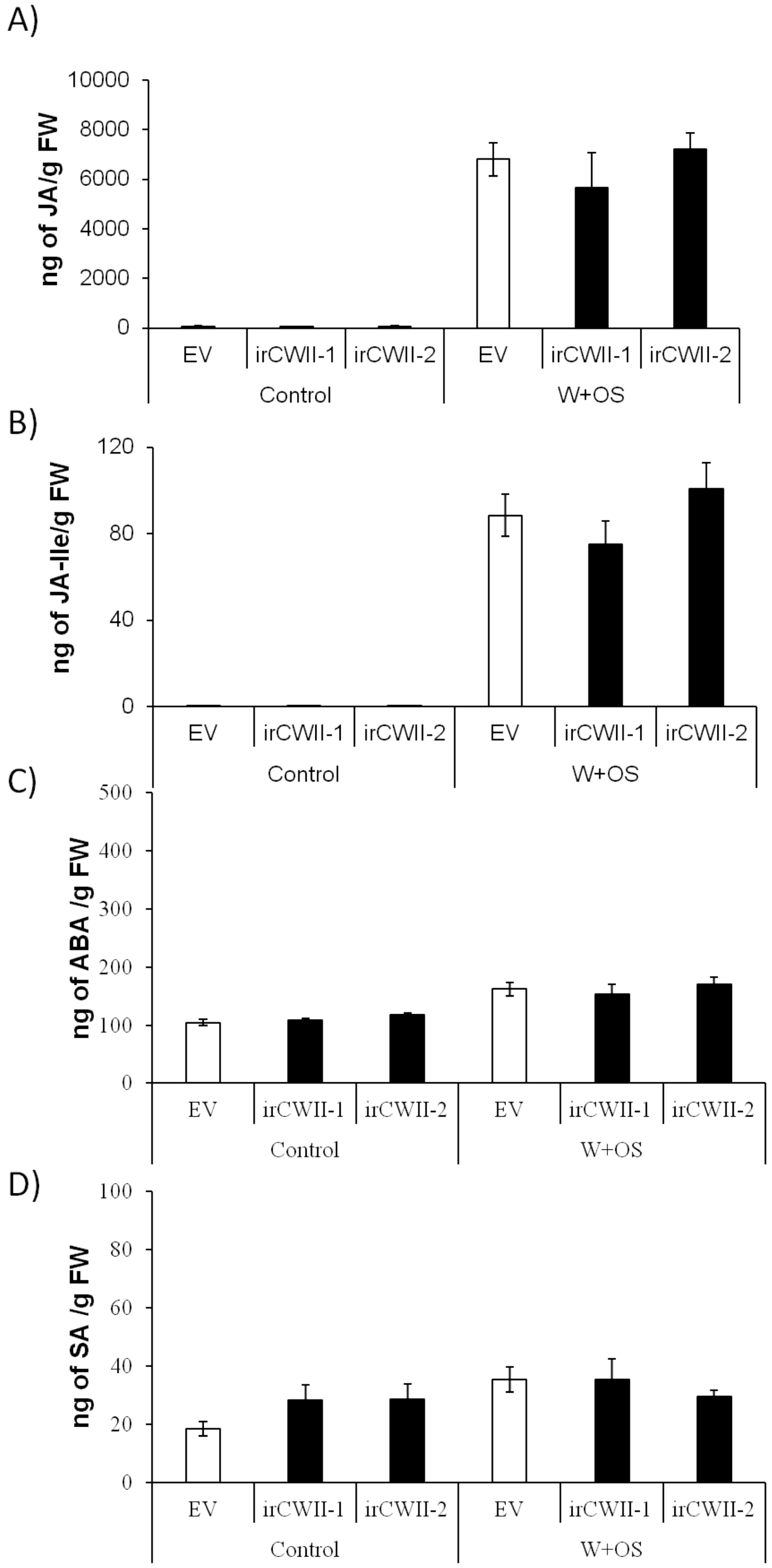

Figure 2.12: Silencing NaCWII does not affect the herbivore-induced accumulation of JA (A), JA-Ile (B), ABA (C), SA (D). Plants of *N. attenuata* (EV, irCWII-1 and irCWII-2) were treated with 1:5 water-diluted OS (W+OS) from *M. sexta* larvae or left as untreated control. JA (jasmonic acid), JA-ile ((+)-7-iso-jasmonoyl-L-isoleucine), ABA (abscisic acid) and AS (salicylic acid). Bars represent average ±SE (n = 5). ($P > 0.05$).

Discussion

We identified NaCWII, a homolog of INVINH1, that is known to specifically inhibit cell-wall invertase activity in tomato (Jin et al., 2009), and used a reverse genetics approach to silence its expression in *N. attenuata* by RNA interference (ir-CWII) in order to understand the role of this putative invertase inhibitor in induced plant defense. Our analysis of invertase activity, downstream changes in primary and secondary metabolites, and NaCWII-silenced lines provide new insights into the post-translational regulation of cell-wall invertase during plant defense and suggests a role for NaCWII in regulating the defense induction in *N. attenuata* following attack by *M. sexta*.

In wild-type *N. attenuata*, we found that the expression of NaCWII increased dramatically (16-fold) following simulated herbivore damage. Analyzing the activity of invertases in wild-type plants confirmed that the inhibitory activity of NaCWII was not only increased upon OS-elicitation, but specific to cell-wall bound invertase. This finding is consistent with others showing that the overexpression of an invertase inhibitor in *A. thaliana* (INVINH1) specifically reduces cell wall invertase activity, while silencing its expression in tomato significantly increased the activity of cell wall invertase, without altering activities of cytoplasmic and soluble invertases (Jin et al., 2009).

The regulatory role of NaCWII was first validated by qPCR, which revealed gene transcripts were significantly attenuated in NaCWII-silenced lines following W+OS. In contrast to wild-type plants which exhibited a significant suppression of cell-wall invertase activity following simulated herbivore attack, the activity of cell-wall invertase of NaCWII-silenced lines remained unaltered. To examine whether NaCWII affects long-term dynamics of plant primary and secondary metabolism, we concurrently measured foliar concentrations of carbohydrates and secondary metabolites known to be induced in *N. attenuata* upon *M. sexta* attack in EV and NaCWII-silenced plants in a time course following repeated damage. As expected under wild-type conditions, we found that the concentrations of soluble sugars and starch were significantly depleted in leaves upon repeated W+OS treatments. This is consistent with previous studies showing reduction in sugar pools following exogenous JA application and herbivore damage (Skrzypek et al., 2005; Machado et al., 2013). As previous studies have also shown, we found OS-induced depletion in carbohydrates response to be driven primarily by a reduction in glucose and fructose and starch, which varied according to time of day: the response was more pronounced at 2:00 and 6:00 AM time points. This finding aligns well with that of previous studies demonstrating a strong endogenous rhythm of carbohydrates in plants, which decline during the dark cycles and peak between 4 and 8h after dawn (Lu et al., 2005; Dalchau et al., 2011).

The accumulation of defensive compounds in plants in response to insect attack can represent a significant metabolic cost in terms of biosynthesis, storage, and eventual fitness impacts (Gershenzon, 1994), and can therefore result in increasing demand for carbon. Indeed, carbohydrates support the production of plant phenolics, which can exceed $>$ 25% of plant dry mass, via the shikimic acid and phenylpropanoid pathways (Harding et al., 2009). Alkaloids (e.g. nicotine) can require upwards of 3.62 g of glucose per gram produced, representing about 17% of a tobacco plant's net carbon gain (Wink & Roberts, 1998). Within this context, the repression of cell-wall invertase and simultaneous depletion in soluble sugars observed in locally damaged leaves may reflect an increase in localized sink metabolism following simulated herbivory which is necessary to satisfy the increased demand for energy for the activation of defense pathways leading to secondary metabolite accumulation.

In contrast to our study, the depletion in carbohydrate pools following herbivory has also been explained by an increase in local invertase (Arnold & Schultz, 2002; Bogatek et al., 2002; Arnold et al., 2004; Ferrieri et al., 2013), which results in higher fructose and glucose necessary for the production of defensive compounds (Machado et al., 2013). However, we have found that constitutive JA-deficiency plants leads to higher sugar levels and higher invertase activity in *N. attenuata* leaves (Machado *et al.*, unpublished). This discrepancy suggests that endogenous JA concentration may dictate sugar levels and invertase activity to a greater extent than exogenous application. Clearly, our understanding of the underlying mechanisms by which endogenous JA regulates invertase and subsequent carbohydrate pools deserves further attention. Although we found that irCWII lines were not compromised herbivory-induced JA and JA-Ile accumulation, these plants display an attenuation in the accumulation of herbivory-induced defensive compounds, in particular the production of dicaffeoylspermidine following repeated W+OS treatments.

Given its strong up-regulation following *M. sexta* damage, we expected our putative CWII gene to play a role in anti-herbivore defense in *N. attenuata*. Yet, in contrast to this expectation, we found that silencing of the CWII gene in *N. attenuata* did not affect the larval performance. Similar results were found in both glasshouse performance assays using intact plants and Waldbauer nutritional assays using previously elicited detached leaves. Interestingly, we found that *M. sexta* larvae had reduced ECI and increased AD when fed irCWII leaves. Despite these observations, all larvae exhibited similar mass gain and excreted the same amount of frass. Independently of plant genotype, larvae consumed more leaf tissue during the day than at night. This feeding pattern not only corresponds to the highest period of activity for this species (personal observation), but also is related to the most exaggerated OS-induced metabolites changes we observed in the plant.

The efficiency of food utilization can be seen as an indicator of nutritional quality of a plant. Thus, it is possible that irCWII tissue represents a poor host in terms of nutritional quality. Thompson & Borchardt (2003) showed that *M. sexta* larvae have growth unaffected by carbohydrate consumption in artificial diet. Similarly, on the carbohydrate-free diets, growth increased with increased protein consumption. *M. sexta* larvae tend to adjust their feeding behavior so that protein ingestion is either equal to or greater than carbohydrate ingestion (1:1 ratio Thompson & Renack, 2005). Carbohydrates are thought to be nonessential because they can be synthesized from fats and/or amino acids, and regulated within the larval midgut when they are limiting (Chapman, 1998). The ability of *M. sexta* to practice gluconeogenesis (Thompson, 1997), an internal regulatory strategy by which amino acids can be converted to energy in the midgut, provides a possible explanation for why we observe no differences in larval weight gain despite greater sugar depletion in irCWII lines following herbivory (Thompson & Renack, 2005). Additional trade-offs between over and under ingesting nutrients on nutritionally imbalanced foods may also act to maintain optimal insect growth (Raubenheimer & Simpson, 1999).

Insect herbivores face the challenge of simultaneously regulating the intake of multiple nutrients. Interactions among secondary plant compounds and nutrients have been demonstrated from tests in which the nutritional content was varied. Duffey et al. (1986) showed that deleterious effect of rutin varies not only with the amount of protein in the food, but also with protein type. Others show that carbon-based tannin secondary metabolites can incur negative effects on locust performance only when the ratio of protein:carbohydrate of the diet becomes more imbalanced (Simpson & Raubenheimer, 2001).

In our study, we found that irCWII plants failed to induced secondary metabolite production to the same extent as their EV counterparts. This attenuation was observed especially for carbon-based phenolic compounds known as phelonamides (DCS dicaffeoylspermidine, and to a lesser extent caffeoylputrescine). Phenolics are found in all plants, play a vital role in survival of the plant, and can function as defensive molecules against herbivores and plant pathogens (Appel, 1993; Matsuki, 1996). When incorporated into artificial diets, some phenolics negatively affects growth and development of several different caterpillar species (Isman & Duffey, 1982; Stevenson, 1993; Stamp & Yang, 1996), but positive and neutral effects have been reported (Reese & Beck, 1976; Lindroth & Peterson, 1988). Eichenseer et al. (1998) suggested that *M. sexta* is insensitive to chlorogenic acid (a type of phenolic). Thus, future work will examine the levels of other nutrients (amino acids, lipids, proteins) within irCWII plants, and their interactions with secondary metabolites using artificial and semi-artificial (plant-based) artificial diet assays to test for interactive effects between primary and secondary metabolites and their impact on *M. sexta* performance.

Digestion and absorption occur along the length of the midgut, with columnar cells secreting digestive enzymes that facilitate absorption. Under wild-type conditions, we found that the activity of plant cell-wall invertase was significantly decreased after simulated herbivory. To test whether plant-derived invertase inhibitor may act directly on invertases of *M. sexta* larvae, we assayed invertase activity of midgut extracts of larvae feeding on irCWII and EV genotypes. We found that invertase activities larval midgut did not differ between irCWII- and EV-fed larvae, suggesting that plant NaCWII may not directly target *M. sexta* invertase. Christopher & Mathavan (1985) showed an analysis on the amylase and invertase activities in the midgut of *Catopsilia crocale* an increased in these enzymes activities is correlated with food intake, but not proportional to an increase in food uptake. Some carbohydrates have a stimulant action that can induce the activity of digestive enzymes in the midgut as amylase, invertase and proteinase of insects (Meisner et al., 1972). Sucrose, glucose, fructose, raffinose and maltose are carbohydrates reported to induce the feeding responses by induction of digestive enzymes activities (Ito, 1960; Yamamoto & Fraenkel, 1960; Augustine et al., 1964; Hsiao & Fraenkel, 1968). Future studies may explore how plant protein inhibitors may regulate other aspects of insect sugar metabolism.

In our study, we found that attenuation in plant primary and secondary metabolism in herbivore-induced irCWII did not correlate to changes in insect aggressiveness, even though previous studies show that food quality ingested by *M. sexta* may directly relate to their survival rate in the field. Schuman et al. (2012) found that larvae fed on irPI plants which have suppressed trypsin proteinase inhibitor defense were more vulnerable to *Geocoris spp.* predation in the field even though *M. sexta* larva showed more aggressive behavior while feeding on these plants. In this study, the authors concluded that changes in the larvae as a nutritious food source for *Geocoris* predators (Kaplan & Thaler, 2011) may better explain the increase in predation rates.

In summary, a clear defensive role for NaCWII remains elusive as *M. sexta* feeding on pre-induced leaves as well as intact rosettes in the glasshouse failed to increase in biomass on irCWII compared to EV controls. Despite the lack of correlation between induced changes in plant primary and secondary metabolism upon silencing of NaCWII, our study is the first of its kind to explore the role of a plant cell wall invertase inhibitor is relation to plant defense against insect herbivores. The use of transgenic plants silenced in a gene of interest coupled with insect performance and nutritional assays provides a robust tool for analyzing the potential defensive role that such gene plays in plant defense against a adapted insect herbivores. While our studies did not point to a clear role of NaCWII in direct defense against *M. sexta*, our work established the foundation to further explore this gene's function within the plant, such as its role in maintaining the balance between growth and defense induction following herbivore attack.

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Chapter 3

Manduca sexta herbivory improve the reproduction of root feeder *Meloidogyne incognita* without strong plant fitness costs in *Nicotiana attenuata*

Abstract

The induction of plant defenses against herbivores is costly and the costs are usually related to the fitness. Moreover, plant responses against herbivores usually change the primary and secondary chemistry of distal plant tissues that might therefore affect the development of organisms sharing the host plant. In this study, we focused specifically on understanding: *(i)* the effect of *Manduca sexta* attack on the reproduction of *Meloidogyne incognita* and *(ii)* the plant fitness costs associated to simultaneous *M. sexta* and *M. incognita* herbivory. To answer these questions, we evaluated the number of eggs produced by *M. incognita* upon a prolonged *M. sexta* attack and the fitness of *N. attenuata* plants that were subjected to simultaneous *M. sexta* and *M. incognita* attack. We found that *M. sexta* herbivory benefit the development of *M. incognita* and the mechanical damage and *M. sexta* simulated herbivory had more impact than *M. incognita* herbivory on plant fitness. In conclusion, our study shows that plant defensive responses against above-ground herbivores might benefit root feeders without short-term fitness consequences. The elucidation of the underlying mechanisms will likely provide elements to understand how plants modulate the interaction between organisms spatially separated.

Keywords: fitness, interaction above- and below-ground, *Manduca sexta*, *Meloidogyne incognita.*

Introduction

How plants defend themselves against attackers and the fitness consequences associated to that response as well as whether and how the induction of plant defense modulates the interaction between organisms spatially separated are long standing questions in ecology. Broadly speaking, it has been suggested that the factors that determine the outcome of the indirect plant-mediated interactions between root and shoot herbivores are: *i)* the feeding guild, *ii)* the degree of host plant susceptibility to herbivores and *iii)* the identity of shoot herbivorous insects, as these may differentially influence the responses of the host plants to attackers (Mateille, 1994; van Dam et al., 2003, 2005; Bezemer & Van Dam, 2005; Wurst & van der Putten, 2007). A growing body of literature reports the outcome of the plant-mediated interactions between nematodes and above-ground insects (Fu et al., 2001; Kaplan et al., 2008, 2009; Russin et al., 1989, 1993; Tiwari et al., 2009). However, no clear patterns have been observed. For example, foliar feeders can reduce (Tiwari et al., 2009), improve (Kaplan et al., 2008, 2009; Russin et al., 1989, 1993) or have no effect (Fu et al., 2001) on nematode performance.

Brown, V.K. & Gange (1990) and Masters et al. (1993) proposed that the negative impact of foliar herbivory on nematode performance may be the result of a slower root growth in plants with damaged foliage. On the other hand, the defense induction hypothesis postulates that herbivores in the opposite compartments negatively influence each other through induction of toxic secondary plant compounds (Bezemer et al., 2003; Bezemer & Van Dam, 2005). Similarly, aboveground herbivory induces changes in the root structure that might reduce nematode penetration, leading to a reduced nematode performance (Tiwari et al., 2009). Conversely, aboveground herbivory induces invertase activity in the roots, which might make available glucose and fructose that in turn, could potentially benefit nematode performance (Russin et al., 1989, 1993; Kaplan et al., 2008, 2009). *Nicotiana attenuata* respond to *Manduca sexta* herbivory by inducing the biosynthesis of defensive compounds in above-ground (Heil & Karban, 2010; Karban & Baldwin, 1997; Paschold et al., 2007; Kallenbach et al., 2012; Machado et al., 2013) and below-ground tissue (Machado et al., 2013) which is accompanied by a depletion of carbon pools in both leaves and roots (Machado et al., 2013). The above suggests that root feeder performance should be negatively affected rather than benefited upon *M. sexta* attack.

Plants respond to foliar herbivory by activating a wide range of different defensive traits. These could be morphological changes (e.g., formation of trichomes), production of toxic compounds (proteins, secondary metabolites), or release of volatiles that either have a repellent effect or attract predators of the attacking herbivores (Bezemer & Van Dam, 2005; Kaplan et al., 2008; Wei et al., 2014). Several defensive secondary compounds have been identified in different plants species as glucosinolates (van Dam et al., 2004; Soler et al., 2009), benzoxazinoids (Classen et al., 1990; Sicker et al., 2000; Erb et al., 2009), terpenoids (van Poecke & Dicke, 2004), defense-related proteins such as arginases, threonine deaminase, vegetative storage proteins, ascorbate oxidases, lipoxygenases, polyphenol oxidases, peroxidases, chitinases, cystein proteases, lectins and leucineaminopeptidases, proteinase inhibitors, cysteine proteinases (Thaler et al., 1996; Thaler, 1999), alkaloids (Baldwin, 1998), phenolics (Keinänen et al., 2001), tannins (Peters & Constabel, 2002), phytoecdysteroids (Schmelz et al., 1999), silica (SiO^2) (Mcnaughton & Tarrants, 1983) and latex (Dussourd, 1995). Upon *M. sexta* attack, *N. attenuata* induces the production of a diverse blend of secondary chemical compounds as proteinase inhibitors (PIs) (Halitschke & Baldwin, 2003; Zavala & Baldwin, 2004; Paschold et al., 2007), nicotine (Steppuhn et al., 2004), diterpene glycosides (DTGs) (Jassbi et al., 2008; Heiling et al., 2010), dicaffeoylspermidine (DCS) (Kaur et al., 2010), caffeoylputrescine (CP) (Kaur et al., 2010) green leaves volatiles (GLVs)(Halitschke et al., 2004) and cis-α-bergamotene (Halitschke et al., 2000; Kessler & Baldwin, 2001). These compounds have been found to act as defense against *M. sexta*. However, their production brings fitness consequences, as their biosynthesis depletes carbon pools and constrains regrowth (Machado et al., 2013). Whether these secondary metabolites act as defense against root feeders is still not clear (Hanounik et al., 1975; Hanounik & Osborne, 1977; Rich & Barker, 1984; Preisser et al., 2007).

Several phytohormones as ethylene (ET), jasmonates (JA) and salicylic acid (SA) have been found to regulate plant responses against biotic stressors. In the case of nematode attack, our knowledge, however, is still very limited. Kyndt et al. (2012) found that three days after infection with *Hirschmanniella oryzaeis*, systemic JA and ET signaling are induced and the SA pathway is suppressed. While *M. graminicola* activate SA and JA, but suppress ET in the systemic root tissue, suggesting that 1) species-specific nematode elicitors might differentially trigger the induction of hormonal pathways, and 2) hormonal crosstalk might play a crucial role in modulating the plant responses against nematode attack. Whether or not the activation of the afore mentioned pathways regulate plant defenses against nematode attack or whether this activation is only part of plant wounding response remains still poorly understood. However, pharmacological and genetic approaches have revealed that *M. graminicola* is sensitive to JA- and ET-induced defense, but only slightly to SA-induced defense, while *H. oryzaeis* sensitive to defenses regulated by JA, SA and ET (Nahar et al., 2011, 2012). On the other hand, pharmacological applications of methyl jasmonate affected negatively *M. incognita* infection (Fujimoto et al., 2011). Upon *M. incognita* attack, many defensive compounds as peroxidases, cell wall modification enzymes, *LOX* genes, and proteinase inhibitors are induced (Wyss et al., 1992; Gheysen & Fenoll, 2002). Another effective strategy used by plants is regulating the R genes, which block the nematode development of the feeding site (Tomczak et al., 2009). Therefore, it is evident that further studies investigating how plants respond to nematodes and the consequences of nematode attack for plant fitness are required.

Upon *M. sexta* attack, carbon pools are depleted and nicotine content is induced in the roots of *N. attenuata* (Machado et al., 2013). The depletion of sugars and the induction of plant defenses create a completely new environment that might potentially affect root feeders. Moreover, the induction of plants defenses is costly and might result in plant fitness costs. In this study, we focused specifically on understanding: *i)* the effect of *M. sexta* attack on the reproductive fitness of *M. incognita* and *ii)* the plant fitness costs associated to simultaneous *M. sexta* and *M. incognita* herbivory. To answer these questions, we evaluated the reproductive fitness of *M. incognita* upon a prolonged *M. sexta* attack and the Darwinian's fitness of *N. attenuata* plants that were subjected to simultaneous *M. sexta* and *M. incognita* attacks. Our results provide evidence that above-ground herbivory affect below-ground feeders and that simultaneous above and below-ground herbivory do not necessarily results in short term fitness costs for the plant.

Material and Methods

Plant growth conditions

Seeds of the 31^{st} generation of an inbred line of *Nicotiana attenuata* Torr. Ex Watts originally collected from Utah, USA, were used as the wild-type genotype in all experiments. Seeds were first surface sterilized and incubated with 1:50 (v/v) diluted liquid smoke (House of Herbs, Passaic, NY, USA) and 0.1 M gibberellic acid (GA3), and germinated on plates containing Gamborg's B5 medium as described in Krugel et al. (2002). Ten days after germination, seedlings were transferred to Teku pots (Poppelmann GmbH & Co. KG, Lohne, Germany) for an additional 10-12 days, followed by a final transplanting to 1 L pots filled with washed sand. Plants were grown under glasshouse conditions at 45-55% relative humidity, 24-26°C, and 16:8h light:dark and 14:14:14 of N:P:K twice per week (Krugel et al., 2002).

Nematode rearing

Meloidogyne incognita (Kofoid & White) Chitwood root-knot nematodes were reared on Tomato (*Solanum lycopersicon*) plants. For this, 1000-2000 eggs were applied to the roots. 45-five to 60 days later the roots were harvested and the nematode eggs were extracted by immersing the roots in a sodium-hypochlorite solution (0.05%) for 30 minutes. The resulting solution was then passed through a 250-μm-mesh sieve and through a 25-μm-mesh sieve according to Boneti & Ferraz (1981). Collected eggs were then re-suspended in distilled water and stored at 4°C for further experiments. The number of collected eggs was estimated by direct counting under a stereomicroscope (Leica S6E; Leica MicrosystemsGmbH; Germany) using an New bauer camera.

Plant-mediated effect of *M. sexta* herbivory on *M. incognita* reproductive fitness

Manduca sexta herbivory decreases soluble sugars and increases the levels of nicotine in the roots of *N. attenuata* plants (Machado et al., 2013). To determine whether these *M. sexta*–induced changes in *N. attenuata* roots and whether there are an effect on the performance of *M. incognita*, we evaluated the reproductive fitness of *M. incognita* females reared on *N. attenuata* plants that were previously subjected to *M. sexta* simulated herbivory. *Manduca sexta* herbivory was simulated essentially as described by Machado et al. (2013). Briefly, a serrated pattern wheel was rolled on three rosette leaves for three times on each side of the midvein. The wounds were immediately treated with 20 μL of a 1:5 diluted (v/v) *M. sexta* oral secretions (W+OS). To distinguish between *M. sexta*-specific and wound-specific effects, an additional set of plants were wounded and treated with 20 μL of water (W+W). The treatments were repeated every other day for three times to obtain 9 treated leaves per plant. Intact plants served as controls ($n = 5$). These treatments induce defenses similar to attacks by *M. sexta* without removing extensive amount of plant tissue and allowing for standardized damage (Halitschke et al., 2001). *Manduca sexta* oral secretions (OS) were collected as described by McCloud & Baldwin (1997). One day after the last treatment, either 5000 or 10000 *M. incognita* eggs were applied directly to the root system. Seven days after *M. incognita* inoculation, a last OS-elicitation treatment was carried out as described before. 28 days after *M. incognita* inoculation, the plants were harvested and the number of galls and eggs laid by *M. incognita* was determined. Eggs were collected as described before. The average length of *M. incognita* life cycle was taken into account to determine the time of harvesting (Moens, M et al., 2009).

Plant fitness upon *M. sexta* and *M. incognita* herbivory

The induction of plant defenses after herbivore attack is costly and it is usually accompanied by fitness costs (Machado et al., 2013). To determine whether simultaneous *M. sexta* and *M. incognita* herbivory affected *N. attenuata* reproductive fitness, we counted the seed capsules of plants that were subjected to simulated *M.*

sexta and actual *M. incognita* herbivory. The number of seed capsules is positively correlated with total seed production, therefore a suitable indicator of plant fitness (Glawe et al., 2003). Simulated *M. sexta* and actual *M. incognita* herbivory were carried out as described before.

Statistical analysis

All data were analyzed using Generalized Linear Models (GLM), performed in R (R Development Core Team, 2012), followed by residual analysis to verify the suitability of error distribution and model fitting. When necessary, model simplification was performed through Pairwise GLM contrast analyses with χ^2 or F tests. When the effects were not significant ($P > 0.05$), treatment levels were combined, as recommended by Crawley (2007). Whether the data were overdispersed, we carried out the analysis using a quasipoisson distribuition with F test. Models used were : (i) the effect of *M. sexta* simulated herbivory ($x - var1$) and the initial number of *M. incognita* eggs inoculated ($x - var2$) on the number of galls formed and eggs laid by *M. incognita* ($y - var$) and (ii) the effect of *M. sexta* and *M. incognita* herbivory ($x - var1$) and the initial number of *M. incognita* eggs inoculated ($x - var2$) on number seed capsules produced (y-var).

Results

M. incognita have higher reproductive fitness on *M. sexta*-attacked *N. attenuata* plants

To determine whether simulated *M. sexta* herbivory affected reproduction of the root knot nematode, we counted the number of galls and eggs laid by *M. incognita* females that were reared on *N. attenuata* plants previously subjected to simulated *M. sexta* herbivory. We observed that the number of galls formed in the roots was affected only by nematode inoculation, the plants that were submited to 5000 eggs/plant produced less galls than plants with 10000 nematode inoculation (Fig. 3.1, $F_{[1,28]} = 8.46$; $p = 0.007$). We observed that *M. incognita* females produced more eggs on plants that were previously subjected to simulated *M. sexta* herbivory (W+OS) than did on W+W-treated or on control plants. Interestingly, this pattern was only observed when 10000 eggs/plant were used as initial inoculum (Fig. 3.2, $F_{[2,26]} = 6.01$; $p < 0.001$). Plants which were inoculated with 5000 eggs/plant the simulated *M. sexta* herbivory had no effect on *M. incognita* fecundity (Fig. 3.2, $F_{[2,12]} = 0.82$; $p = 0.461$).

M. sexta but not *M. incognita* herbivory reduces *N. attenuata* fitness

To evaluate whether *M. sexta* herbivory and *M. incognita* herbivory affected *N. attenuata* fitness, we determined the number of seed capsules of plants that were under both simulated *M. sexta* and actual *M. incognita* herbivory. Plants of *N. attenuata* produced significantly lower number of seed capsules upon wounding (W+W) and simulated *M. sexta* herbivory (W+OS) independently of the initial *M. incognita* inoculum level: 5000 (Fig. 3.3, $\chi^2_{[2,12]} = 11.01$; $p = 0.03$) or 10000 (Fig. 2, $F_{[2,12]} = 5.06$; $p = 0.02$).

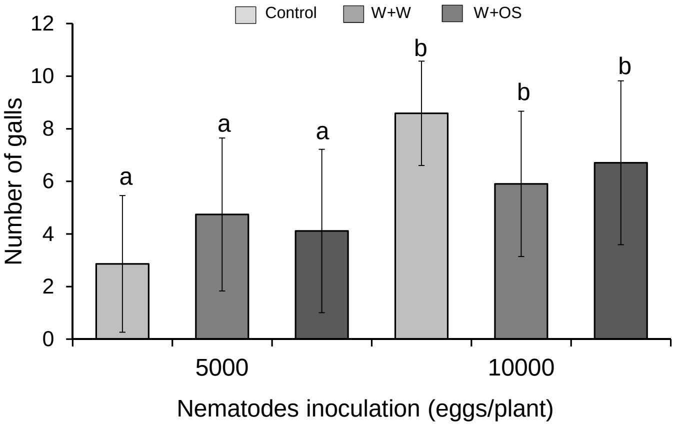

Figure 3.1: The number of galls produced by *Meloidogyne incognita* were not affected in *Nicotiana attenuata* plants upon *Manduca sexta* herbivory. Number of galls in *N. attenuata*, under simulated herbivory by OS-elicitation (W+OS), mechanical damage (W+W) and untreated (control) plants. Bars represent average±SE ($n = 5$). Different letters indicate significant differences ($P < 0.05$) between nematode inoculation; two-way ANOVA with Pairwise GLM comparisons under Poisson distribution.

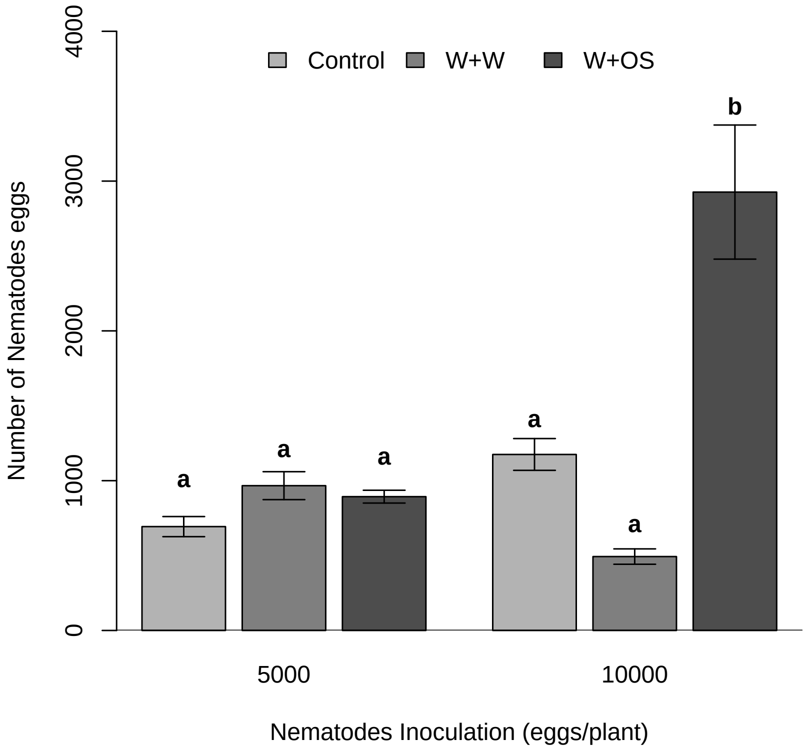

Figure 3.2: *Meloidogyne incognita*increased their fecundity in *Nicotiana attenuata* plants upon *Manduca sexta* herbivory. Number of nematodes eggs in *N. attenuata*, under simulated herbivory by OS-elicitation (W+OS), mechanical damage (W+W) and untreated (control) plants. Bars represent average±SE ($n = 5$). Different letters indicate significant differences ($P < 0.05$) between treatments within each density of nematode eggs; two-way ANOVA with Pairwise GLM comparisons under Poisson distribution.

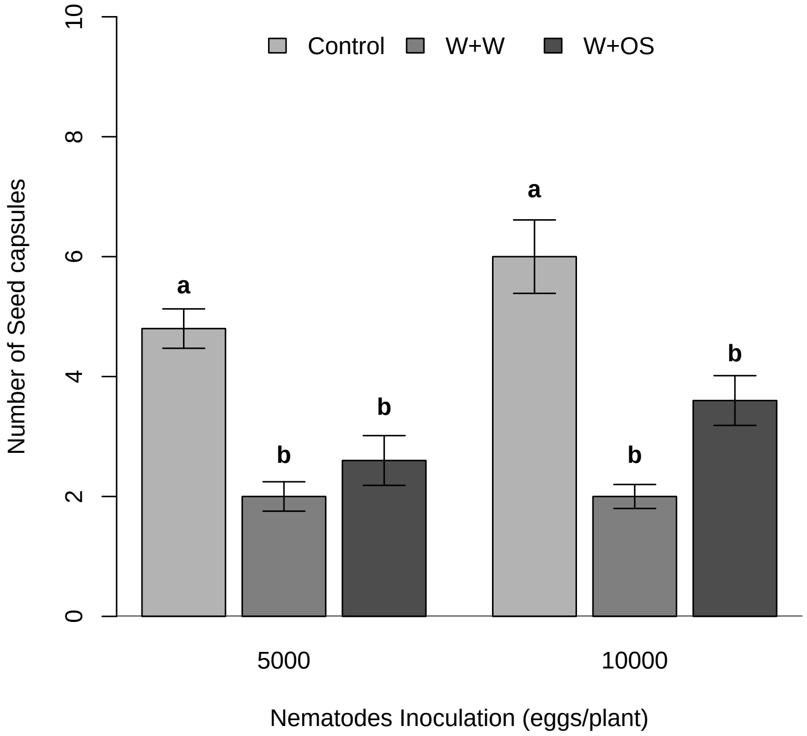

Figure 3.3: *Nicotiana attenuata* plants have their fitness reduced after wounding and *Manduca sexta* herbivory. Number seed capsules of *N. attenuata* plants, under simulated herbivory by *M. sexta* OS-elicitation (W+OS), mechanical damage (W+W) and untreated (control) plants. Bars represent average±SE ($n = 5$). Different letters indicate significant differences ($P < 0.05$) between treatments within each density of nematode eggs; two-way ANOVA with Pairwise GLM comparisons under Poisson distribution.

Discussion

N. attenuata-mediated effect of *M. sexta* on *M. incognita* reproduction

Contrary to our expectation, we observed that females of the root-knot nematode reared on *N. attenuata* plants subjected to a prolonged simulated *M. sexta* herbivory produced more eggs than did the females reared on control plants. However, the number of galls produced was not affected by simulated herbivory. Several studies have reported that aboveground herbivory positively impacts belowground herbivores leading to increased belowground herbivory (reviewed by Wondafrash et al., 2013). For example, similar to what we observed, Kaplan et al. (2008) found that the root-knot nematode, *M. incognita*, laid more eggs on *M. sexta* attacked-Nicotiana tabaccum plant that did on control plants. The authors argued that the increased nematode fecundity was due to 1) higher invertase activity in the roots and 2) higher photoassimilate transport to the roots. The higher photoassimilate transport to the roots has been widely observed in other plant systems (Dyer et al., 1991; Briske et al., 1996; Holland et al., 1996; Babst et al., 2005, 2008; Bazot et al., 2005; Schwachtje et al., 2006; Gómez et al., 2010, 2012) as well as the increased invertase activity in the roots (Schwachtje et al., 2006). It is widely considered that these two phenomena will result in higher sugar pools in the roots, which benefits the development of root feeders. However, none of the above mentioned studies have documented an increase in sugar pools in the roots. To the contrary, soluble sugars are even depleted in the roots upon above-ground-herbivory (Gómez et al., 2012; Machado et al., 2013). One explanation for this is that the increased resources in the roots might be used for the synthesis of secondary compounds to support aboveground defense (Arnold & Schultz, 2002; Arnold et al., 2004; Appel et al., 2012; Ferrieri et al., 2012, 2013). Apart from that, increased photoassimilate transport to the roots does not necessarily result in increased carbon pools since the translocated photoassimilates may be used for other processes like exudation into the rhizosphere (Holland et al., 1996;

Frost & Hunter, 2008) or respiration (Clayton et al., 2010). Taken together, we propose that a different mechanism explain higher root nematode fecundity upon foliar herbivory.

Vascular parasites such as root-knot nematodes induce metabolic sinks in their host plant that alter normal translocation patterns of sugars and amino acids, redirecting assimilate flow toward feeding sites (Peel & Ho, 1970; Forrest, 1971; Rehill & Schultz, 2003). The root-knot nematode *M. incognita* induces the formation of galls that act as sinks for assimilates from both the phloem and xylem (McClure, 1977; Hussey, 1985; Sijmons et al., 1994). Specific transport processes are altered (Hammes et al., 2005), including the induction of sink transporter which may be involved in transporting nutrients required for nematode development (Hammes et al., 2006). The above suggests that *M. incognita* fecundity might not be affected by the *M. sexta*-induced sugar depletion in the roots. A conclusive test for that hypothesis might be to evaluate *M. incognita* fecundity on plants that constitutively transport more photoassimilates to the roots i.e.: *N. attenuata* asGAL83 (Schwachtje et al., 2006).

Machado et al. (2013) have also observed increased levels of nicotine in *N. attenuata* roots upon *M. sexta* attack. Nicotine is a nitrogen-rich compound and might serve as nitrogen source for root feeders. Therefore, increased levels of nicotine in the roots might benefit *M. incognita* reproductive output by providing a source of nitrogen. Evidence to support this hypothesis is still controversial. Preisser et al. (2007) found that *M. incognita* reproductive fitness was similar when developed on *N. tabaccum* genotypes that differed in their nicotine biosynthesis capabilities. However, nicotine has also been reported that negatively affect nematodes (Hanounik et al., 1975; Hanounik & Osborne, 1977; Rich & Barker, 1984). This suggest that increased levels of nicotine might not explain the higher nematode performance. To conclusively test whether nicotine content in the roots affect the development of *M. incognita*, their reproductive output should be assessed on plants impaired in the biosynthesis of nicotine i.e.: *N. attenuata* irPMT (Steppuhn & Baldwin, 2007).

Mi-1 is a single dominant gene that confers resistance against the three major species of root-knot nematodes: *M. incognita*, *M. javanica*, and *M. arenaria* (Milligan et al., 1998; Rossi et al., 1998; Nombela et al., 2003). Induction of *Mi*-mediated nematode resistance is correlated with increased activity of several defensive enzymes (Brueske, 1980; Bajaj et al., 1985; Zacheo et al., 1993). In addition, resistance is associated with rapid localized cell death around the invading nematode (Riggs & Winstead, 1959; Dropkin, 1969; Paulson & Webster, 1972). Many studies of nematode infested plants have shown that *Mi*-mediated resistance can dramatically reduce root-knot nematode survival, reproduction and gall induction (e.g. Dropkin, 1969; Milligan et al., 1998; Goggin et al., 2004). To date, this gene has been identified in many other cultivars such as potato, peach and wheat (Barbary et al., 2014; Cao et al., 2014). Taken into account the above, we propose as an alternative explanation for the better performance of *M. incognita* on *M. sexta*-attacked plants, that *M. sexta* negatively affect *N. attenuata* capability to effectively defend against the root knot nematode *M. incognita* by, for example, interfering with the *Mi*-1-mediated resistance. Gene expression of *Mi*-1 homologue genes upon *M. sexta* attack might shed lights on whether or not this hypothesis holds true. Alternatively, the generation of transgenic *N. attenuata* plants silenced in the expression of *Mi*-1-homologue genes might help to conclusively test whether such genes confer resistance against *M. incognita* and whether or not are involved in this plant-mediated *M. sexta*- *M. incognita* interaction.

Effect of above and belowground herbivory on *N. attenuata's* fitness

In this study, we found that plants under machanical damage and a prolonged *M. sexta* simulated herbivory produce less number of seed capsules compared to undamaged plants. This effect seems to be governed by mechanical damage, once that the reduced seeds capsules produced were observed on both treatments. On the other hand, *M. incognita* has little effect on *N. attenuata's* fitness. Current optimal plant defense theory predicts that one benefit of induced defenses is to allow a plant to optimize the allocation of limiting resources to defense, growth, and reproduction (Karban & Baldwin, 1997). When plants are challenged by herbivory, it induces the synthesis of defensive compounds to minimize the herbivore damage, thereby limiting the resources that could be otherwise used for growth and/or reproduction (Mc Key, 1974; van der Meijden et al., 1988; Schwachtje & Baldwin, 2008; de Jong & van der Meijden, 2000; Anten & Pierik, 2010; Orians et al., 2011). In response to *M. sexta* attack, *N. attenuata* plants induce the synthesis of a complex blend of secondary compounds as 17-Hydroxygeranyllinalool diterpene glycosides (DTGs), nicotine, proteinase inhibitors (TPIs) and phenolamides (Steppuhn & Baldwin, 2007; Heiling et al., 2010; Kaur et al., 2010) that impose a metabolic cost for the plant, and might consequently limit the resources for growth and reproduction. The perception of fatty acid-amino acid conjugates (FACs) present in the oral secretion of *M. sexta*, results in growth reductions of the plant (Hummel et al., 2009) and reduced number of flowers, seed capsules and lifetime (Baldwin, 1998; van Dam & Baldwin, 1998; Zavala & Baldwin, 2006; Machado et al., 2013).

On the other hand, we did not observe fitness costs imposed by *M. incognita* herbivory on *N. attenuata* plants. This observation is not supported by the current literature. For example, Corbett et al. (2011) observed that tomato plants did not suffer any fitness cost under low nematode herbivory pressure (plants inoculated with 20.000 eggs/plant). However, when plants were inoculated with 200.000 eggs/plant, a drastic reduction in fruit production was observed. Similar observation was done by other authors (Sorribas et al., 2005; Lopez-Perez et al., 2006). We propose four possible explanations for this divergence: 1) since the fitness cost associated with nematode herbivory is population-dependent (Corbett et al., 2011), the number of eggs used in our study might have been low and therefore the nematode damage might have not been sufficient to impose a fitness cost; 2) Although capsule number is a suitable parameter to measure plant fitness (Machado et al., 2013), the actual fitness cost associated to *M. incognita* herbivory might only be visible in the following generations, for example by reducing the number of seeds per capsule or their viability; 3) the virulence of the nematode population used in this study might differ to the one of the other studies. It might be then plausible that *N. attenuata* poses tolerance/resistance mechanisms against nematode infestation. However, to test the hypothesis mentioned above, further experiments with different *M. incognita* strains, at different initial inoculum densities are required.

In conclusion, we have shown that *M. sexta* herbivory promote facilities to *M. incognita* reproduction. Moreover, we found that *M. incognita* herbivory has little impact on *N. attenuata* fitness. Although some of the underlying mechanisms that drive above- and below-ground interactions have been documented in the current literature, the experimental evidence is still controversial. Our study adds evidence for potentially new mechanisms that modulate plant-mediated above- and belowground herbivore interactions and opens new research avenues on this topic.

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Chapter 4

The root feeder *Meloidogyne incognita* in tomato plants affects oviposition and offspring development of leaf-mining *Tuta absoluta*

Abstract

Herbivores have been shown to interact indirectly through the plants' response. In the case of systemically induced plant responses, this interaction extends to herbivores inhabiting different parts of a plant, for example, above- and below-ground. Plant-induced responses elicited by root herbivores have been shown to affect feeding and development of above-ground herbivores. We assessed whether the presence of a below-ground nematode, *Meloidogyne incognita*, in the roots of *Solanum lycopersicon* influenced the oviposition decisions and development of an above-ground insect, *Tuta absoluta*. Moreover, we also checked if the period of damage by the below-ground herbivore (i.e. 10 and 20 days after the infestation) affected the oviposition and development of the above-ground insect. In oviposition choice-tests females of *T. absoluta* deposited more eggs on healthy plants than plants with *M. incognita*. The result was the same for 10 and 20 days after infestation by *M. incognita* in the roots. The developmental parameters of *T. absoluta* were negative affected by the root feeder. Our results show that indirect interactions between below-ground and above-ground herbivores extend to behavioral avoidance, in terms of oviposition. Moreover, given the negative effect on development, *T. absoluta* seems to avoid this root infested plants to attempt to choose the "best" for their offspring.

Keywords: Interaction above- and below-ground, *Tuta absoluta*, *Meloidogyne incognita*, "PPH", induced defense.

Introduction

Host plant selection is a crucial aspect in the ecology and evolution of interactions between plants and phytophagous insects (Thompson & Pellmyr, 1991). In specialist insect herbivores, the female preference oviposition for specific plant species, or even different phenological stages and physiological states of given species, often correlates well with offspring survival (Gripenberg et al., 2010; Jaenike, 1978; Mayhew, 2001; Rajapakse & Walter, 2007). The “preference-performance hypothesis” (PPH) for herbivorous insects states that females choose to oviposit on the host with the best suitability for the offspring, especially when the offspring have no or limited ability to relocate after hatching (Jaenike, 1978). Factors directly relating to the nutritional quality of the food plant include the levels of primary as well as secondary metabolites (Bezemer et al., 2003). Plant compounds may act directly by deterring oviposition and repelling lepidopteran herbivores (De Moraes et al., 2001; Kessler & Baldwin, 2001).

Inter- and intraspecific competitive interactions have recently been identified as important drivers of the performance and distribution of phytophagous insects (Kaplan & Denno, 2007). These interactions can be direct, for example, through interference and exploitative competition. However, many interactions between herbivores were found to be indirect, often mediated by plant responses (Ohgushi, 2005; Kaplan & Denno, 2007). Studies of plant-insect interactions have focused on organisms present in the same domain, mainly above-ground (van Dam et al., 2003). However, root herbivores and pathogens interfere with basic below-ground plant functions, and they can thereby affect plant fitness and spatial and temporal patterns in natural plant communities (Rasmann et al., 2011). Several studies reported that root herbivory, can result in both local (root) and systemic (foliar) increases in defensive compounds similar to what has been observed for foliar-feeding chewing insect (reviewed by Bezemer & Van Dam, 2005; Kaplan et al., 2008; Erb et al., 2008; Rasmann & Agrawal, 2008). Experiments using cultivated and wild species have shown that feeding by root feeders induces notable changes in the levels of primary and secondary non-volatile plant compounds in roots and leaves (Hopkins et al., 1998; Soler et al., 2005; Van Dam & Bezemer, 2006) as well as changes in the plant volatile blend (Neveu et al., 2002; Soler et al., 2007; Pierre et al., 2011).

Root-induced systemic responses may improve the defensive status of the shoot. Consequently, this response can also influence the performance of other organisms that are feeding from the same plant, but at other locations or later in time (Gange & Brown, 1989; Bezemer et al., 2003; Hol et al., 2004; Turlings & Wäckers, 2004). Many studies suggest that induced plant responses can also drive interactions between herbivores below- and above-ground (e.g., van der Putten et al., 2001; Wardle et al., 2004; Bezemer & Van Dam, 2005; Erb et al., 2008). There are many examples showing that the growth and development, and example where oviposition decisions (Soler et al., 2010), of above-ground herbivores are affected by belowground herbivory. Furthermore, it has been shown that below-ground herbivory can induce changes in the plant that influence the behavior and performance of natural enemies of above-ground herbivores (Wäckers & Bezemer, 2003; Rasmann & Turlings, 2007; Soler et al., 2007).

Ecological linkages between soil organisms and above-ground insect herbivores, mediated by effects on the quality and quantity of the plants compounds as primary resource for both subsystems (Bezemer et al., 2003; Wurst & Jones, 2003; Wardle et al., 2004; Soler et al., 2007). Recent studies show that the presence of soil organisms can affect host plant selection by shoot insect herbivores for feeding and oviposition (Soler et al., 2009, 2010; Wurst & Forstreuter, 2010). Variations in the health status of a plant may potentially be perceived by the olfactory system of an insect before and after it lands on the plant. Among the possible biotic factors, infections by soil microorganisms are known to influence the nutritional content of the plant, with positive, neutral or detrimental effects on the fitness of foliar insects. Therefore it is hard to predict the effects of root induced response on foliar herbivores without experimental assessments. Root herbivory affects distal above-ground plant organs and might consequently affect the performance of other plant herbivores (Wondafrash et al., 2013). In this study, we evaluated whether the root feeder *Meloidogyne incognita* herbivory on tomato plants (*Solanum lycopersicon*) affects the oviposition behavior and development of the leaf miner *Tuta absoluta*. Moreover, we also checked if the period of damage by root-knot nematode (i.e. 10 and 20 days after the infestation) affected oviposition and development of the aboveground insect. To this end, we carried out oviposition choice-tests, evaluated the performance of larvae that developed on and assessed the fecundity and longevity of females that emerged from *M. incognita* infested plants. Our results show that root feeder influence the oviposition preference of *T. absoluta* females and their offspring development.

Material and Methods

Tomato plants growing conditions

Solanum lycopersicon (cultivar Santa Clara I-5300) seeds were germinated in Teku pots filled with commercial substrate (Plantmax, Eucatex Agro). 15 days later, the seedlings were transplanted to 1L pots filled with 1:1 soil and washed sand sterilized with methyl bromide. Fertilizer (15-15-20 N-P-K) was added every week and plants were watered daily. Plants were grown under greenhouse conditions at 55±5% relative humidity, 27,5±1,5°C, and 12h of photoperiod. 25-days-old plants were used to run the the experiments.

Tomato leaf miner insect rearing

The tomato leaf miner *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) was reared according to Miranda et al. (1998). In short, male and female *T. absoluta* adults were maintained in a cage (25°C, 70% relative humidity and 12:12 hour light-dark cycles). Detached tomato leaves were provided for oviposition daily. Eggs were collected every day and placed in a new cage. Larvae were fed with leaves of tomato *ad libitum*. Adults were used for the experiments.

Tomato root herbivore rearing

Meloidogyne incognita (Tylenchida, Heteroderidae) root-knot nematodes were extracted from tomato roots and inoculated on other tomato plants (*Solanum lycopersicon*, cultivar Santa Clara I-5300) where the nematodes were reared. After 53±7days , the tomato roots were harvested and immersed in a 0.05% sodium hypochlorite solution for 30 minutes. This solution was then filtered through a 200 mesh sieve and then through a 500 mesh sieve, roots were washed several times with distilled water according to Boneti & Ferraz (1981). The eggs collected were suspended in distilled water and stored at 4°C.

Tomato plants infection with *M. incognita* - Plant treatments

The nematode eggs (4000) were suspended in a water solution, the number of eggs were estimated by counting them using a New Bauer camera under a stereomicroscope. This solution was applied to the root system of 25-day old tomato plants that comprised the nematode treatments. Control treatment was constituted by non-infested tomato plants. Plants were used either 10 days (treatment 1) or 20 days (treatment 2) after inoculation. These time points were selected since 10 and 20 days after infestation it is the beginning of gall formation and all galls are fully formed, respectively (Arce, 2010).

Oviposition Choice-tests

To test whether *M. incognita* herbivory of the roots affected the oviposition preference of the tomato leaf miner *T. absoluta*, we recorded oviposition preference of females by infested and non-infested plants by nematodes. The choice-test was evaluated independently in plants that were subject to either 10 days or 20 days *M. incognita* herbivory. Intact non-infested plants were used as controls. Three mated 2-day-old *T. absoluta* females were released in each cages (90x90x90cm) (n=12) contained two plants: one infested and another non-infested control. The cages were maintained at environmental conditions (± 26°C, and 12:12 hour light-dark cycles), 12h after the release (overnight), females of *T. absoluta* prefer to lay their eggs during the night according to Proffit et al. (2011). After this period the eggs laid on each plant were counted (see Scheme 4.1 A).

Development of *T. absoluta* on *M. incognita* infested plants

To determine whether *M. incognita* herbivory affected the development of *T. absoluta* and whether the mothers' choice were good for its progeny, we evaluated the survival and developmental time of *T. absoluta* on *M. incognita* infested plants. For this, we used the plants from the above experiment (choice-test). After counted the eggs laid by females of *T. absoluta*, we standardized the number of eggs in all plants. When necessary the eggs were removed with a paintbrush and the same procedure was performed on plants that need not have eggs removed. The plants were kept in laboratory conditions (± 26°C, R.H. ± 70% and 12L:12D). The plants were monitored daily and the time of development and survival from egg to adult was assessed. Additionally, pupae were weighed and incubated separately (see Scheme 4.1 B). Pupae were sexed according to França & Castelo Branco (1992). Additionally we recorded the number of healthy and deformed pupae one day after pupation (see Fig. 4.2).

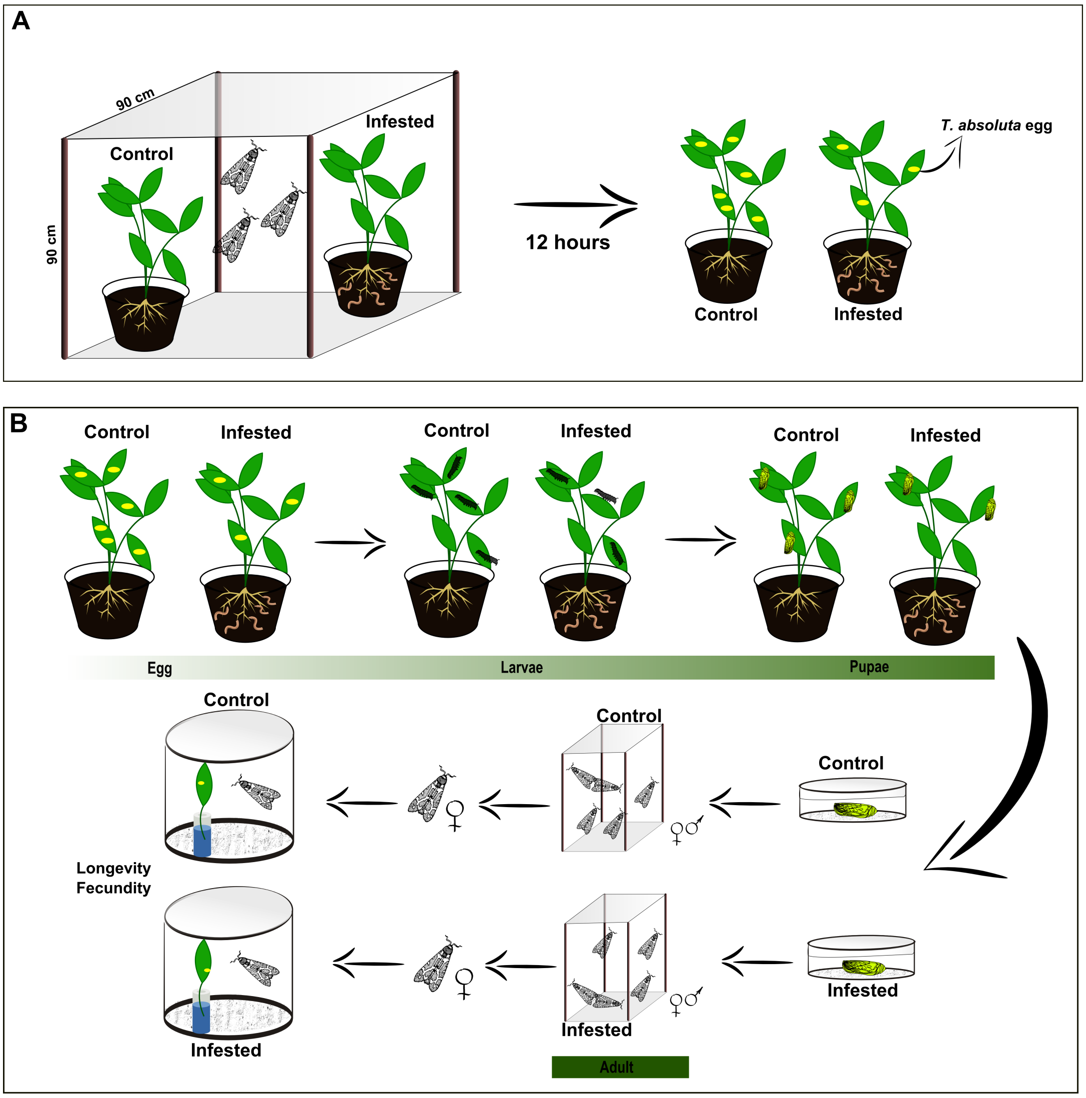

Figure 4.1: **A**. Experimental design used in oviposition choice-test (more details see Materials and Methods). Three mated females of *T. absoluta* chosen between control and infested plants in the root by root-knot nematode. After 12 hours the number of eggs on both plants were counted. **B**. Developmental parameters measured in *T. absoluta* on non-infested and infested tomato plants in the roots by *M. incognita*: time of development of egg into adulthood, longevity and fecundity of females. The experiments were repeated to 10 and 20 days after root infestation.

Figure 4.2: (A) Healthy *T. absoluta* pupae from non-infested tomato plants; (B) Deformed *T. absoluta* pupa from infested tomato plants in the root by *M. incognita*.

Fecundity and longevity of females of *T. absoluta*

To investigate whether *M. incognita* affected the longevity and fecundity of *T. absoluta*, we evaluated the duration of adult phase and the number of eggs laid by females during the adulthood of *T. absoluta* emerged from *M. incognita* infested tomato plants. Females and male adult emerged from infested plants were paired and allowed to mate. Adults emerged from non-infested plants were used as controls. 24 hours after mating, individual females were placed in transparent plastic pots (500 mL). Fresh tomato leaflets collected from non-infested plants were available for oviposition. Eggs were counted and fresh leaves were placed daily (see Scheme 4.1 B). This procedure was repeated until the females died or when the females stopped laying eggs for more than three consecutive days. The females were fed with a honey solution (10%) on a moist piece of cotton wool.

Statistical analysis

All data were analyzed using Generalized Linear Models (GLM), performed in R (R Development Core Team, 2012), followed by residual analysis to verify the suitability of error distribution and model fitting. When necessary, model simplification was performed through Pairwise GLM contrast analyses with χ^2 or F tests. When the effects were not significant ($P > 0.05$), treatment levels were combined, as recommended by Crawley (2007). To evaluate the oviposition preference by females of *T. absoluta* among infeted and non-infested plants, we carried out a Binomial distribution to each time of infestation separately. To assess the effect of *M. incognita* infestation on developmental time (days) of *T. absoluta* (from egg to adult), we used a Weibull distribuitiona, the plants entering in the model as a block, since they had more than one larva per plant. The effect of *M. incognita* infestation on insect survival (number of emerged adult per egg) was evaluated under Binomial distribution. The pupal weight of *T. absoluta* was carried out using a Normal distribution. The proportion of deformed pupae was analysed with a Binomial distribution. The sex ratio was calculated by an index (N° of females / [N° of females + N° of males]). To assess the effect of *M. incognita* on *T. absoluta* fecundity, it was evaluated under Poisson distribution. *Meloidogyne incognita* effect on *T. absoluta* longevity was tested under Poisson. distribution.

Results

Females of *T. absoluta* oviposit preferentially on non-infested plants by *M. incognita*

We found that *T. absoluta* females laid more eggs on non-infested plants than did on plants that were subject to both 10 days (Fig. 4.3, $F_{[1,6]}= 39.72$, $p < 0.001$) or 20 days (Fig. 4.3, $\chi^2= 83.82$, d.f.=8, $p < 0.001$) *M. incognita* herbivory.

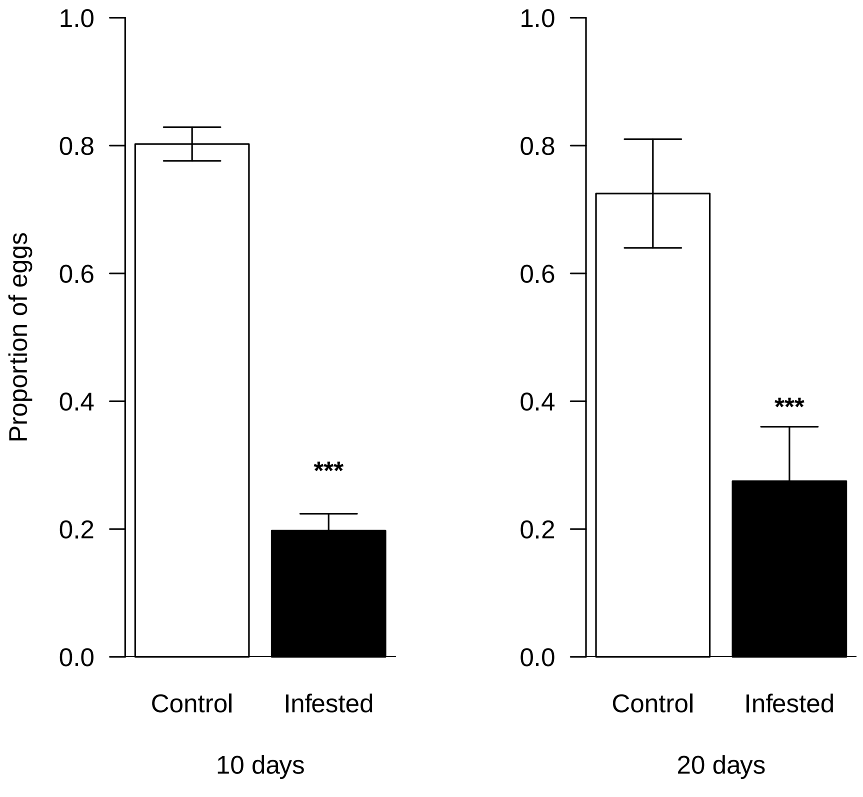

Figure 4.3: The proportion of eggs laid by females of *Tuta absoluta* in a oviposition choice test was higher in healthy plants than infested plants in the roots by *Meloidogyne incognita* 10 and 20 days after inoculation. Each bar represent the average ±SE (n=4), control (white) and infested (black). One-way ANOVA, GLM under Binomial distribution (P<0.005).

T. absoluta **develops faster in healthy plants than on** ***M. incognita*** **infested plants**

We observed that *T. absoluta* developed faster on non-infested plants than on *M. incognita* infested plants, independently of the duration of *M. incognita* herbivory. The development time of 50% of the eggs became adults was on average 27.20 days when *T. absoluta* developed on healthy non-infested plants while it was 28.17 days when developed on plants that were subjected to 10 days of *M. incognita* herbivory (Fig. 4.4, χ^2=2975.20, d.f.= 545, α =6.07, p=0.004). Similarly, we found that *T. absoluta* developed slower (28.64 days) when developed on plants that were subject to 20 days of *M. incognita* herbivory than did on healthy plants (27.58 days)(Fig. 4.5, χ^2=1897, d.f.= 363, α = 4.81, p< 0.001). We did not observe a significant effect on the survival rate (calculated as number of adults emerged per total number of eggs laid) when *T. absoluta* developed on plants that were subjected to 10 days of *M. incognita* herbivory (64%) compared to when did on control plants (74%) (Table 1, $F_{[1,21]}$= 1.43, p=0.243). Similarly, we observed only a marginal effect of *M. incognita* herbivory in survival rate when *T. absoluta* developed on plants subject to 20 days nematode herbivory (45%) compared to when did on control plants (52%)(Table 1, $F_{[1,19]}$= 0.48, p=0.496). *Meloidogyne incognita* herbivory did not affect either the pupal weight or the sex ratio (Table 1). Interestingly, we found that a greater proportion of deformed pupae were originated from *T. absoluta* larvae that developed on plants subjects to 20 days of *M. incognita* herbivory (Table 1, χ^2=114.9, d.f.=341, p=0.054) and a slightly higher proportion, although not significant, when did on plants upon 10 days of *M. incognita* infestation (Table 1, χ^2=359.5, d.f.=543, p=0.113). Taken together, our data suggest that, when co-occurring, *M. incognita* herbivory retard the development of *T. absoluta* larvae, potentially decreases the survival rate and increases the proportion of deformed pupae, which all together might result in a detrimental effect of *M. incognita* herbivory on *T. absoluta* population dynamics.

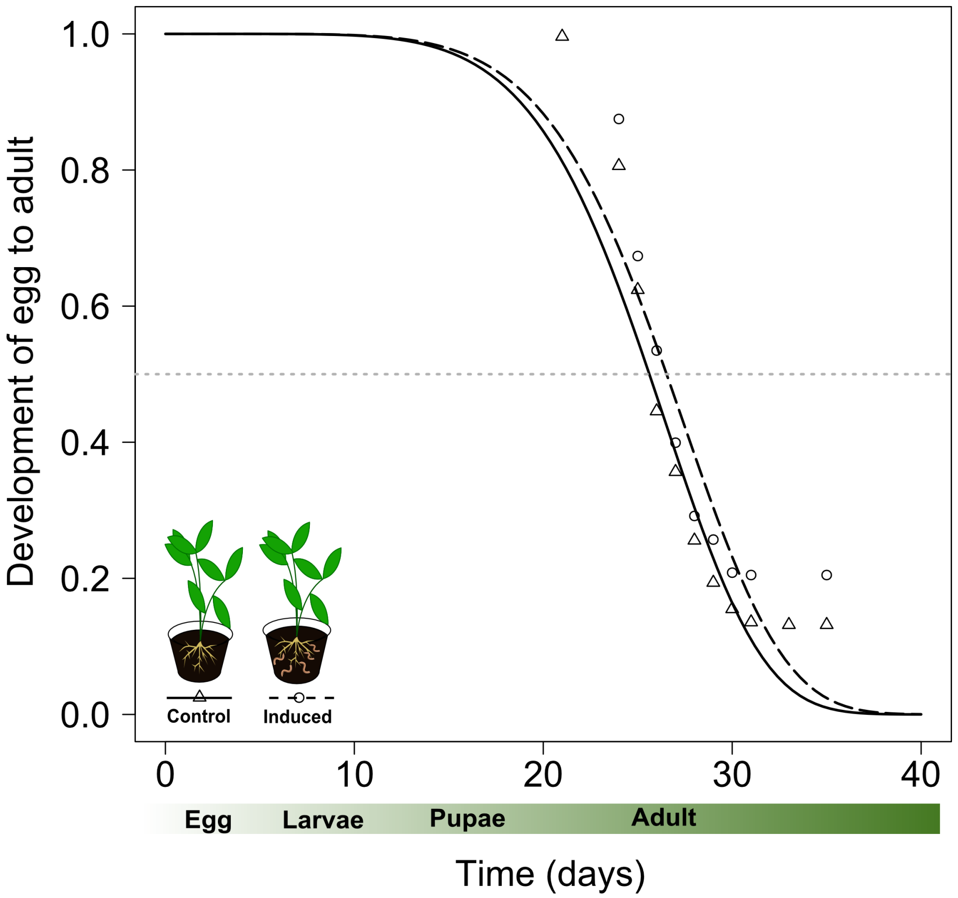

Figure 4.4: *Tuta absoluta* developed faster when fed in healthy tomato plants than induced plants in the root by nematode 10 days after inoculation. Lines represent in 1.0 when all insects were eggs and 0.0 when they became adults. Control (continuous line) and induced (dashed line). Two-way ANOVA, GLM under Weibull distribution ($p < 0.005$).

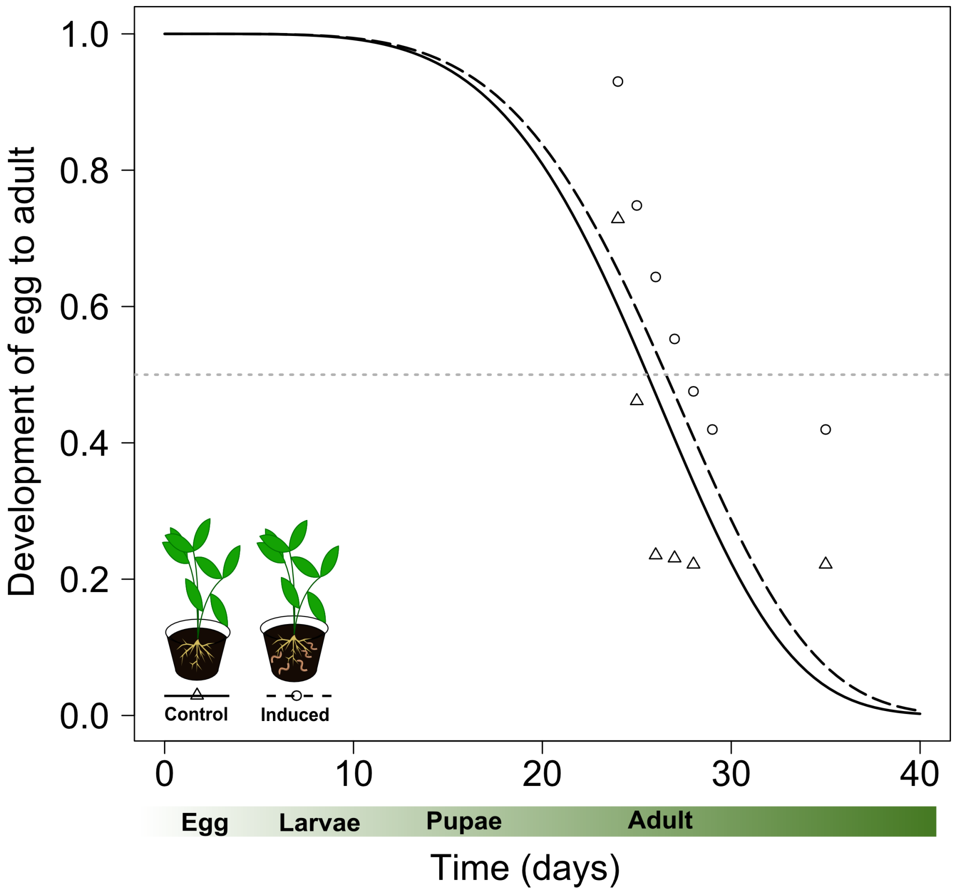

Figure 4.5: *Tuta absoluta* developed faster when fed in healthy tomato plants than induced plants in the root by nematode 20 days after inoculation. Lines represent in 1.0 when all insects were eggs and 0.0 when they became adults. Control (continuous line) and induced (dashed line). Two-way ANOVA, GLM under Weibull distribution ($p < 0.005$).

Table 4.1: Developmental parameters measured in *T. absoluta* feeding on non-infested (control) and infested tomato plants in the roots by nematodes. The results are from 10 and 20 days after root infestation by *M. incognita*. Survival (%), pupal weight (mg), pupal deformation (%), females longevity (days) and fecundity (number of total eggs laid) are shown ± SD. ($*$ P = 0.05)

Parameters	**10 days after infestation by *M. incognita***				**20 days after infestation by *M. incognita***			
	Control	**Infested**	***F*/χ^2 *values***	***P values***	**Control**	**Infested**	***F*/χ^2 *values***	***P values***
Survival (%)	73.84 ± 6.42	64.3±4.49	[1,21] 1.43	0.243	52.94±7.55	45.52±7.1	[1,19] 0.48	0.496
Pupal weight (mg)	3.74±0.04	3.78±0.05	[1,544] 0.41	0.519	3.91±0.06	3.95±0.08	[1,362] 0.14	0.702
Pupal deformation (%)	7.75±0.01	11.8±0.01	[1,544] 349.8	0.110	2.26±0.01	6.29±0.01	[1,362] 114.9	0.054*
Females longevity (days)	12.8±0.38	13.1±0.55	[1,45] 23.45	0.719	11.5±0.44	11.7±0.48	[1,55] 36.55	0.948
Female fecundity (total eggs)	58.1±7.02	66.7±8.74	[1,45] 0.47	0.492	65.03±9.2	67.29±7.18	[1,55] 20.49	0.977

Fecundity and longevity of *T. absoluta* females were not affected by *M. incognita* herbivory

We found that the females emerged from healthy (no deformed) pupae that were originated from larvae that developed on plants subject to 10 days of *M. incognita* herbivory laid similar number of eggs than females that emerged from pupae developed on control non-infested plants (Table 1. $F_{[1,45]}= 0.47$, P=0.49). Similar results were observed for the fecundity of females emerging from plants subjected to 20 days of *M. incognita* herbivory (Table 1, χ^2=2049, d.f.=56, P=0.97). Female longevity of *T. absoluta* was not influenced by infestation of the plants for 10 days (Table 1, χ^2=23.45, d.f.=46, P=0.77) and 20 days of nematodes infestation (Table 1, χ^2=36.55, d.f.=56, P=0.94). These results show that the root infestation by root-knot nematode changes some parameters of *T. absoluta* however, not the reproduction as fecundity neither the longevity of females.

Discussion

This study shows that leaf herbivores can detect healthy host and choose oviposite on leaves of plants that do not have their root colonized by below-ground herbivores. Also, the developmental time was faster when larvae were fed on healthy plants. Nevertheless, the percentage of deformed pupae, the survivorship and the fecundity of these above ground herbivore were not affected when they were reared on root herbivore infested plants.

We observed that *T. absoluta* preferred to oviposit on leaves of tomato plants whose roots were not infested by *M. incognita.* The moth can distinguish between infested and non-infested plants before the complete formation of the root-knot. Although adults of *T. absoluta* choose healthy plants to lay their eggs, larvae reared on tomato plants infested with *M. incognita* where not affected in their fecundity and survivorship. These outcomes were observed even for those adults originated by the development of larvae fed on infested plants whose pupae were deformed.

The "preference-performance hypothesis" (PPH) predicts that females of insect herbivores choose to oviposit on host with the best suitability for their offspring (Jaenike, 1978). Several studies investigating oviposition preferences and larval performance have reported positive correlations consistent with the PPH (e.g. Craig et al., 1989; Heisswolf et al., 2005; Staley et al., 2009). Our data suggest that females of *T. absoluta* confirm this hypothesis, corroborating the additional reported data, as they follow this pattern to avoid nematodes infested plants to oviposit.

The pattern of choice observed during the oviposition of *T. absoluta* females seems to be due to changes in the blend of emitted volatiles by the tomato plants as physiological response to the root damages caused by *M. incognita.* It was already reported that females of *T. absoluta* use volatiles of tomato plants to distinguish between resistant and susceptible hosts to herbivory attack (Ataíde, 2009; Proffit et al., 2011). Herbivore-induced plant volatiles (HIPVs) are emitted by plants after damage and have been used as an efficient signal to evaluate food resources by herbivores (Arimura et al., 2009; Dicke & Baldwin, 2010; Heil & Karban, 2010). Undamaged plants seems to be more attractive to herbivores than damaged ones (see Dicke & van Loon, 2000; De Moraes et al., 2001; Heil, 2004). Moreover, root herbivory altering the eggs distribution of above-ground herbivores has also been reported (Simelane, 2006; Soler et al., 2010; Anderson et al., 2011).

The ability to perceive between plants already infested by herbivores from the non-infested ones before the oviposition has the important effect to avoid interspecific competition between the larvae (Kaplan & Denno, 2007). This is an adaptive trait of some species of herbivorous insects through which they can choose in advance the best quality of food for their offspring, with positive effect on their survivorship and, consequently, on their fitness. Thus, being able to perceive plants under root herbivore attack and to do it as earlier as possible is an extra advantage contributing to the reproductive success of *T. absoluta*.

Our results show that the development of *T. absoluta* (egg-adult) on tomato plants infested by *M. incognita* was slower than on non-infested ones. It can be explained by the increases in the amount of trypsin protein inhibitors (TPIs) in the tomato leaves after root damage by nematodes (Arce, 2010). These proteins present have deleterious effect on insects by reducing the plant tissues digestibility by insects. Consequently, there is less energy available than the necessary to support a high metabolism demanded by a fast development.

Ecologically, fast development can be an advantage. Species that grow and reproduce fast can reach large population in short period of time and it might mean explore and dominate the available resources against interspecific competitors. Individual fitness depend on that and so its population dynamics, especially to species that explore ephemeral or seasonal resources that are distributed in patches (Shorrocks et al., 1990). Thus, avoiding infested tomato plants *T. absoluta* not only are choosing the best place for its offsprings but also contributing with the enlargement of its population.

Studies has been shown that shoot quality, as food resources, may change when the plant is infested by root herbivores, which may affect the performance of foliar insects (Soler et al., 2007; van Dam & Oomen, 2008). It has been reported that root herbivory can increase the concentration of shoot defensive compounds like terpenoids in *Gossypium herbaceum* and *Zea mays* (Bezemer et al., 2003, 2004; Rasmann et al., 2005), phenolics compounds in *Brassica nigra* (van Dam et al., 2005), pyrrolizidine alkaloids in *Senecio jacobea* (Hol et al., 2004), certain glucosinolates in *Brassica spp.* (van Dam et al., 2004; Soler et al., 2005, 2007; Van Dam & Bezemer, 2006), phytoectosteroids in *Spinacia oleracea* (Schmelz et al., 1998), and proteinase inhibitors in *Nicotiana attenuata* (van Dam et al., 2001). Although some compounds are characteristically synthesized by a botanical family or some group of plant species, there is not a clear pattern of which chemical defensive compound could be activated when a plant is under herbivore attack because it depends on the herbivore species, the plant species and environmental conditions where it is growing, the intensity and duration of the damage (Gange & Brown, 1989; Bezemer et al., 2003).

Nevertheless, when plants are attacked by insects, independently of the tissue type - root or shoot system- jasmonic acid (JA) chemical pathway are systemically triggered and its related chemical responses mediate a negative impact from the initial to the subsequent species (Karban & Baldwin, 1997). These JA-related responses constitute the main route of proteinase inhibitors activation and synthesis induction of volatiles compounds as terpenes. It is probable that this JA-related responses induced defense modulated the interaction of *M. incognita* and *T. absoluta* via tomato plant.

In the present work we obtained data showing that a larger number of deformed pupae and lower survival rate was observed for *T. absoluta* individuals reared on infested tomato plants compared with those reared on non-infested ones. Interestingly, we did not find differences in the fecundity and longevity between *T. absoluta* females originated from larvae fed with leaves of infested tomato plants from those reared on tomato leaves of non-infested plants. Although these data were not statistically significant, it is important to point out that individuals of *T. absoluta* fed on *M. incognita* infested plants presented unexpected negative effects in its biological features under the conditions that were run the experiments. These outcomes indicate that there is a high probability that these effects observed on *T. absoluta* biology might be caused by the biological activity of *M. incognita*, as consumption of plant resources, on tomato roots.

Thus, it seems that *T. absoluta* individuals are able to effective and efficiently use the plant chemical reaction against the root herbivore as a cue to perceive between root herbivore infested and non-infested tomato plants. This ability has a positive effect on its developmental time and on the probability of survivorship of its offspring. Consequently, avoiding host plants previously infested by *M. incognita* is an adaptive strategy of *T. absoluta* that contribute to individual reproductive success and to maintain its large populations and to match the characteristics of its resources distribution.

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Chapter 5

General Conclusions

Given the essential role that cell-wall invertase plays in carbon allocation processes, it is intriguing as to why there are inhibitory proteins such as NaCWII that limit CWI activity. In the chapter 2 we propose a resource allocation model where modulation by CWII may function to control and optimize carbon distribution under plant defense. This carbon allocation strategy ensures efficient utilization of limited resources for active growth and defense. In this context, we propose that the dramatic increase in expression of NaCWII in leaves following herbivore damage, upon which the activity of CWI is suppressed, may act to dictate the timing of defense induction thereby guaranteeing optimal carbon allocation to growth and defense induction during herbivore attack. When plants are attacked simultaneously in above- and below-ground, it is possible to observe a different patterns of interactions between herbivores. A positive effect on nematode reproduction after foliar herbivore and the costs to the plant to sustained the balance between growth and defense were observed in the chapter 3. Therefore the opposite way turn the interaction negative, in tomato plants where the foliar herbivore was impacted in its preference, showing that the host plant status in important to make good decisions to their progeny.

In this thesis we showed some relevant points: 1)the CWII involvement in regulating growth and metabolisms within the context of plant defense systems against insect herbivores has never been addresses; 2) the interactions among above- and below-ground herbivores have been focused more on leaf-chewing insects, works with leaf miner and nematodes are scarce. The findings generated by this thesis adds a new dimension not only to entomology, but also to other important fields of science. I expected that the presented results can be considered an important contribution to our efforts to the better understanding the plant induced defense mediating interactions between above- and below-ground herbivores and can generate new hypothesis to future works.