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# Induced defense in tomato damaged by *Spodoptera littoralis* larvae



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# Induced defense in tomato damaged by *Spodoptera littoralis* larvae

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# 1. Summary

Plants defend themselves against feeding insects via both a constitutive; always existing defense, and/or via an induced defense that is activated in response to the feeding damage. The aim of this study was to investigate the induced defense in tomato after damage by insect larvae. The hypothesis was that if larvae chose to feed on undamaged plants over damaged plants, they would do so due to an induced defense in the damaged plants. Moreover, the aim was to examine temporal aspects of induced defense, the difference in constitutive defense between genetically improved cultivars and wild tomato plants, the possibility of interplant communication and if induced defense compounds reduce the growth of larvae.

Food preference bioassays and a larval growth experiment were made to test the hypotheses. In feeding choice experiments, the larvae of the moth *Spodoptera littoralis* fed more on leaves from undamaged plants over leaves from damaged plants, which suggest that there was an induced defense in the damaged tomato plants that affected the larval behavior. The strongest effect of induced defense was found two days after the initial damage, but it was present until at least eight days after the initiation of the damage. Also in wild tomato plants, the induced defense was strong in recently wounded plants. There were no significant differences in constitutive defense between wild tomato and genetically improved tomato cultivars. When interplant communication was tested by larval feeding choice experiments, no difference was found between damaged or undamaged plants neither when these had grown together nor separately, which indicate that both airborne and root borne signaling can occur. Finally, when the larvae were forced to feed on either damaged or undamaged plants in a larval growth experiment, the larvae feeding on leaves from damaged plants showed reduced growth rate. This result indicated an induced defense of the damaged plants that affected the larval physiology.

## 2. Sammanfattning

När insekter äter på växter så kan växterna försvara sig både med ett konstitutivt försvar; som alltid finns i växten, och/eller med ett inducerat försvar som aktiveras efter skada orsakad av insekten. Syftet med den här studien var att analysera tomatplantans inducerade försvar mot insektslarver. Hypotesen var att om larverna valde att äta av oskadade plantor istället för av skadade plantor så skulle det bero på ett inducerat försvar i de skadade plantorna. Därefter var syftet även att studera efter hur lång tid som det inducerade försvaret var som mest effektivt, skillnaden i konstitutivt försvar mellan förädlade och vilda tomater, möjligheten för tomatplantor att kommunicera och slutligen att se om larvers tillväxt hämmas av att äta av inducerade plantor.

Tvåvals försök och larvtillväxtförsök har gjorts för att testa hypoteserna. När larver av nattflyet *Spodoptera littoralis* fick välja mellan skadade och oskadade plantor så valde den de oskadade plantorna, vilket tyder på ett inducerat försvar i de skadade plantorna. Effekten var tydligast två dagar efter första skadan men fanns kvar mer än åtta dagar efter initiering av skadan. Det var ingen skillnad i valet mellan vilda och förädlade oskadade tomater, vilket indikerar på ett lika konstitutivt försvar, trots hypotesen om större resistens i vildtomater. Larverna föredrog oskadade vilda tomater framför vilda tomater som skadats två dagar tidigare, vilket tyder på ett snabbt verkande inducerat försvar även i vildtomater. Det var ingen skillnad i larvers födoval mellan oskadade eller skadade tomater som hade grott i samma kärl eller som grott separat, vilket kan tyda på att försvarssignaler skickats både via luft och via rötter i jorden. Slutligen, i ett larvtillväxtförsök där larver tvingades äta på antingen skadade eller oskadade plantor under en vecka, hämmades larvernas tillväxt om de åt av skadade plantor. Resultatet från larvtillväxtförsöket indikerar att det skadade plantorna hade ett inducerat försvar som påverkade larvernas fysiologi.

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## **4. Introduction**

### **4.1. Constitutive and induced defense mechanisms in plants**

Plants have evolved a great number of defense mechanisms to protect themselves against herbivores and pathogens (Dicke et al., 2001; Gatehouse, 2002; Kennedy, 2003; Orians et al., 2000; Stout et al., 1994; Zangerl, 2003). Some of these mechanisms are constitutive, which means that they are always present in the plant, others are induced after that the plant is damaged. Herbivores and pathogens induce plants with different elicitors and the defense mechanisms against each attacker involve different signal transduction pathways (Dicke et al., 2001). Moreover, there are experiments that have shown that artificial damage induces defense responses in plants, which may indicate that the defense may be induced without a specific elicitor. The knowledge about elicitors and their mechanisms of induction is not yet fully understood.

In this report I have studied the induced defense in tomato plants, elicited by larvae of the Egyptian cotton leafworm larvae *Spodoptera littoralis* (Lepidoptera: Noctuidae). The elicitors of these larvae are thought to originate from their oral secretions and/or from the gut of the larvae (Dicke et al., 2001).

### **4.2. Direct and indirect induced defenses**

An herbivore that feeds on or searches for an egg-laying site on an induced plant may change to another host or feeding source since it otherwise could experience reduced fitness or mortality from the induced plant tissue (Dicke, 2003; Ament et al., 2004). This induced defense is referred to as a direct induced defense.

If the induced defense attracts natural enemies of the herbivores to the plant, it is referred to as an indirect induced defense (Dicke, 2003). Different types of herbivores; e.g. sucking, chewing, leaf mining, stem borers, root feeders or herbivores that use the plant for oviposition; induce specific responses in plants (Stout et al., 1998; Dicke, 2003). In the indirect induced defense, herbivore-induced plant volatiles (HIPV) attract specific parasitoids and parasites as these natural enemies have developed responses to odor blends that are emitted in specific ratios.

Another form of indirect defense is to produce extrafloral nectar as alternative food for the natural enemies on the plant (Bruin & Sabelis, 2001).

### **4.3. Interplant communication**

After herbivore attack, an herbivore-damaged plant may send the HIPVs to neighboring undamaged plants (Bruin & Dicke, 2001; Bruin & Sabelis, 2001; Farmer, 2001). The damaged plant would thus induce defense into the undamaged plants. The damaged plants benefit from the signaling as the HIPVs attract more natural enemies and the undamaged plants benefits from the signaling as they get induced defense before severe insect attacks. The plant-plant information may either be conspecific (intraspecific communication) or between different plant species (interspecific communication). Bruin and Sabelis (2001) showed positive evidence for interspecific communication between tomato and lima beans, where lima beans had been exposed to spider mites and the tomato plants received defense signals from the infested lima beans. In another study by Farmer and Ryan (1990), the HIPV methyl jasmonate was discussed to be involved in intraspecific communication between tomato plants, as tomato plants next to damaged tomato plants also showed induced accumulation of defense related proteins.

The volatile signals from a damaged plant are thought to move with the wind to undamaged plants (Bruin & Dicke, 2001). At the undamaged plant, the volatiles may enter the leaf through stomata and diffuse into the leaf cytoplasm. Alternatively, the volatiles bind to signal-receptors after entering stomata, and in that way the signal is transported within the vascular system. Thus, an air signal communication is dependent on open stomata and that receptors are present on cell surfaces within the receiving plant.

The plants are also thought to communicate in the rhizosphere by the roots and this transfer works in the absence of wind (Bruin & Dicke, 2001). Thus, the root-root communication makes the plant able to reach even “upwind” plants. The belowground transfer of information has been shown in vitro to occur via direct root-root contact in soil or in water solution (Bruin & Dicke, 2001; Chamberlain et al., 2001). In theory it is also discussed to occur via mycorrhizal fungi (Bruin & Dicke, 2001).

#### **4.4. Costs of plant defenses**

Plants are attacked by insects at different rates; depending on time, space and the quantity of insects (Dicke & Hilker, 2003). In an environment where there are large insect communities present, plants may have developed a mechanical and/or a chemical constitutive defense through the evolution. Constitutive defenses are not as common in annual plants and where there are small and unpredictable insect communities. An explanation to this is that constitutive defenses are biosynthetically and ecologically costly for plants that rather invest energy in growth and reproduction (Karban & Myers, 1989; Dicke & Hilker, 2003). The high costs of constitutive defenses is therefore suggested to be a reason to the evolution of inducible defenses since these are produced only when needed and hence may be considered as cost saving strategy of the plant (Karban & Myers, 1989; Zangerl, 2003). However, even inducible defenses may result in fitness costs such as decreased fruit production (Redman et al., 2001) and a drawback with the induced defense is that the plant remains susceptible to the herbivore in the time period between the initial damage and the peak of defense response (Zangerl, 2003).

#### **4.5. Defense strategies in tomato**

For many years, researchers have been using tomato (*Lycopersicon esculentum*) as a model plant for induced defense (Edwards et al., 1992; Orians et al., 2000; Stout et al., 1994; Duffey & Stout, 1996). A local damage in the tomato plant causes both a production of defense-related proteins and an allocation or production of secondary metabolites (Stout et al., 1998). The compounds are toxic both to the tomato pests and to the plant itself. Thus, the secondary metabolites and the defense-related proteins are stored within plant tissues such as the vacuoles and the trichome heads (Kennedy, 2003).

The constitutive defense of the tomato plant is associated with the trichomes that either make up a physical barrier (a mechanical defense) or act as releasers of toxic exudates when crushed by the insect (a chemical defense) (Kennedy, 2003). The secondary metabolites; acyl sugars, several phenolics (primarily rutin) and sesquiterpenes, are stored within the trichome heads and become toxic to the insect when they are oxidized after tissue damage (Bernays and Chapman, 1994). The trichomes may cause mortality to small insect species such as the two-spotted spider mites (Chatzivasileiadis & Sabelis 1997), and in experiments performed by

Hoffman and McEvoy (1986), 1<sup>st</sup> and 2<sup>nd</sup> instars were not able to feed on plants tissue if trichomes were present.

In the tomato, there is also a constitutive non-trichome defense that includes rutin, chlorogenic acid and the glycoalkaloid tomatine (Duffey & Stout; 1996, Kennedy, 2003), which are present in the vacuoles of both leaves and unripe fruits (Kennedy, 2003).

The induced defense of the tomato includes elevated levels of plant hormones and the toxic secondary metabolites tomatine, chlorogenic acid and acyl sugars that are allocated within the plant after local damage (Duffey & Stout, 1996; Edwards & Wratten, 1983). In addition, after an insect damages the tomato plant, the induction results in a production of proteinase inhibitors (PIs) and oxidative enzymes, which both are different defense-related proteins (Kennedy, 2003).

The PIs seem to be the main defense-related proteins in response to larval feeding (Stout et al. 1998) and they accumulate in the vacuoles of uninjured cells of damaged plants (Karban & Myers, 1989). PIs and the oxidative enzymes polyphenol oxidase (PPO), peroxidase (POD) and lipoxygenase (LOX) are induced by jasmonic acid, which pathway is elicited by the herbivore feeding damage.

As the larva consumes the induced tissue, the PIs bind to and inhibit digestive enzymes in the larval midgut, which leads to reduced growth of the larvae (Chen et al., 2005). PPO, POD and LOX catalyze an oxidation of the toxic tomato substances chlorogenic acid and rutin into quinones, which are associated with free radicals (Stout et al., 1994). Quinones are also alkylating amino acids of the tomato plant, and therefore reduce the plant's nutritional value.

#### **4.5.1. The systemic transport of defense molecules**

As insects damage a leaf at one position of the tomato plant, a signal is transported in the phloem vascular bundles to other leaf positions (Stout et al., 1996). In experiments made by Edwards et al. (1992), the induced responses of tomato were shown to be stronger in younger leaves than in mature fully developed leaves. This finding follows the Optimal defense theory (ODT), that the plant should defend its more valuable developing leaves, such as the top leaves, rather than the fully developed leaves (Anderson & Agrell, 2005). According to a study by Edwards et al. (1992), the stronger induced responses in younger tomato leaves may be due to competition as these leaves are the most important leaves in the competition for light. Orians et al. (2000) refers to the small sinks of young tissue that thus are able to receive more defense

signals. However, findings by Orians et al. (2000) and Stout et al. (1998), have shown that leaves at diverse positions respond differently due to the complex vascular architecture of tomato plants and that the result may be a non-uniform spatial distribution of the defense signals.

#### **4.5.2. The role of jasmonic acid for the wound signaling**

The major herbivore-mediated signals in tomato plants that arise in response to a wounding are the ester-derived jasmonic acid (JA) and the airborne methyl-jasmonate (JMT) (Edwards & Wratten, 1983; Dicke & Hilker, 2003; Ament et al., 2004; Wasternack et al., 2006). These wound signals are synthesized via the octadecanoid pathway and as they have been activated, an expression of genes coding for PIs and PPO is induced. In addition, there is also an accumulation of the precursor signal systemin, which leads to formation of JA and PIs within the vascular system (Wasternack et al., 2006).

#### **4.5.3. Variation in defense between genetically improved cultivars and wild tomato plants**

Insect resistance has not been an important breeding objective when developing new tomato cultivars (Heuvelink, 2005). Today, the insects that are pests of tomato are controlled biologically with natural enemies or by spraying insecticides. According to tomato breeders at the World Vegetable Center (AVRDC), some of the wild relatives of tomato possess insect resistance but the resistance has been difficult and time consuming to transfer into genetically improved cultivars (Hanson, 2007). When breeding for new cultivars, it takes long time to reduce the size of the wild DNA and eliminate factors such as the reduced fruit size of wild species. However, some of the new tomato cultivars have low levels of resistance as more active genes of acyl sugars, 2,3-tridecanone and tomatine are crossed into them (Hansson, 2007). In the wild tomato species *L. hisutum*, *L. glabratum* and *L. pennellii*, the trichome-mediated defense has significantly been shown to directly affect herbivores, parasites and parasitoids as these touch the trichome covered tissue (Kennedy, 2003). The herbivores are also hosts or preys for parasites and parasitoids, which results in a significant effect of the indirect defense in such plants.

In a study by Chatzivasileiadis and Sabelis (1997), the naturally occurring toxic methyl ketone 2-tridecanone was shown within the trichomes of both wild and cultivated tomato.

## **4.6. Experimental aims and hypothesises**

My main aim was to investigate the induced defense in tomato after herbivore damage. The herbivore *Spodoptera littoralis* was chosen for the experiments since it was easily maintained in the laboratory and since it was documented to be a pest of tomato in many countries (Edwards et al., 1992; Heuvelink, 2005).

My main hypothesis was that the *S. littoralis* larvae in two-choice bioassays would choose to feed on leaves from undamaged plants over damaged plants, due to the induced defense in the damaged plants.

### **4.6.1. Experiment 1: Temporal aspects of induced defense**

The aim of the first feeding experiment was to investigate if the induced defense was active at two, four, eight and thirteen days after the initial damage. From each damaged or undamaged plant, leaves from different leaf positions were taken, in order to observe a systemic plant response.

My hypothesis was that I would see an induced defense after all time periods since the production of PIs previously had been shown to occur within hours and last more than 23 days (Stout & Duffey, 1996). However, my hypothesis was that the induced defense two days after initial damage would be less expressed in the younger leaves but more in the leaves closer to the damaged 2<sup>nd</sup> true leaf, due to the short time for the systemic transport of signal molecules and PI production. Further, I believed that the peak of induced defense would occur at four or eight days after the initial damage, since four days after induction had been shown successful in a previous tomato experiment (Rodriguez et al. 2005). At thirteen days after the initial damage I expected that the quality of defense had decreased due to plant age (Stout & Duffey, 1996) and therefore I believed that these plants would have less effect on the larval feeding choice than the previous time periods.

### **4.6.2. Experiment 2: Induced defense in wild tomato**

In the third experiment, I aimed to test the hypothesis that there was an induced defense also in wild tomato. Leaflets from all leaf positions were chosen for investigating if the possible induced defense were systemically transported within the plant.

### **4.6.3. Experiment 3: The constitutive defense of wild vs. genetically improved tomato**

In the second experiment I aimed to see if wild tomato species were more resistant to insects than genetically improved tomato cultivars. In larval feeding choice experiments, the difference in constitutive defense was studied between fully developed leaves and developing leaves of plants with different genetic background.

My hypothesis was that *S. littoralis* chose to feed on genetically improved tomato leaves rather than wild tomato leaves due to the greater resistance properties of the wild tomato. Moreover, my hypothesis was that the larval choice of genetically improved cultivars would be most evident in the feeding choice experiment between top leaves because of the observation that the smaller leaves had higher densities of trichomes per leaf area and hence increased amounts of constitutive defense compounds.

### **4.6.4. Experiment 4: Interplant communication**

The aim of this experiment was to investigate if there was an interplant communication between tomato plants. By having damaged and undamaged plants grown together, I would test if the damaged plants sent signals through the roots to the undamaged plants or if the undamaged plants received airborne signal volatiles from the damaged plants. Moreover, undamaged plants that grew in separate pots were placed next to the plants that grew together, in order to test airborne signaling.

My hypothesis was that the undamaged plant that had grown together with the damaged plant would receive defense signals through the roots and therefore be equally chosen as the damaged plant in a larval feeding choice experiment. When undamaged and damaged plants were grown separately, my hypothesis was that the larvae would choose the undamaged plants because of the non-windy condition in the greenhouse that could limit the air signal transmission.

Moreover, my hypothesis was that the difference in feeding would be most obvious between the top leaves since it could be possible that the top leaves received more defense signals than fully developed leaves.

#### **4.6.5. Experiment 5: The effect on larval growth**

The fifth experiment was a larval growth experiment, where the aim was to show that induced defense compounds had effect on the larval physiology.

My hypothesis was that the larvae feeding on leaves from damaged plants would have a lower growth rate than the larvae feeding on leaves from undamaged plants. This because of the induced compounds in damaged plants that may have degraded essential amino acids in the midgut of *S. littoralis*.

### **5. Materials and methods**

#### **5.1. Plants**

Seeds from genetically improved tomato cultivars (*L. esculentum* cv. Aromata, Armada, Elin, Dalton) and seeds from a wild tomato, *L. esculentum* var. *cerasiforme* var. Summer Cherry (PI 290856) were sown in a greenhouse with 20°C. Natural light was supplemented with ZonT lamps (high pressure sodium, 10:14 hr light-dark cycle). When the plants had three to four developing leaves they were planted individually in 12 cm diameter pots and were watered daily and fertilized every second day with Superba T 1-5-3. When the plants had 8-9 true leaves, the plants were placed in a greenhouse at 15 °C. The true leaves were numbered from the base to the top, with the first true leaf as leaf number 1.

#### **5.2. Insects**

Egyptian cotton leafworm larvae (*Spodoptera littoralis* Boisduval) were taken from a laboratory culture, where they had been reared on artificial diet.

#### **5.3. Induction of plants**

To induce the plants, two or three 3<sup>rd</sup> instar larvae of *S. littoralis* were placed to damage the 2<sup>nd</sup> true leaf on each plant. To prevent the larvae from feeding elsewhere, the leaf and larvae were enclosed in a plastic bag that was sealed with a metal thread. The larvae were then allowed to feed for 24 hours and thereafter the plastic bag and larvae were removed. All damaged and the same number of undamaged plants were maintained in the greenhouse until the experiment started.

## **5.4. Two-choice bioassays**

After removal of leaflets from the plants, the leaflets were first photocopied and then put on moist filter paper in plastic boxes. One *S. littoralis* larva was put in the middle of the box with leaflets and left for 24 hours. If the larvae had not eaten after this period, they were replaced and the experiment continued for another 24 hours. Thereafter, the leaflets were photocopied again on squared paper. These copies were later on used to calculate the amount and percentage leaf surface eaten.

## **5.5. Experiments**

### **5.5.1. Temporal aspects of induced defense**

Leaflets from the 4<sup>th</sup>, 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> true leaf and the 2<sup>nd</sup> top leaf and the top leaf from damaged and undamaged common tomato plants were cut off with a razor blade and placed in boxes for the two-choice bioassays. This removal of leaves was done at two, four, eight and thirteen days after the initial induction of the plants.

### **5.5.2. Induced defense in wild tomato**

Two days after induction of wild plants (PI 290856), the 4<sup>th</sup>, 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> true leaves and the 2<sup>nd</sup> top and top leaves were cut off with a racer blade. These were then used in larval two-choice bioassays.

### **5.5.3. The constitutive defense of wild vs. genetically improved tomato**

Leaflets from the 6<sup>th</sup> and 7<sup>th</sup> true leaf, the 2<sup>nd</sup> top and top leaf were cut off with a racer blade from either a wild (PI 290856) or a genetically improved tomato cultivar (*L. esculentum* cv. Elin). In two-choice bioassays, each leaf position was compared.

### **5.5.4. Interplant communication**

In a plastic container (20 x 30 cm), one damaged and one undamaged plant (*L. esculentum* cv. Elin) were grown together to make root contact possible. Next to each container, one undamaged plant was placed. The air distance between all plants was set to 5 cm, but all plants unavoidably had leaf contacts at some of the leaf positions.

Two days after the first damage, two different experiments were performed with two-choice bioassays. In the first experiment, leaflets from the 6<sup>th</sup> true leaf and the top leaf were cut off from both the damaged plant and from the undamaged plant that had grown together. Thereafter the two-choice bioassays started. In the other experiment, the 7<sup>th</sup> true leaf and the 2<sup>nd</sup> top leaf were cut off from both the damaged plant and from the undamaged separately grown plant. The leaflets were then compared in larval consumption by two-choice bioassays.

#### **5.5.5. The effect on larval growth**

Twenty 3<sup>rd</sup> instar larvae were transferred into each of two boxes; one box with leaves from damaged plants and another with leaflets from control plants. The leaflets were taken from 4<sup>th</sup> true leaves and were placed on moist filter paper in the boxes together with the larvae. Weighing of larvae was made every day during a one week period and as the old leaves wilted during this period, the larvae were supplied with new fresh leaflets.

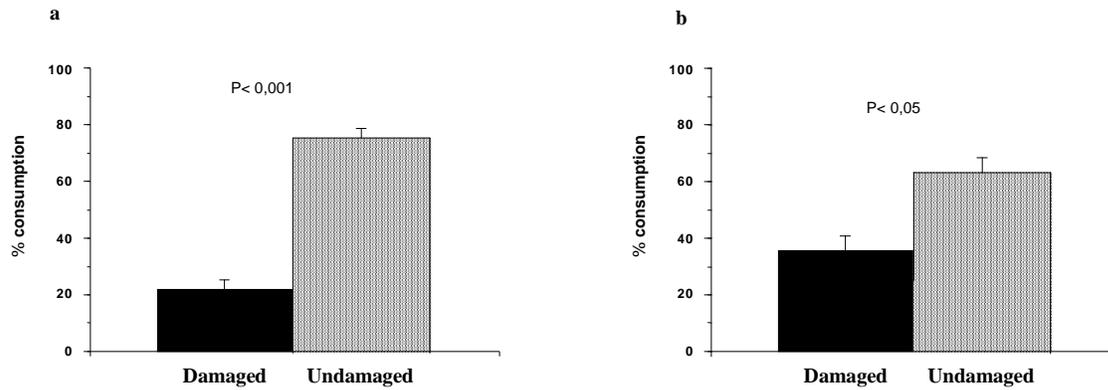
#### **5.6. Statistical analyses**

The larval feeding bioassays were tested with Wilcoxon's signed rank test for paired differences. The larval growth, when forced to feed on either undamaged or damaged tomato leaves, was analyzed by One-way ANOVA.

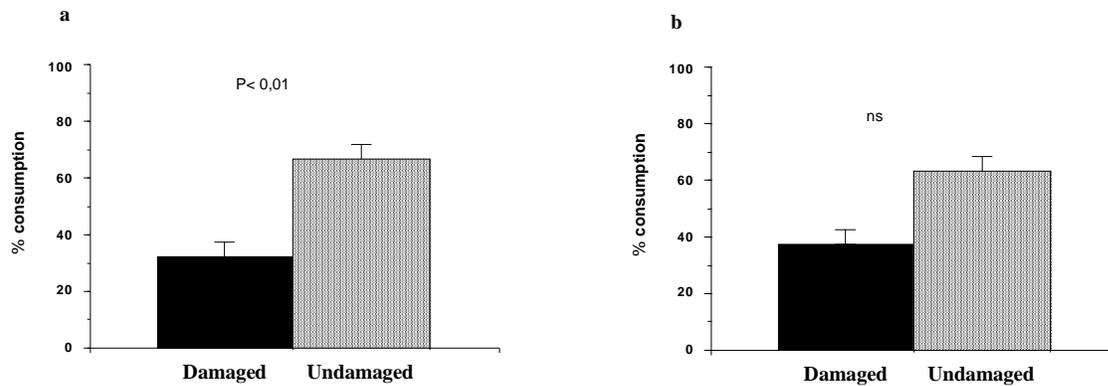
### **6. Results**

#### **6.1. Temporal aspects of induced defense**

Two days after initial damage, the *S. littoralis* larvae fed more on leaves from undamaged plants than on leaves from damaged plants (difference significant;  $P < 0.001$ ) (Figure 1a). Even at four days after first damage, the larvae significantly ( $P < 0.05$ ) chose leaves from undamaged plants (Figure 1b) and at eight days after initial damage, there was a strong significance ( $P < 0.01$ ) for the choice of leaves from undamaged plants (Figure 2a). At thirteen days after initial damage, a tendency was seen for choice of leaves from the undamaged plants (Figure 2b).



**FIG. 1.** Mean leaf area consumed in percentage + SE of *S. littoralis* larvae in feeding choice experiment with leaflets from the 4<sup>th</sup>, 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup>, 2<sup>nd</sup> top and top leaf. The leaves were taken from damaged plants (filled bar) and from undamaged plants (dotted bar): **a** two days after first damage (n= 78) and **b** four days after first damage (n= 90). Wilcoxon's signed rank test for paired differences was used for statistical analysis.



**FIG. 2.** Mean leaf area consumed in percentage + SE of *S. littoralis* larvae in feeding choice experiment with leaflets from the 4<sup>th</sup>, 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup>, 2<sup>nd</sup> top and top leaf. The leaves were taken from damaged plants (filled bar) and from undamaged plants (dotted bar): **a** eight days after first damage (n= 78) and **b** thirteen days after first damage (n= 78). Wilcoxon's signed rank test for paired differences was used for statistical analysis.

Two days after initial damage, the larval choice of undamaged plants over damaged plants was shown at different leaf positions (Table 1): at leaf position 4 (difference significant;  $P < 0.01$ ), leaf position 6 ( $P < 0.05$ ), leaf position 7 ( $P < 0.01$ ) and at the 2<sup>nd</sup> top leaf ( $P < 0.05$ ). There was a tendency for choice of 5<sup>th</sup> true leaves and top leaves from undamaged plants over damaged plants.

Four days after initial damage, there was significant choice of undamaged top leaves over damaged top leaves ( $P<0.05$ ) and a tendency was seen for the choice of leaf 4 and 6 from undamaged plants (Table 1). There was no difference in feeding between 2<sup>nd</sup> top leaves, 5<sup>th</sup> true leaves and 7<sup>th</sup> true leaves from undamaged and damaged plants.

Eight days after initial damage, there was a significant choice for top leaves of undamaged plants ( $P<0.05$ ) and a tendency for the choice of leaf 6, leaf 7 and 2<sup>nd</sup> top leaves from undamaged plants over damaged plants (Table 1). No difference was seen in feeding at leaf position 4 and 5 from damaged and undamaged plants.

Thirteen days after initial damage, the larvae significantly chose to feed on 2<sup>nd</sup> top leaves from undamaged plants rather than from damaged plants ( $P<0.05$ ) (Table 1). A tendency was shown for the choice of leaf 5, leaf 6 and top leaves from undamaged plants over damaged plants. No difference was seen at leaf position 4 and 7 from damaged and undamaged plants.

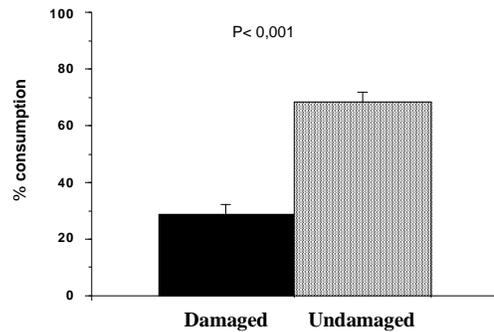
**Table 1. Larval consumption of different leaf positions from damaged or undamaged plants.**

Days after initial damage	2		4		8		13	
	D	U	D	U	D	U	D	U
n	13	13	15	15	13	13	13	13
True leaf 4	11	89**	36	64	43	57	42	58
True leaf 5	36	64	42	58	45	55	26	74
True leaf 6	16	84*	34	66	28	72	27	73
True leaf 7	13	87**	43	57	34	66	54	46
2 <sup>nd</sup> top	18	82*	49	51	30	70	31	69*
Top	38	62	27	73*	29	71*	40	60

% average consumed leaf areas are shown. n, number of damaged or undamaged plants; D, damaged; U, undamaged. Wilcoxon's signed rank test for paired differences was used for statistical analysis; \*,  $p<0.05$ ; \*\*,  $<0.01$  compared with damaged.

## **6.2. Induced defense in wild tomato**

In the larval feeding choice experiment between wild damaged and wild undamaged plants, there was a strong significant choice ( $P<0.001$ ) of leaves from undamaged plants (Figure 3).



**FIG. 3.** Mean leaf area consumed in percentage + SE. Comparison between leaf preferences in a two-choice experiment of leaves from wild damaged tomato plants (filled bar) and leaves from wild undamaged tomato plants (dotted bar). Wilcoxon’s signed rank test for paired differences was used for statistical analysis. (n = 90).

At different leaf positions, the larvae chose to feed on undamaged plants over damaged plants (Table 2): at true leaf 4 (difference significant;  $P < 0.05$ ), true leaf 6 ( $P < 0.05$ ), the 2<sup>nd</sup> top ( $P < 0.01$ ) and the top leaf ( $P < 0.05$ ). There was a tendency for choice of true leaf 7 from undamaged plants, rather than from damaged plants and no difference was seen in feeding at leaf position 5.

**Table 2. Larval consumption of different leaf positions from damaged or undamaged wild tomato plants.**

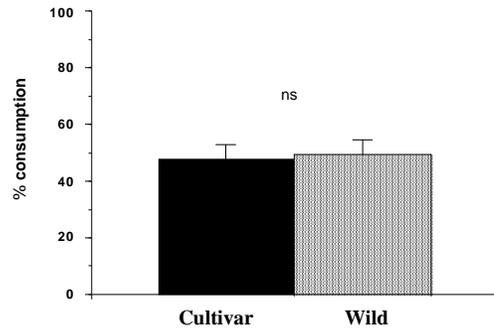
	D	U
True leaf 4	27	74*
True leaf 5	52	48
True leaf 6	24	76*
True leaf 7	40	60
2 <sup>nd</sup> top	17	83**
Top	18	82*

% average consumed leaf areas are shown. D, damaged; U, undamaged.

Wilcoxon’s signed rank test for paired differences was used for statistical analysis; \*,  $p < 0.05$ ; \*\*,  $< 0.01$  compared with damaged.; n, number of damaged or undamaged plants: n = 15

### 6.3. The constitutive defense of wild vs. genetically improved tomato

In the food choice experiment between leaves from genetically improved cultivars and wild tomato plants, no significant difference was shown in the larval feeding (Figure 4).



**FIG. 4.** Mean leaf area consumed in percentage + SE of *S. littoralis* larvae in feeding choice experiment with leaflets from the 6<sup>th</sup>, 7<sup>th</sup>, 2<sup>nd</sup> top and top leaf. The leaves were taken from genetically improved cultivars (filled bar) and from wild plants (dotted bar). Wilcoxon's signed rank test for paired differences was used for statistical analysis; n, number of wild and cultivated tomato plants: n = 60.

When different leaf positions of cultivars and wild tomato plants were compared in larval feeding choice, the larvae fed more on top leaves from wild plants (difference significant;  $P < 0.05$ ) (Table 3). No difference in feeding was seen at leaf position 6, 7 and the 2<sup>nd</sup> top from wild and genetically improved plants.

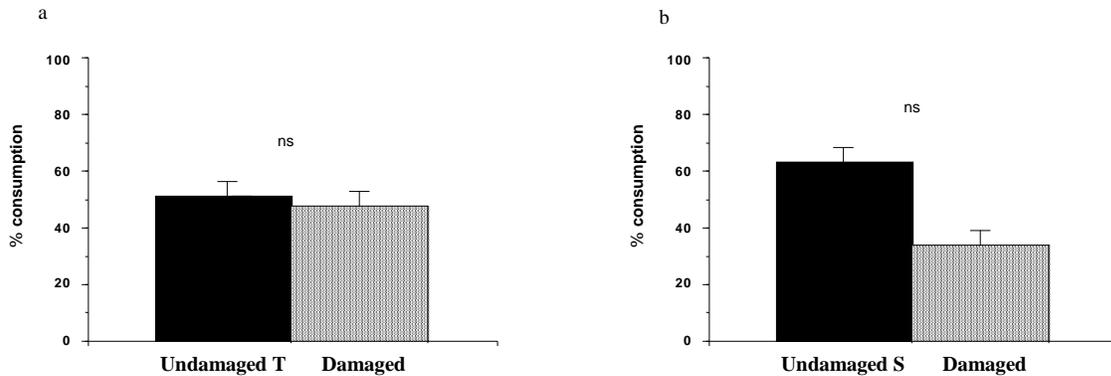
**Table 3. Larval consumption at different leaf positions from genetically improved cultivars or wild tomato plants.**

Leaf position	6		7		2 <sup>nd</sup> top		Top	
	C	W	C	W	C	W	C	W
Consumed leaf area (%)	59	41	52	48	47	53	32	68*

% average consumed leaf areas are shown. C, cultivar; W, wild. Wilcoxon's signed rank test for paired differences was used for statistical analysis. \*,  $p < 0.05$  compared with cultivar; n, number of wild or cultivated tomato plants: n = 15

## 6.4. Interplant communication

When larvae were able to choose between leaves from a damaged plant and an undamaged plant that had been grown in the same container, no statistical difference was seen (Figure 5a). A tendency was shown for choice of the undamaged plants over damaged plants when the undamaged plants were grown in separate pots (Figure 5b).



**FIG. 5.** . Mean leaf area consumed in percentage + SE of *S. littoralis* larvae in feeding choice experiment with leaflets from the 6<sup>th</sup>, 7<sup>th</sup>, 2<sup>nd</sup> top and top leaf. **a** True leaves 6 and 2<sup>nd</sup> top leaves were taken from undamaged plants (filled bar) and from damaged plants (dotted bar) that had grown together (n= 30) and **b** true leaves 7 and top leaves were taken from undamaged plants (filled bar) and from damaged plants (dotted bar) that had grown in separate pots (n= 30). Wilcoxon's signed rank test for paired differences was used for statistical analysis.

At leaf position 6, there was no significant difference in larval feeding choice between damaged and undamaged plants that had grown together (Table 4). Between damaged plants and the undamaged plants that had grown in separate pots, there was a tendency for choice of leaf 7 and top leaves from undamaged plants over damaged plants. No difference was shown in larval feeding between 2<sup>nd</sup> top leaves from damaged and undamaged plants that had grown together.

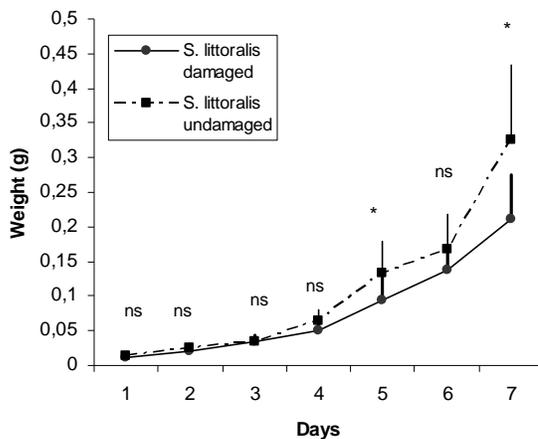
**Table 4. Larval consumption of different leaf positions from damaged plants, undamaged separately grown plants and from undamaged plants grown together with damaged plants.**

Leaf position	6		7		2 <sup>nd</sup> top		Top	
	Ug	D	Us	D	Ug	D	Us	D
Consumed leaf area (%)	46	54	62	38	57	43	65	35

% average consumed leaf areas are shown; Ug, Undamaged plant grown together with damaged plant; Us, Undamaged separately grown plant; D, Damaged plant; Wilcoxon's signed rank test for paired differences was used for statistical analysis; n, number of damaged, separately grown undamaged or undamaged plants grown together with damaged: n = 15.

### 6.5. The effect on larval growth

In the larval growth experiment, the larvae feeding on leaves from damaged plants had a lower growth rate compared to larvae feeding on leaves from undamaged plants (Figure 6). The change in growth occurred after five days when larvae feeding on leaves from undamaged plants increased more in weight (difference significant;  $P < 0.05$ ). After seven days there was a significant difference ( $P < 0.05$ ) in average weight between the two treatments.



**FIG. 6.** Growth curves of *Spodoptera littoralis*. Average weight (g) after feeding during seven days of damaged (circles) vs. undamaged (squares) tomato leaves. One-way ANOVA was used for statistical analyses. n = 10.

## 7. Discussion

The larvae experienced reduced growth when they were forced to feed on previously herbivore-damaged tomato plants (Figure 6) and they chose to feed on undamaged plants over recently wounded plants (Figures 1-2a; Table 1). These results suggest that there was an induced defense in the damaged tomato plants that affected both the behavior and the physiology of *S. littoralis* larvae. Also in wild tomato plants there was an induced defense already two days after initial damage (Figure 3, Table 2).

In both wild and genetically improved cultivars, the larvae chose to feed on undamaged top leaves rather than damaged top leaves (Tables 1 & 2), which indicated that the top leaves may have been induced from a systemic wound signaling. In a previous study of cotton (*Gossypium hirsutum*), a systemic wound signaling within plants was seen (Anderson & Agrell, 2005), where the herbivores were more concentrated on lower less valuable mature leaves as the young developing leaves showed increased resistance. However, in tomato, the induced defense is dependent on a complex vascular system that makes even some younger leaves unconnected to the systemic signaling (Orians et al. 2000). In a study of hybrid poplar, (*Populus deltoides x nigra*) unconnected leaves were induced by airborne volatiles from herbivore-damaged plants (Frost et al. 2007). Thus, based on the previous results from Orians et al. (2000) and Frost et al. (2007), further studies would be needed to confirm whether the induced defense in the top leaves in my experiment was caused by a systemic signaling or by volatiles emitted from the damaged plants. As no difference was seen in the feeding neither between damaged and undamaged plants that had grown separately (Figure 5b, Table 4), nor between the undamaged and damaged plants that had grown together (Figure 5a, Table 4), both root- and airborne signals could have been sent from the damaged plants to the undamaged plants.

There was no difference in constitutive insect resistance properties between wild and genetically improved cultivars (Figure 4), which was in contrast to my hypothesis that there should be stronger resistance properties in wild tomato plants.

Experiments 1-5 are more thoroughly discussed in the following text.

### **7.1. Temporal aspects of induced defense**

In the experiment studying temporal aspects affecting the induced defense, the strongest induced defense was seen already two days after the first damage, which was different from the

hypothesized peak between four or eight days after the initial damage. In previous studies, younger plants had shown significant results for induced defense at four days after initial damage (Rodriguez et al. 2005; Stout & Duffey, 1996). In cotton, *S. littoralis* larvae fed less on damaged plants than undamaged plants after three days, with a peak after seven days and the effect was found until fourteen days after initial damage (Anderson et al., 2001).

Thirteen days after first damage, there was no statistical differences in the feeding, although there was a tendency for a weak induced defense left in the damaged plants. The result confirmed my hypothesis that the quality of defense may have declined with plant age (Stout & Duffey, 1996). In addition, after thirteen days the plants had started to suffer from nutrient deficiencies and stressed plants with low nitrogen levels could be less able to produce defense-related proteins and allocate the secondary metabolites (Stout et al., 1998).

The highly significant larval choice of the true leaves 4, 6 and 7 from undamaged plants over damaged plants two days after the initial damage, indicated a rapid response in fully developed leaves. It is possible that the allocation of secondary metabolites and defense-related proteins may have been most active in the leaf positions closest to the wounded leaf, due to the short reaction time of the plant. In cotton, Anderson et al. (2001) observed differences in the induced defense between small (4-5 true leaves) and large plants (8-10 true leaves). In small cotton plants, the induced defense that affected larval feeding was mainly found in the youngest top leaves, while the large cotton plants showed effect in both the youngest and second youngest top leaves. In my experiment, old plants were used and it is possible that these plants could have developed different defense abilities than the younger plants would have done and that the larger size of the older plants allowed them to allocate more resources to defense. However, two days after initial damage, 2<sup>nd</sup> top leaves from undamaged plants were chosen over damaged plants. Furthermore; four, eight and thirteen days after the first damage, the mature leaves showed a weaker defense than the top leaves. The stronger induced defense in top leaves confirmed the hypothesis that the plant would aim to allocate the resources to defend its most valuable parts (Anderson & Agrell, 2005). Undefended young leaves should be the most attractive for larval feeding as they contain more nutrients per leaf compared to fully developed leaves (Orians et al., 2000). The tomato is also a competitive plant where the top leaves are important since they compete for light (Edwards et al., 1992). Fortunately, the top leaves have small sinks that are able to receive more defense signals than the mature leaves (Orians et al., 2000).

## **7.2. Induced defense in wild tomato**

Also the wild tomato plants require an effective induced defense against herbivore feeding to avoid reduced fitness. The induced defense was found in the damaged wild plants (Figure 3) at leaf position 4, 6, 2<sup>nd</sup> top and top (Table 2). Further studies would be needed to investigate whether wild tomato plants were better than genetically improved tomato cultivars in protecting their leaves by the induced defense.

## **7.3. The constitutive defense of wild vs. genetically improved tomato**

No difference in feeding was found when leaves from undamaged wild and genetically improved cultivars were compared. This was in contrast to my hypothesis and may be explained by earlier findings of Chatzivasileiadis et al., (1997) that showed no significant differences in the levels of toxic methyl ketones between top and middle leaves of wild and genetically improved tomato plants. The trichomes were important for this experiment, since the constitutive mechanical and chemical defense (Kennedy, 2003) were involved. In earlier comparative studies of tomato responses to herbivore damage, both improved cultivars and wild tomato plants were shown to release toxic constitutive methyl ketones from the trichomes but in wild tomatoes fewer contacts that broke the trichomes were needed for reducing the insect's fitness (Chatzivasileiadis et al, 1997). One possible explanation to why the larvae in my experiment did not significantly choose the genetically improved plants, may be that those plants (*L. esculentum* cv. Elin) had been bred with more active genes of acyl sugars, 2,3-tridecanone and tomatine. Another possible reason may be that my wild tomato plant was a wild breeder's variety and could resemble the resistance properties of improved tomato cultivars more than of the wildest relatives *L. hirsutum*, *L. glabratum* and *L. pennellii*. Moreover, no difference was observed in trichome density between the cultivar and the wild tomato (A. Eriksson, unpublished).

## **7.4. Interplant communication**

The undamaged plants may have received both airborne and root borne signals from the damaged plants. Previous studies on interplant communication have both showed positive evidence for root signaling (Chamberlain et al., 2001) and for airborne signaling (Bruin & Sabelis, 2001; Chamberlain et al., 2001). No significant difference was shown neither between damaged and undamaged plants at the top leaf position, which may be the most attractive,

(Orians et al., 2000) valuable and protected leaves (Anderson & Agrell, 2005). However, although the non statistical significance, there was a tendency that larvae fed more on top leaves from undamaged plants that had grown separately from the damaged plants. It is possible that the damaged plants transferred defense signals more successfully to the undamaged plants through the roots than through the air, due to the little wind in the greenhouse. Interplant communication is still under discussion as the successful tests so far have been carried out in laboratory where the right conditions may be missed (Bruin & Sabelis, 2001). This topic needs further behavioral, chemical and molecular biological bioassays and the information of variation in wind, the impact on competition and the cost of information production require in vivo experiments.

As the tomato plant is a competitive plant (Edwards et al., 1992), it is probable that damaged or undamaged tomato plants would aim to enhance their own fitness rather than helping the neighbor. The undamaged plants would thus parasitize on the induced defense from the damaged plant, whereas the damaged plants would send signals to attract more natural enemies or to induce defense in their leaves that were unconnected to the systemic defense signaling.

### ***7.5. The effect on larval growth***

The physiology of the larvae feeding on damaged plants was negatively affected as they showed reduced growth (Figure 6). The reduction of growth was probably caused by the defensive chemical compounds within the damaged plants. For instance, in a previous study, the PIs were shown to degrade essential amino acids in the larval midgut after feeding on induced plants (Chen et. al. 2005).

According to Hoffman and McEvoy (1986), the youngest instars would not be able to feed in the presence of trichomes due to the mechanical trichome defenses. In my experiment, the instars were old enough to handle the mechanical defense and further studies would be needed to confirm that trichomes cause mortality of young instars. Moreover, in order to understand the total effect on larval growth, another experiment should examine the development from egg to pupa.

The larval growth experiment and the two-choice bioassays showed that the induced defense can be investigated through larval behavior experiments. Further behavioral studies could be combined with molecular studies, by using mutants with signal deficiencies that not would be able to express the induced defense. Moreover, the behavioral studies could be combined with

chemical studies, by adding JA to the plants or by extracting the PIs from the plant. Behavioral, chemical and molecular experiments of induced defense are important for understanding the plant-insect interactions and the plant-plant interactions. Experiments investigating the inducible defenses are therefore essential for the development of plant protection by integrated pest management.

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