



Swedish University of Agricultural Sciences
The Faculty of Natural Resources and Agricultural Sciences
Department of Plant Biology and Forest Genetics

Plant defensive mechanisms against insect pests

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Abstract

This study has focused on testing the following two hypotheses:

- Pest resistant plants have higher levels and/or several different kinds of secondary metabolites.
- Induced defensive systems in resistant plants are activated more rapidly than those of susceptible plants.

As a background for the test of the hypotheses a short literature study was performed concerning plant defensive mechanisms in *Arabidopsis thaliana*. This survey describes basic defenses like trichomes, constitutive defenses like the glucosinolate-myrosinase system, which can create toxins, and inducible defenses like the JA-, ET- and SA- defenses, which result in different chemical responses. Feeding experiments with the plant *A. thaliana*, the generalist insect pest *Spodoptera littoralis* and the specialist insect pest *Plutella xylostella* have been conducted. The results showed that the *Spodoptera* generalist is greatly affected by the plant's defensive system, while the *Plutella* insect is not. A qPCR analysis of *LOX 2* gene expression was conducted to examine the defensive strategy of plants against attacks by *Plutella*, which showed that all plants except one activated their inducible defenses. A feeding experiment with primed (*Bacillus amyloliquefaciens*) *Arabidopsis* plants and *Spodoptera* larvae was conducted and showed that primed plants, which trigger their defenses faster, not necessarily counteract pests better than non-primed plants.

Sammanfattning

Denna studie har fokuserat på att testa två hypoteser:

- Insektsresistenta växter har högre halter och/eller har flera olika sorters sekundära metaboliter.
- Inducerbara försvar aktiveras snabbare i resistent växter än i mottagliga växter.

Som bakgrund till hypotesprövningen gjordes en mindre litteraturstudie om försvarsmekanismer i växten *Arabidopsis thaliana*, vilken beskriver grundläggande försvar som trikomer, konstitutivt försvar som glukosinolat-myrosinassystemet som kan bilda gifter och inducerbara försvar som JA-, ET- och SA- systemen som kan stimulera flera olika typer av kemiska reaktioner. Flera matningsförsök har gjorts med växten *A. thaliana*, generalistinsekten *Spodoptera littoralis* och specialistinsekten *Plutella xylostella*. Resultaten visade att *Spodoptera* påverkades starkt av växtens försvarssystem medan *Plutella* inte gjorde det. För att undersöka närmare hur plantorna försvarade sig mot angrepp av *Plutella* genomfördes en qPCR- analys för att analysera *LOX 2*-genens uttryck, vilket visade att alla plantor, utom en, aktiverade sina inducerbara försvar. Ett matningsförsök utfördes med primade (*Bacillus amyloliquefaciens*) *Arabidopsis* plantor och *Spodoptera* larver, vilket visade att primade plantor (som aktiverar sitt inducerbara försvar snabbare) inte nödvändigtvis försvarar sig bättre än icke primade plantor.

Introduction

The project concerns defenses of *Arabidopsis thaliana* against insect pests. My study was focused on defensive mechanisms operating against specialist and generalist herbivore insect pests. Two hypotheses were tested:

1. Pest resistant plants have higher levels and/or several different kinds of secondary metabolites.
2. Induced defensive systems in resistant plants are activated more rapidly than those of susceptible plants.

The project was conducted as a Bachelor thesis project in my agronomist program. I chose this topic because of the importance of proper understanding of plants defensive systems to improve pest management for efficient crop production. Each year different kinds of pests are estimated to reduce crop yields with 30-80% for farmers around the world (Oerke 2006). Understanding the defensive systems of plants enables development of resistant crops or pest management systems reducing the need of hazardous pesticides and supporting safer crop production. Another positive effect would be a reduction of the development of pesticide resistant pest strains.

The defensive mechanisms in plants operate at different levels. They vary from external defenses like thorns to complicated chemical responses leading to poisoning of the attacker or plant senescence (Lev-Yadun et al. 2004, Pegadaraju et al. 2005). In the case of *Arabidopsis*, a major defensive strategy is to produce compounds that are toxic for its attackers. These compounds emanate from several chemical defensive systems where phytoalexins, such as camalexin and glucosinolate products, are the most studied. Several hormones trigger different defenses including jasmonic acid (JA), salicylic acid (SA) and ethylene (ET). There are other more poorly understood and unknown protection systems in *Arabidopsis* that are yet to be discovered. I will briefly discuss some of the known defensive systems.

Background

Trichomes

Arabidopsis plants have small hairs on their leaves, so called trichomes. These trichomes are the first line of defense against insects. They are sharp and make the insect feel uncomfortable moving on the plant surface. Many plants also secrete noxious chemicals by the trichomes but Arabidopsis is reported to lack such secretory cells. Although the trichomes are a primitive defense it should not be underestimated. It has been shown in experiments that Arabidopsis plants with higher density of trichomes are disliked by insects (Mauricio 1997). The density of trichomes also seems to be positively correlated with the glucosinolate-myrosinase system, so if trichome density is high, glucosinolate levels also tend to be elevated as well (Mauricio and Rausher 1997, Clauss et al. 2006).

The glucosinolate-myrosinase system

One of the best known defensive systems in Arabidopsis plants is the glucosinolate-myrosinase system. The basic components of this binary system are the secondary metabolites glucosinolates and the enzyme myrosinase. Upon tissue damage these compounds react and form toxic and bad-tasting products like isothiocyanates. The glucosinolates are stored in certain giant cells, “S-cells”, which are adjacent to the phloem and release their contents upon tissue damage, “wounding” (Wittstock and Burow 2010, Koroleva et al. 2010). Myrosinase is stored in idioblast cells (myrosin cells) close to the phloem and is released when the tissue is wounded (Andréasson 2000, Andréasson et al. 2001). This “mustard bomb” system (glucosinolates were earlier referred to as mustard oils) is activated when an aggressor has damaged both the S-cells and the myrosin cells (Fig. 1) while under normal conditions the separation makes this system inactive. This system is effective against generalist herbivores because they will choose a less toxic plant instead of Arabidopsis (or any other glucosinolate containing plant) thus providing antixenosis in addition to antibiosis. On the other hand, specialist herbivores are attracted to plants containing these products, because they have a metabolism which has coevolved with the Arabidopsis plant defenses and become tolerant and even use glucosinolates (or more often the products of glucosinolates) as feeding cues (Nehlin and Mörner 1991). The reason why the specialists have coevolved with this plant defensive system might be that they had fewer alternative plants to feed on.

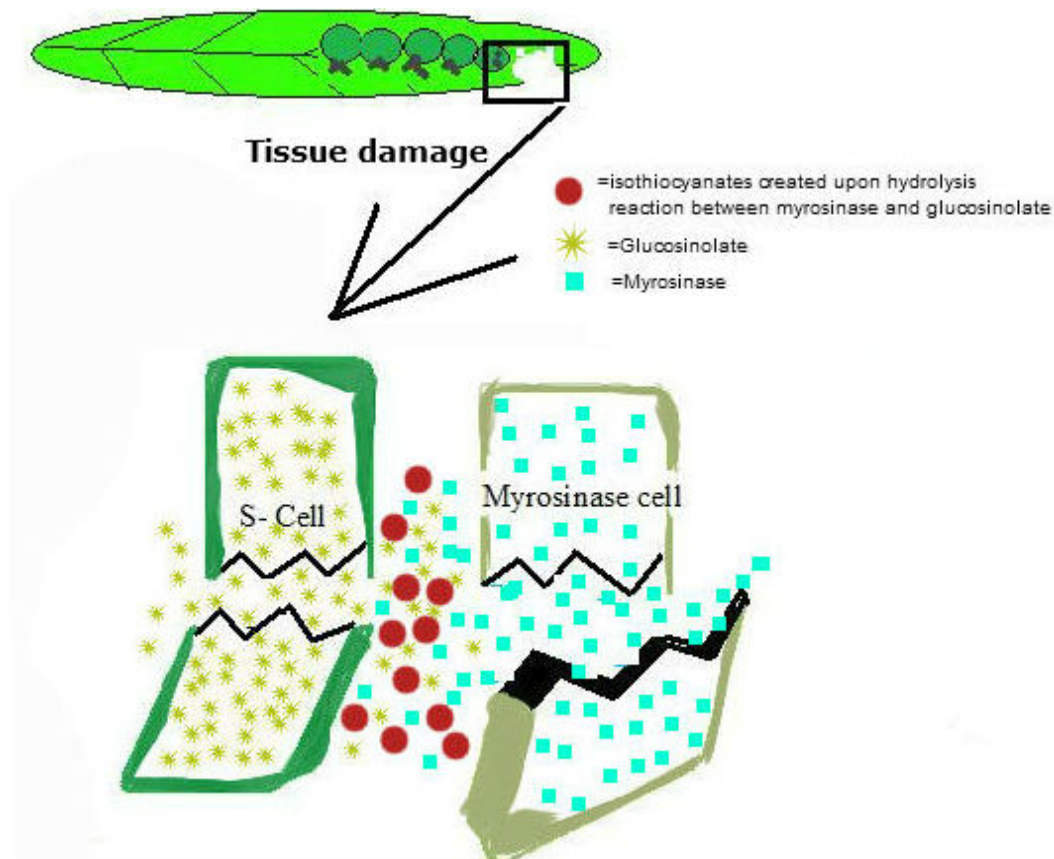


Figure 1. Schematic illustration how tissue damage due to larval feeding leads to the destruction of a myrosin and a S-cell resulting in the formation of toxic glucosinolate products such as isothiocyanates

Jasmonic acid induced defensive system

JA is important for triggering inducible defenses systemically in plants but this process takes some time to reach full power. When the plant is attacked by an aggressor, JA synthesis is initiated in plastids in cells of the attacked tissue (Acosta and Farmer 2009). The attack is recognized as a result of the tissue damage but also by elicitors e.g. in the attacker's saliva (Alborn et al. 1997). The JA is released and transported through the plant and acts as a hormone to activate several defensive mechanisms (Karban and Baldwin 1997). These mechanisms include defensive proteins (lectins and protease inhibitors), defensive chemicals and compounds which will reduce the damage caused by the aggressor. JA and degradation products like certain glucosinolate products may contribute to indirect defense by attracting natural enemies to the pest (Thaler 1999). Furthermore plants use methylated JA (MJ) to alert nearby plants that they are under attack (Fig. 2). This will activate the JA systems in the neighbouring plants so they are prepared for potential coming attacks (Matthes et al. 2011).

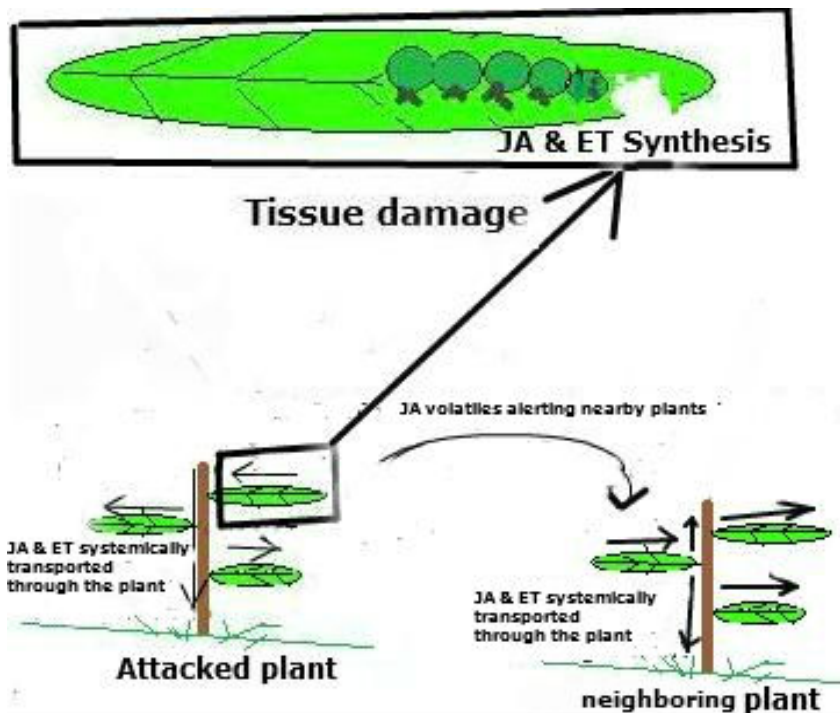


Figure 2. Illustrating that both the JA and ET systems are activated upon pest attack and transported systemically through the plant. JA volatiles (MJ) may also alert neighboring plants

The salicylic acid system

The SA system is similar to the JA system. It is an induced system resulting in systemic resistance. But what differs from the JA system is that it mainly results in pathogen defenses by forming extra thick cell walls, producing hydrogen peroxide and specific pathogenesis related proteins (PR proteins) as well as inducing senescence (Fagard et al. 2007, Van Wees et al. 1997). Cross-talk occurs between the signal transduction pathways and SA can interfere with JA signaling and vice versa. It has been speculated why the SA and JA systems are competing and one theory is that when an aggressor has been identified, e.g. as a pathogen, it is unnecessary to turn on the JA system and instead focus plant resources on one (or the strongest) aggressor. There are even insects that manage to fool the plant to mount a pathogen response that allows the insect to continue to feed on the plant. It is mainly phloem-feeding insects like silverleaf whiteflies that manage to activate the SA system instead of the JA system (Kempema et al. 2007). Zarate et al. (2007) made an experiment where they overexpressed the SA system in a group of Arabidopsis mutants, silenced the SA system in another group of Arabidopsis mutants and had an untreated control group of Arabidopsis and released silverleaf whiteflies over the plants. The results showed that the SA silenced plants fought off the whiteflies much better than both the control group and the SA over expressing plants suggesting that the JA system was counteracting the whiteflies once operating.

The ethylene system

The ET system is the one of the signaling systems we know least about regarding plant defenses. It is similar to the JA system and can also cross talk with the JA system, but is not competing and it rather has synergetic effects (De Vos et al. 2005, Lorenzo et al. 2003). The ET system is induced by tissue wounding when the plant is under attack and ET is produced and transported systemically through the plant acting like a hormone (Fig. 2). ET can act like a volatile signal molecule, which attracts natural enemies to the aggressor, adjusts the levels of glucosinolates and induces defensive proteins. But ET can also change the priority for energy distribution in the plant. For example if the plant is under attack by many insects it is better to save energy rather than produce flowers, which results in abortion of flowering buds (Dahl and Baldwin 2007, Mewis et al. 2005).

Priming

Priming refers to a state when a plant is prepared to respond more efficiently when being challenged by some sort of stress. In priming JA/ET and SA systems are not induced but become activated faster by stress because some defensive genes are triggered in advance. Triggering mechanisms include exposure of the plant to a microorganism or to low levels of chemicals like JA, SA and beta-amino butyric acid (BABA), molecules that upon biotic stress stimulates production of defensive compounds (Conrath et al. 2006). However, it is still not known how the priming mechanisms operate, but probably they include both genetic and epigenetic changes (Conrath 2011). However, it is known that priming, at least in pathogen related cases, only seems to infer minor costs for the plant (low fitness cost) but when attacked that small amount of energy invested in priming can repay the plant several times (van Hulten et al. 2006). This is in contrast to strategies using resistance inducers based on stimulation of induced defenses which result in high energy costs.

Spodoptera littoralis

Spodoptera littoralis (Egyptian cotton worm) is a moth belonging to the family Noctuidae. The life cycle starts with an egg hatching to a larva after five days. The larva then has six instar stages before becoming a pupa, but can under certain circumstances (in colder environment) have seven instar stages. The larva pupates close to the soil and is a pupa for approximately 10 days. Thereafter it reaches adult stage and becomes a night active moth. The female lays up to 2000 eggs after mating and that combined with the insect's short lifecycle rapidly expand the *S. littoralis* population. In warmer and tropical climates up to twelve generations occur per year. The larvae are generalists and eat both wild and cultivated plants. They are a big economical problem and can lead to total defoliation of the crop if no countermeasures are taken (Bayer crop science 2011, Follin 1970).

Plutella xylostella

Plutella xylostella (Diamond back moth or cabbage moth) belongs to the family Plutellidae. It has a life cycle which starts with an egg laid on the host plant. The egg hatches to a larva with four instar stages. The larva begins to eat on the host plant immediately after hatching. The larva is a specialist on cruciferous plants and seems to be attracted to higher levels of glucosinolates. After four weeks the larva evolves to a pupa and it stays as pupa for two weeks. Thereafter the pupa reaches the adult stage and becomes a night active moth. After mating the female individuals can lay 11 to 200 eggs. *P. xylostella* is spread all over the world and causes huge economical problems each year in crucifers like oilseed rape, cabbages and mustards (Talekar and Shelton 1993, Nehlin and Mörner 1991)

Arabidopsis thaliana

Arabidopsis thaliana is a small plant common on sandy oligotrophic soils (Mossberg and Stenberg 2008). The plant is a popular model organism in plant science for several reasons such as:

- Short life cycle.
- Small and easy to handle.
- The genome has been fully sequenced (The Arabidopsis genome initiative 2000) and is relatively small.
- Belongs to the family Brassicaceae and is closely related to agricultural important crops like *Brassica napus*, *Brassica rapa* and *Brassica oleracea*.
- Self pollinating.
- Prolific seed production.
- Many mutants available.

Hypothesis testing

To test the two hypotheses several tests were conducted by using the plant *A. thaliana*.

- The first experiment was to test four Arabidopsis ecotypes to see how resistant they were against a generalist insect. For that purpose the chewing generalist insect *S. littoralis* was used, and allowed to feed on the Arabidopsis ecotypes Can-0, Ler-0, Mt-0 and EH-0. This addressed the first hypothesis because the *S. littoralis* larvae are sensitive to glucosinolates and the plants would probably not have time to activate their JA/ET or SA defensive systems.
- An additional test to the first experiment was conducted to calculate the trichome density on the investigated ecotypes since trichome density has been reported to be positively linked with glucosinolate levels.
- The second experiment was to try and find out, how resistant the ecotypes used in the first experiment were against the specialist insect *P. xylostella*. This experiment will test the second hypothesis because the specialist should not be affected by the glucosinolate-myrosinase system, but rather by the inducible JA/ ET systems. After the feeding experiment the plants were analyzed for their *LOX 2* gene expression, which is known to be activated when they are attacked by *S. littoralis*. This gene is involved in the JA defense in the plant and might answer the second hypothesis (Bell et al. 1995, Moran and Thompson 2001).
- In the third experiment plant defenses were primed by inoculation with the beneficial bacterium *Bacillus amyloliquefaciens* UCMB 5113. The tested ecotypes were Mt-0, Ta, Ms-0, Ler-0, N13, Shahdara, Edi-0, and Can-0. The plants were then challenged with the Spodoptera generalist. This experiment addressed the second hypotheses, because primed plants should activate their inducible defensive system much faster than the non-primed plants. If the groups with the same treatment are compared with each other it is possible to compare the strength of the different ecotype's inducible defenses.
- In the fourth experiment a choice feeding assay was carried out. Spodoptera larvae were allowed to freely choose between the Can-0, Ler-0, Mt-0 and Eh-0 ecotypes. This experiment answers the first hypothesis because Spodoptera larvae prefer low levels of glucosinolates.

Materials

Some of the materials used are listed, just to show what is needed for anyone wanting to conduct similar experiments:

Artist brush (to transfer larvae)

Autoclave

B. amyloliquefaciens strain 5113

Bottles, flasks, measuring cylinder

Burner, Platinum loop

Chemicals: peptone, yeast extract, sodium chloride (NaCl), sodium hydroxide (NaOH)

Ethanol

Filter paper

Microscope

Miller and metal bullets

MilliQ-water (ultrapure water)

Oligonucleotide primers; LOX2F, 5' – cttaccgcggatctc -3', and LOX2R, 5' – actccatgttctgcggtctt – 3'; TubulinF, 5' – CGATGTTGTTTCGTAAGGAAGC – 3', and TubulinR, 5'-

TCCTCCCAATGAGTGACAAA -3'

Omega bio-tek RNA kit, Qiagen cDNA synthesis kit

PCR machine and PCR plates

Petri dishes

Petri dishes with LB media

Pipettes and tips

P. xylostella insects, first instar larvae

Pots, soil

QGENE software

Seeds for *A. thaliana* ecotypes Can-0, Ler-0, Eh-0, Mt-0, Edi-0, N13, Ta-0, Shadara, Ms-0,

Shaker

Stirrer plate

S. littoralis 1st instar larvae

Syringe and spray bottle

SYBR Green

Methods

Non-choice feeding experiment with *S. littoralis*.

In this experiment the plants were grown in a growth chamber with a temperature of 25°C and 18h light every day.

Several pots with soil were prepared and sown with many seeds to ensure germination for the experiment. Table 1 lists the plan for each prepared pot.

Table 1. List of ecotypes sown in each pot, germination status one week after sowing, and transplantation two weeks after germination.

Pot nr	Ecotype seeded	Germination	Number of troughs for plants transplanted
1	Can-0	Yes (discarded)	0
2	Ler-0	No (discarded)	0
3	Can-0	Yes (discarded)	0
4	Ler-0	No (discarded)	0
5	Can-0	Yes	2
6	Ler-0	Yes	2
7	Can-0	Yes (discarded)	0
8	Ler-0	Yes (discarded)	0
9	Mt-0	Yes	1
10	Eh-0	Yes	1
11	Mt-0	Yes (discarded)	0
12	Eh-0	Yes (discarded)	0

Pot 5 and 6 were transplanted to an additional tray each, in order to practice the transplanting procedure and to ensure that a sufficient number of plants survived the transfer.

Pots 1-4 were discarded due to bad germination. Pots 11, 12, 7, 8 were discarded because they were only planted as a backup in case of germination problems.

One week after replanting the plants from pot 5 and 6 were used in a feeding experiment. Ten petri dishes were prepared with a lightly moist filter paper and one single plant each (Fig. 3). The root and flowering bolt of the plant were cut off and discarded. Half of the petri dishes contained Ler-0 plants and the other half five Can-0 plants. Four larvae were put in each petri dish.



Figure 3. An Arabidopsis plant in a petri dish during a feeding experiment.

When the plant had been consumed by the larvae, a new plant of the same ecotype was placed in the petri dish.

After 8 days the experiment was ended and the larvae were weighed. Dead larvae were included in the weighing process if bodies were found. The larvae were then stored in a fridge for 5 days before being photographed.

Two weeks after replanting, an identical experiment was conducted for the plants from pots 9 and 10. The larvae from this experiment were stored at 4°C for 7 days before being photographed.

Non-choice feeding experiment with *P. xylostella*.

In this experiment the plants were grown in a growth chamber with a temperature of 25°C and 18h light every day.

In the experiment four pots were prepared with soil and sown with seeds of one ecotype each:

Pot 1, Can-0

Pot 2, *Ler*-0

Pot 3, *Eh*-0

Pot 4, *Mt*-0

After two weeks, plants from each pot were transplanted into one trough with 40 minor pots each. The rest of the plants were discarded.

After further eight days the *Plutella* larvae hatched and a feeding experiment similar to the non-choice feeding experiment with *Spodoptera* was conducted but using three *Plutella* larvae in each petri dish, the experiment was conducted for seven days. The larvae were then stored at 4°C for 5 days and in a freezer for one day at -20°C before being photographed.

In the end of the feeding experiment three larvae were collected from each ecotype (except for *Ler*-0 where only two larvae were collected) and were put on one plant each with the same ecotype as they were fed on earlier. Five hours later the larvae were returned to their petri dishes. The chewed leaves and a couple of systemic leaves were collected from the plants (Fig. 4). Leaves from a control group with plants not exposed to insects were also collected. A qPCR analysis of the *LOX 2* gene was conducted on the collected leaves with RNA extracted according to the manual (Omega Biotek Plant RNA kit). The columns used for cleaning the RNA on stage 6 in the first protocol were clogged for the Can *Plutella* treated and Can control leaves and an additional centrifugation step was included, in totally 10 minutes at 10,000 rpm, without greater effect but this was dissolved by itself on a later stage. cDNA synthesis was carried out using Qiagen cDNA synthesis kit. The cDNA concentration was measured and all samples were diluted with deionised water to a concentration of 50 ng/μl. The samples were thereafter handled in sterile and dark environment. The samples (5 μl) were transferred to a PCR plate together with 15 μl of master mix containing SYBRGreen, forward primers, reverse primers and water (Table 2). The plate was then put in a PCR thermocycler (Table 3) and the samples were analyzed. Tubulin was used as a control gene to normalize the gene expression. QGENE software was used to calculate the normalized gene expression of the herbivory treated and non-treated samples.

Table 2 Real time PCR protocol

	Stock Conc.	Per Reaction	Vol./ reaction (µl)
PCR SYBR Green Master Mix	2X	1X	10
F primer	5 µM	0.3 µM	1.2
R primer	5 µM	0.3 µM	1.2
Milli Q water			2.6
cDNA			5
Total Volume			20

Table 3 Real time PCR conditions

Step	Temperature °C	Time	Number of cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	40
Annealing/Extension	60	60 sec	



Figure 4. Plutella larvae feeding on an Eh-0 (A) or a Mt-0 (B) Arabidopsis plant.

Experiment with Bacillus effect on Spodoptera feeding behavior

In this experiment the plants were grown in a growth chamber with a temperature of 25°C and 18h light every day.

Four pots were prepared with soil. Each pot was sown with one ecotype.

Pot 1: Can-0

Pot 2: Ler-0

Pot 3: Edi-0

Pot 4: Mt-0

Pot 2 was discarded later on in the experiment due to bad germination.

After two weeks 40 plants from each pot were transplanted into troughs with 40 single pots.

After a further 10 days period half of all plants were inoculated with Bacillus bacteria through soil drenching.

After an additional 15 days a Spodoptera feeding experiment started. The feeding experiment was similar to the non-choice feeding experiment with Spodoptera. But instead of five petri dishes per ecotype there were three petri dishes per ecotype and treatment, and there were five larvae in every petri dish instead of three larvae and the experiment was conducted for ten days instead of eight.

The larvae were kept at 4°C for seven days before being photographed.

One liter of LB medium was prepared from 5 g yeast extract, 10 g peptone, 10 g NaCl and 900 ml water. The pH was adjusted to 7 with NaOH. The solution was then poured into a measuring cylinder and adjusted to 1 liter, distributed into two 1 liter bottles and autoclaved. The sterilized LB media were stored at 4°C.

The *Bacillus* bacteria were grown by transferring a colony with a loop into a small volume of LB media. The sample was put in a shaker at a constant temperature of 28°C in darkness for one day. The culture was then transferred into two larger sterile flasks with 400 ml of LB and shaken for three days at 28°C in darkness. After that the bacterial culture was transferred to bottles and centrifuged for 15 minutes at 5,000 rpm. The pellet was kept and the suspension discarded. The bacterial pellet was dissolved in deionised water and applied to half of the plants by drenching the soil with a syringe. Approximately 10 ml of bacterial spore suspension was applied to each plant.

The concentration of bacteria was calculated by making a dilution series where 100 µl of the culture was diluted with 100 µl LB and the resulting solution was further diluted in the same way were 100 µl

of solution is diluted with 100 µl of LB until a dilution of 10^4 was achieved. The 10^2 to 10^4 dilutions

were applied to LB plates and stored in darkness and a constant temperature of 28°C for 1 day before colony counting. The colony forming units (CFU) for the 10^4 dilution were calculated to be 2.0×10^8 .

A similar experiment was conducted on the ecotypes Shadara, N13 and Ta-0. There was also a second attempt to make an experiment on the *Ler-0* ecotype but it failed again due to bad germination. But this experiment had an additional treatment where the *Bacillus* bacteria were applied by spraying the leaves. A total number of 80 plants per ecotype were involved in the experiment and a third of that was used for each treatment. Four larvae were put in each petri dish instead of five. The bacterial concentration was calculated through spectroscopy at the wavelength 600 nm and using LB medium as reference. The bacterial concentration was intentionally diluted to $OD_{600}=0.5$. Due to lack of time the bacteria were cultivated for 24h instead of three days before centrifugation.

Trichome analysis

Three leaves were taken from a *Can-0* and a *Ler-0* plant. The leaves were studied in a microscope and four zones with an area of 3.15 mm², 4.45 mm², 5.21 mm² and 2.06 mm² were used to count the number of trichomes to be able to calculate trichome density.

The *Eh-0* and *Mt-0* ecotypes were studied in the same way as the *Can-0* and *Ler-0* ecotypes but the examined areas were 5.83 mm², 6.1 mm² and 7.9 mm².

Free choice feeding experiment

Three petri dishes were prepared with dry filter papers. One leaf from the ecotypes *Can-0*, *Ler-0*, *Mt-0* or *Eh-0* were placed on the edge of each petri dish. All petri dishes were photographed and five *Spodoptera* larvae were put in the middle of each petri dish. Two hours later the experiment was terminated and each petri dish was photographed again.

One day later the experiment was repeated but using five petri dishes.

Statistical methods

A t-test was used to analyze the results from the different experiments. Following formulas were used to compare two different treatments at a time:

$$d = \frac{\sum x_1 - x_2}{n} \quad S_d^2 = \frac{\sum d^2 - \frac{(\sum d)^2}{n}}{(n-1)} \quad t = \frac{d - 0}{\sqrt{\frac{S_d^2}{n}}} \quad t > t_{(1-\frac{\alpha}{2}, n-1)}$$

x = sample data (mean value of all larvae in the same petri dish)

d = difference between treatments

n = degrees of freedom

S_d^2 = variances

t = t-variable

α = possibility

In those cases where too many larvae were put in the petri dish those larvae with the most extreme weight were excluded.

Results

Non-choice feeding experiment with *S. littoralis*

Larvae that fed on the *Ler-0* ecotype seemed bigger than those that fed on the *Can-0* ecotype (Fig. 5). This difference in body mass was also supported by a t-test that showed statistical significance (Fig. 6). During the weighing process it was noticed that some of the larvae that had been kept on the *Can-0* ecotype had died (probably due to starvation or partial cannibalism) or managed to escape during the experiment.

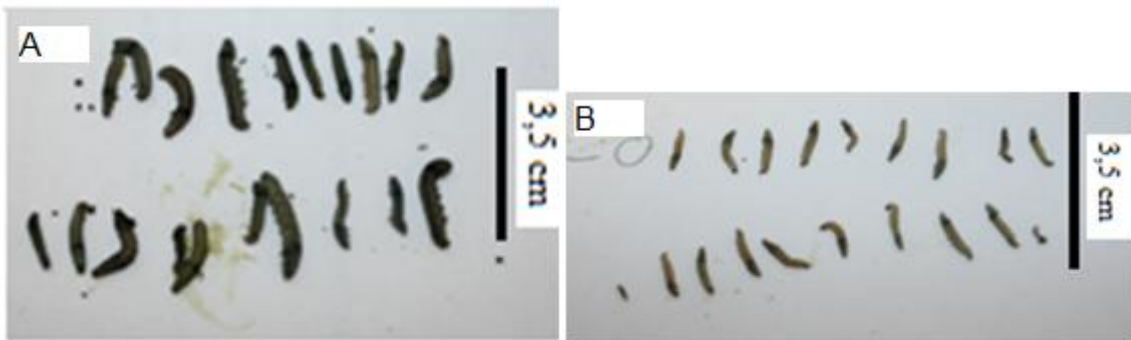


Figure 5. Spodoptera larvae after feeding on the *Ler-0* ecotype for eight days (A). Spodoptera larvae after feeding on the *Can-0* ecotype for eight days (B).

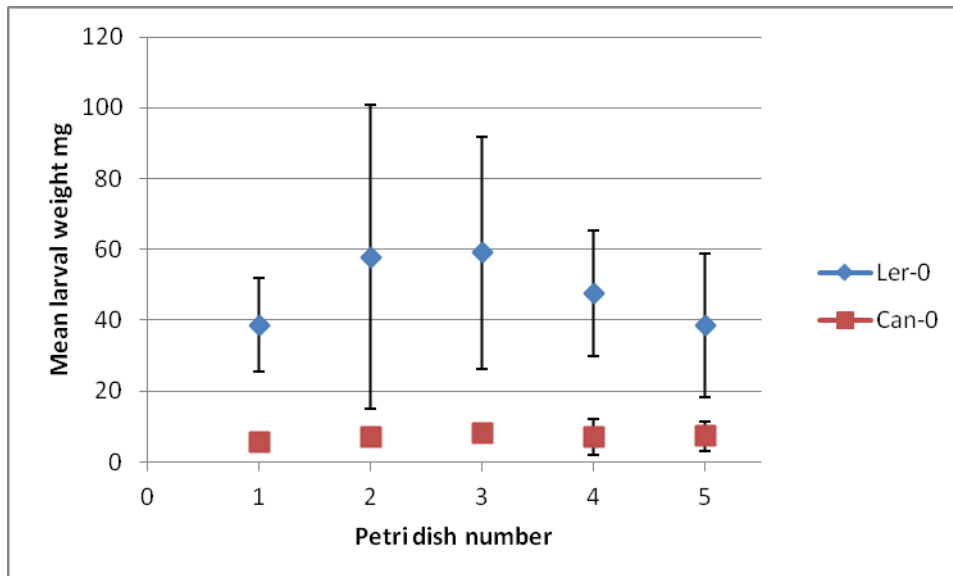


Figure 6. Spodoptera larvae weight after feeding on *Can-0* or *Ler-0*. The results are shown as means and standard deviation. $Ler-0$ and $Can-0$ t-test $t > t_{(1-\alpha/2; n-1)} = 6.44 > 2.093$ $\alpha = 0.025$ statistical significance.

Experiments conducted with *Spodoptera* feeding on the *Mt-0* and *Eh-0* ecotypes showed that the larvae fed on the *Mt-0* ecotype were much bigger than those fed on the *Eh-0* ecotype (Fig. 7). Like in

the previous experiment some larvae were missing but in this case on the Eh-0 ecotype. The t-test showed that differences recorded in larval body mass were statistically significant, i.e. larvae fed on the Mt-0 ecotype were bigger compared to those fed on the Eh-0 ecotype (Fig. 8).

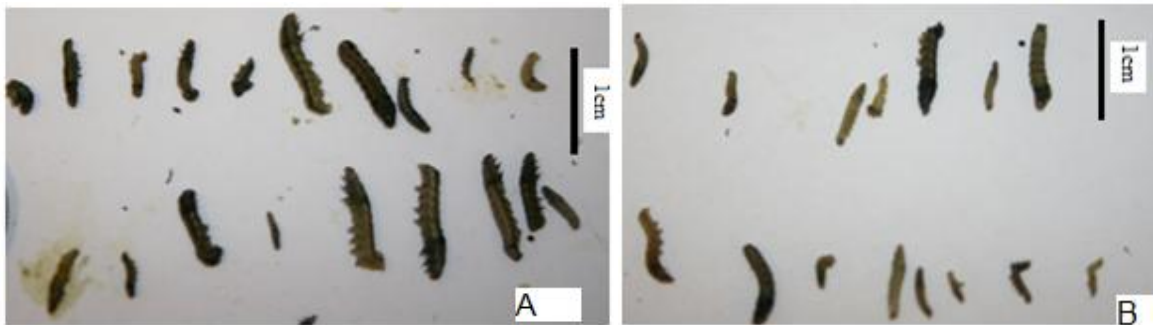


Figure 7. Spodoptera larvae fed on the Mt-0 ecotype for eight days A. Spodoptera larvae fed on the Eh-0 ecotype for eight days B.

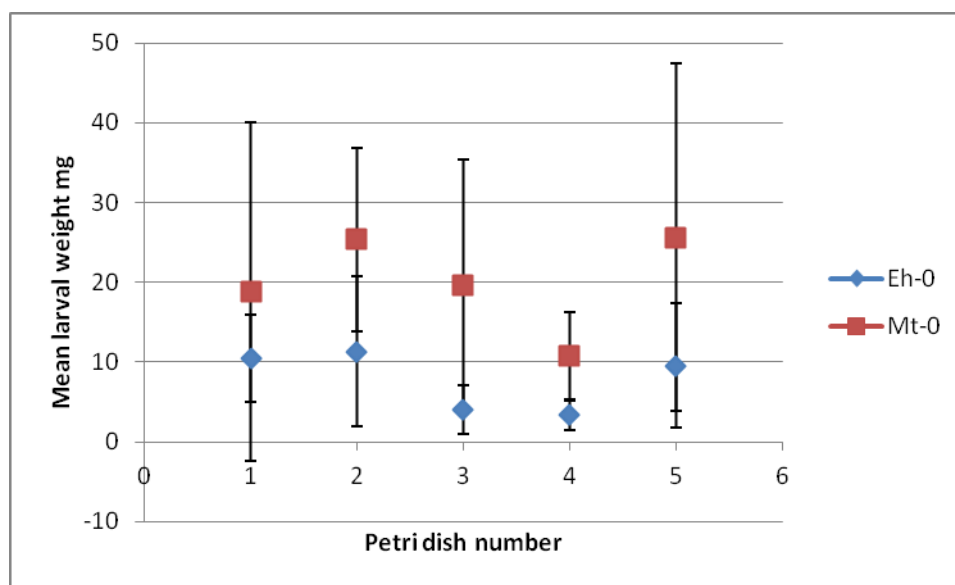


Figure 8. Body mass values of Spodoptera larvae fed on Eh-0 or Mt-0. The results are shown as means and standard deviation. Eh-0 and Mt-0 t-test $t > t_{(1-\alpha/2; n-1)} = 6.73 > 2.093$ $\alpha = 0.025$ statistical significance

Non-choice feeding experiment with *P. xylostella*

The *P. xylostella* feeding experiment showed that there were no significant feeding preferences between the tested ecotypes (Fig. 9, 10 and 11). But some larvae seemed to have escaped from the experiment since a couple of larvae were missing during the weighing process. It was suspected that the larvae could escape from the closed petri dishes because larvae were detected outside the petri dishes even if no petri dish had been opened.

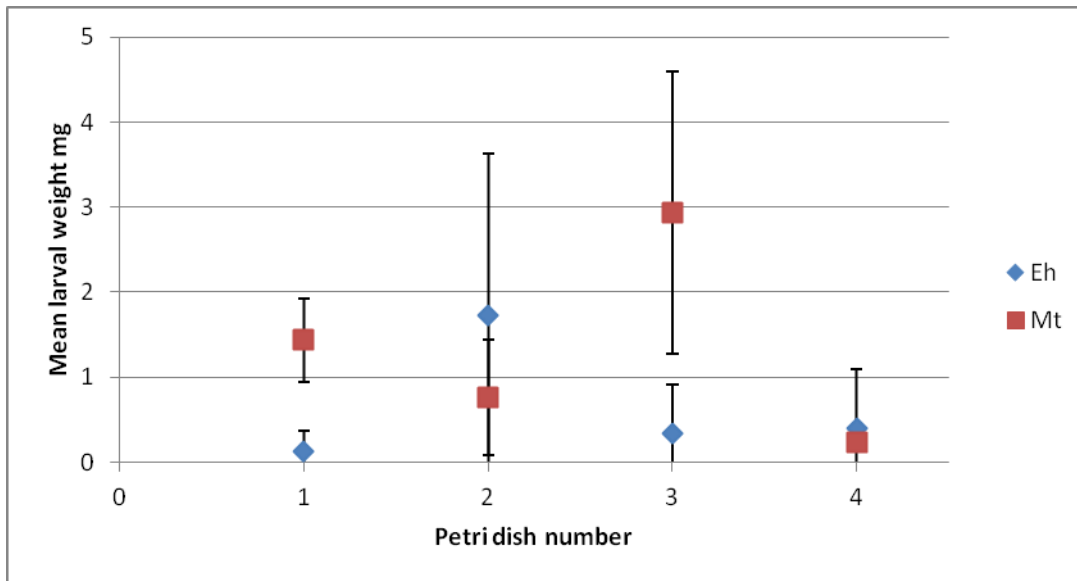


Figure 9. Weight of *Plutella* larvae fed on Eh-0 or Mt-0. The results are shown as means and standard deviation. Eh-0 and Mt-0 t-test $t > t_{(1-\alpha/2; n-1)} = 0.67 < 1.796$ $\alpha = 0.05$ no statistical significance.

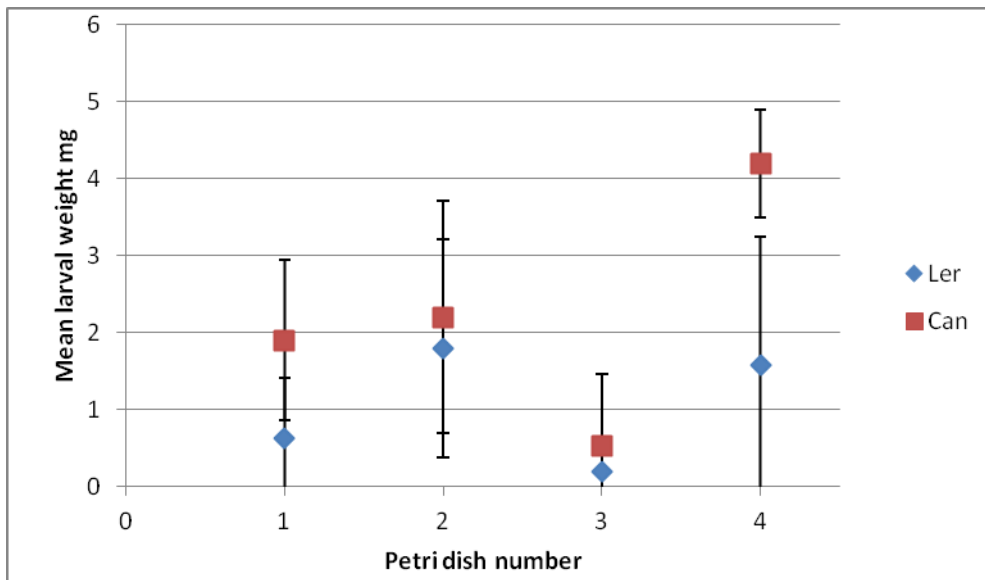


Figure 10. Weight of *Plutella* larvae fed on Ler-0 or Can-0. The results are shown as means and standard deviation. Ler-0 and Can-0 t-test $t > t_{(1-\alpha/2; n-1)} = 0.91 < 1.796$ $\alpha = 0.05$ no statistical significance.

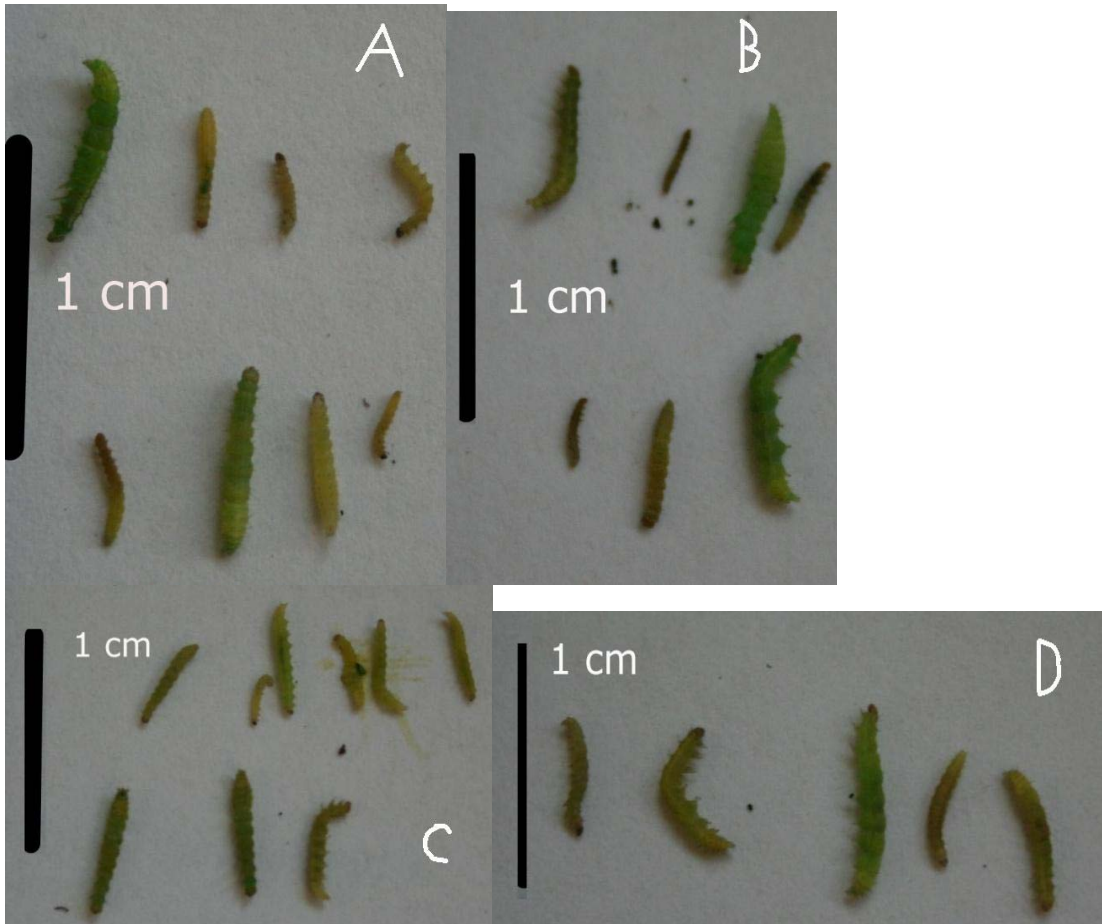


Figure 11. The picture shows *Plutella* larvae fed on *Ler-0* (A), *Eh-0* (B), *Mt-0* (C) or *Can-0* (D).

The *LOX 2* analysis (Fig. 12) showed that the *Ler-0* ecotype had a strong induction of the *LOX 2* gene expression when compared with the control. *Mt-0* and *Eh-0* ecotypes had a weak induction of expression while the *Can* ecotype decreased *LOX 2* expression when attacked by *Plutella* larvae.

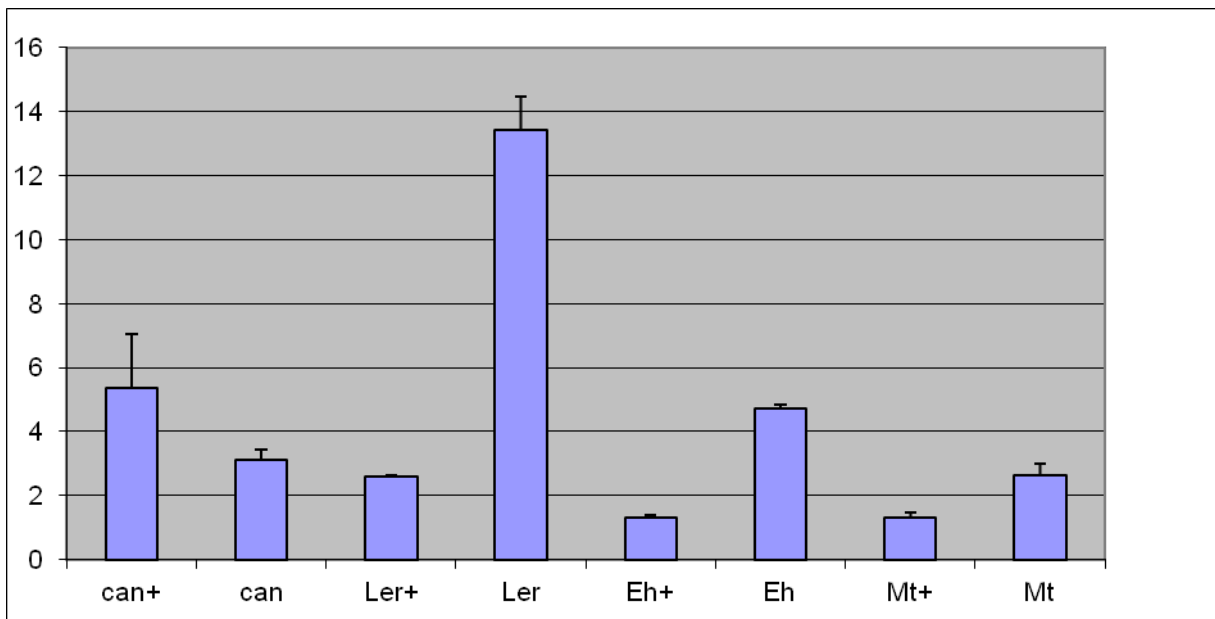


Figure 12. The *LOX 2* gene expression in the ecotypes *Can-0*, *Ler-0*, *Eh-0* and *Mt-0*. Ecotypes marked with a plus are corresponding controls.

Experiment with Bacillus priming for effects on Spodoptera feeding behavior

In this experiment plants were treated with beneficial Bacillus bacteria to prime plant defenses against insect herbivory.

The Mt-0 ecotype was discarded from the experiment because a mold fungus was detected in the control group.

No obvious body mass differences were recorded between Spodoptera larvae fed on the untreated Can-0 ecotype vs. those that fed on Bacillus treated plants Can-0 (Fig. 13, 14). However, it was noted that several larvae had perished when the experiment was terminated. This was especially pronounced for the larvae that fed on the Can-0 ecotype. In one of the petri dishes with untreated Can-0 all larvae had perished.

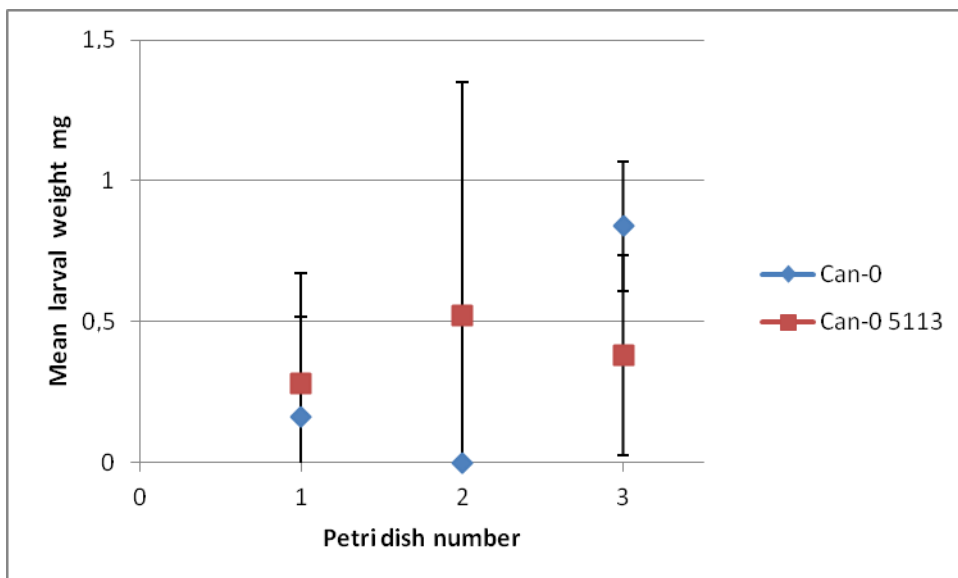


Figure 13. Weight of Spodoptera larvae fed on Can-0 control or plants treated with Bacillus 5113 through soil drenching. The results are shown as means and standard deviation. Can-0 control and Can-0 5113 t-test and $t > t_{(1-a/2; n-1)} = -0.35 < 2.145$ $\alpha = 0.025$ no statistical significance

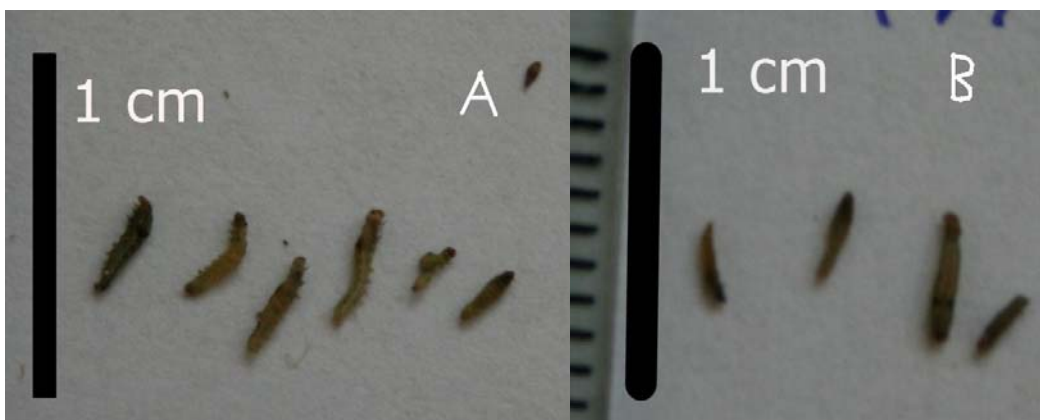


Figure 14. Spodoptera larvae fed on Can-0 control (A) or Bacillus 5113 treated Can-0 plants (B).

In the case of the Edi-0 ecotype larvae that fed on the untreated plants were somewhat heavier than those fed on plants with Bacillus treated soil (Fig. 15, 16) and this difference was also statistically significant. But because accidentally more than five larvae were put in petri dish 1 and 2 containing the untreated samples, there is no information if all larvae had survived the test or if some larvae had perished. It was noticed that five larvae had perished among those that had been fed on the plants with Bacillus treated soil.

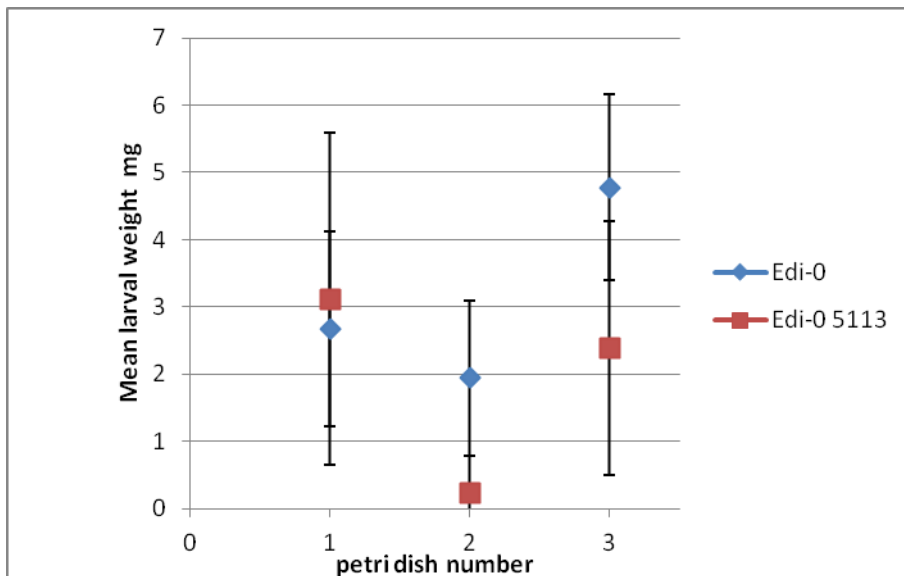


Figure 15. Body mass values of *Spodoptera* larvae fed on Edi-0 control or plants treated with *Bacillus* 5113 through soil drenching. The results are shown as means and standard deviation. Edi-0 control and Edi-0 5113 t-test $t > t_{(1-\alpha/2; n-1)} = 3.3296 > 2.145$ $\alpha = 0.025$ statistical significance.



Figure 16. *Spodoptera* larvae fed on Edi-0 control (A) and Edi-0 plants treated with *Bacillus* 5113 (B).

The experiment shows that the *Spodoptera* larvae that fed on the Edi-0 ecotype were significantly heavier than those that fed on the Can-0 ecotype (Fig. 13 and 15).

In the second test with the ecotypes Shadara, Ta-0 and N13 slightly different results were obtained. The larvae that fed on the Shadara and the Ta-0 ecotypes did not show any statistical significance between the controls and the treatments (Fig. 17 and 18). However, a difference was detected between larvae fed on the N13 *Bacillus* treated plants and the control. Larvae fed on *Bacillus*-treated N13 plants were significantly heavier than those fed on the untreated control (Fig. 19).

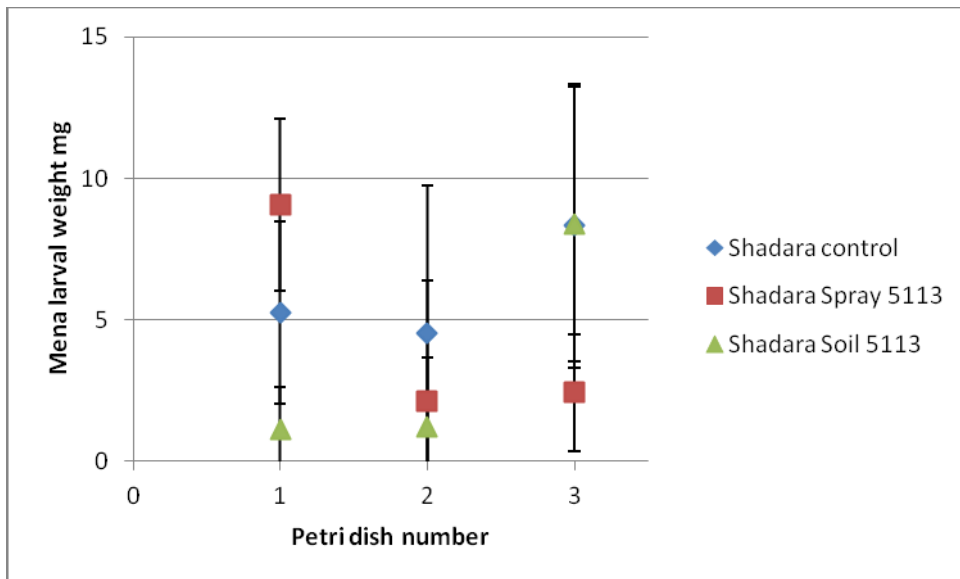


Figure 17. Body mass values of Spodoptera larvae fed on Shadara control or plants treated with Bacillus 5113 using soil drenching or spraying on leaves. The results are shown as means and standard deviation. The value for the shadara control in petri dish 3 is behind the value for the Shadara Soil 5113.

Shadara control and Shadara spray t-test $t > t_{(1-\alpha/2; n-1)} = 0.735 < 2.201$ $\alpha = 0.025$ no statistical significance.

Shadara control and Shadara soil t-test $t > t_{(1-\alpha/2; n-1)} = 1.438 > 2.201$ $\alpha = 0.025$ no statistical significance

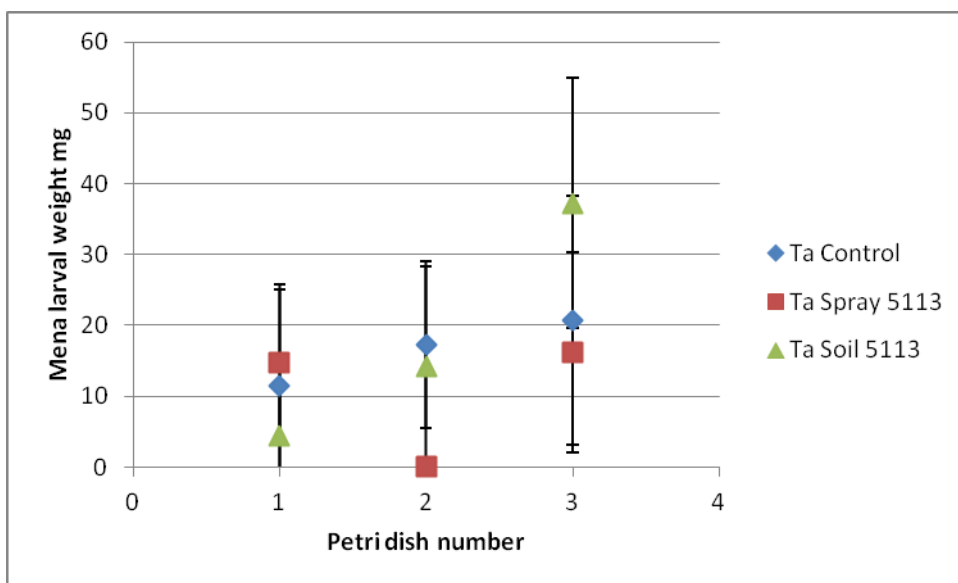


Figure 18. Weight of Spodoptera larvae fed on Ta-0 control or plants treated with Bacillus 5113 using soil drenching or spraying on leaves. The results are shown as means and standard deviation. Ta control and Ta spray

t-test $t > t_{(1-\alpha/2; n-1)} = 1.4451 < 2.201$ $\alpha = 0.025$ no statistical significance, Ta control and Ta spray t-test

$t > t_{(1-\alpha/2; n-1)} = -0.461 < 2.201$ $\alpha = 0.025$ no statistical significance

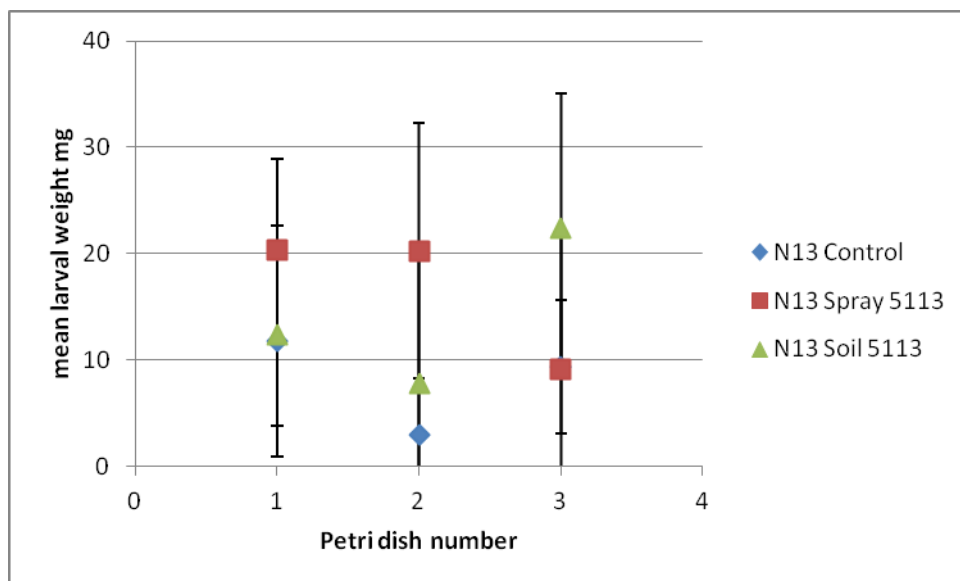


Figure 19. Body mass values of Spodoptera larvae fed on N13 control or plants treated with Bacillus 5113 using soil drenching or spraying on the leaves. The results are shown as means and standard deviation. The control value in petri dish 1 is hidden behind the N13 soil 5113 value. The value for petri dish 3 control is hidden behind the N13 Spray 5113 value.

N13 control and N13 spray t-test $t < -t_{(1-\alpha/2; n-1)} = -2,408 < -2,201$ $\alpha = 0,025$ statistical significance, N13 control and N13 soil t-test $t < -t_{(1-\alpha/2; n-1)} = -2,29 < -2,201$ $\alpha = 0,025$ statistical significance.

When comparing the treatments of the ecotypes Ta-0, Shadara and N13 (Table 4), there was a significant difference between Ta-0 and Shadara in the treatment Bacillus treated soil, between Shadara and N13 in both Bacillus treatments and between Ta-0 and N13 for the control indicating that the different ecotypes are not equally resistant.

Table 4. Comparison between ecotypes and treatments for the ecotypes Shadara, Ta-0 and N13.

Ecotypes	Treatment	Statistical significance
Ta-0 and Shadara	Control	No
Ta-0 and Shadara	Bacillus treated soil	Yes. Ta-0 larvae were bigger
Ta-0 and Shadara	Bacillus treated leaves	No
Ta-0 and N13	Control	Yes. Ta-0 larvae were bigger
Ta-0 and N13	Bacillus treated soil	No
Ta-0 and N13	Bacillus treated leaves	No
N13 and Shadara	Control	No
N13 and Shadara	Bacillus treated soil	Yes. N13 larvae were bigger
N13 and Shadara	Bacillus treated leaves	Yes. N13 larvae were bigger

Trichome analysis

The results of trichome analysis of the plants showed that the mean densities of the Can-0 and Eh-0 ecotypes were significantly higher than for the Mt-0 and *Ler-0* ecotypes (Fig. 20 and 21).

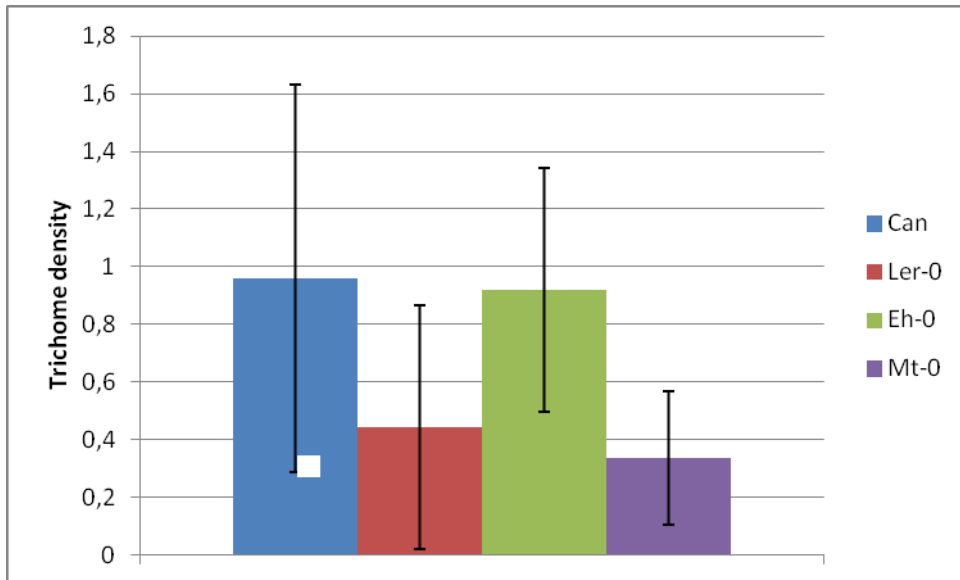


Figure 20. Trichome density for the ecotypes Can-0, *Ler-0*, Eh-0 and Mt-0. Can-0 and *Ler-0* t-test $t > t_{(1-\alpha/2; n-1)} = 2.649 < 2.201$ $\alpha = 0.025$ statistical significance. Eh-0 and Mt-0 t-test $t > t_{(1-\alpha/2; n-1)} = 4.015 > 2.306$ $\alpha = 0.025$ statistical significance.

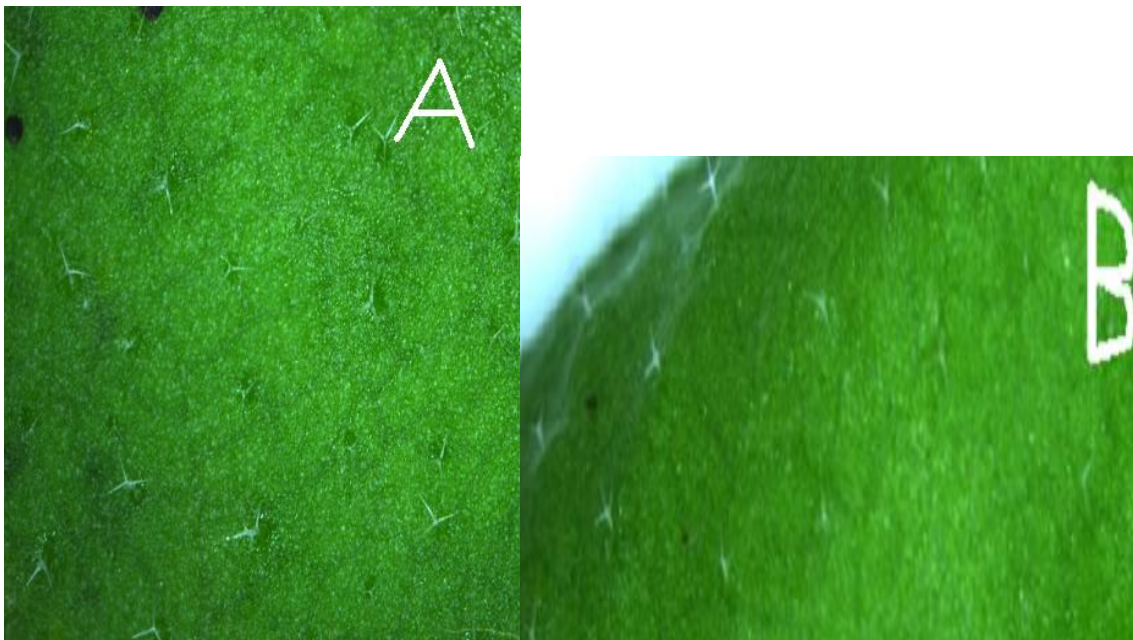


Figure 21. Trichomes on an Eh-0 leaf surface (A) or on Mt-0 (B). The pictures have different scales and the final magnification size is unknown.

Free choice feeding experiment

The larvae preferred to feed on the Mt-0 ecotype followed by the *Ler*-0 ecotype. The Can-0 and Eh-0 ecotypes were less favored with least consumption of the Can-0 (Table 5 and Fig. 22).

Table 5. Number of eaten leaves per ecotype. Every ecotype had totally eight leaves each.

Ecotype	Number of damaged leaves
Mt-0	6
Ler-0	5
Eh-0	3
Can-0	2

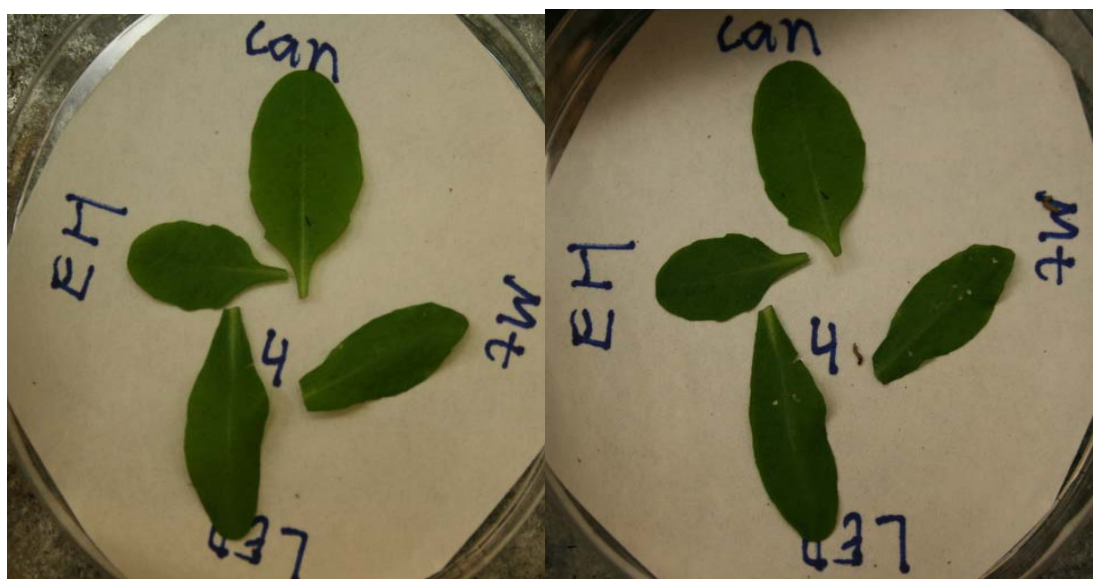


Figure 22. Spodoptera free choice feeding experiment with Can-0, Ler, Eh-0 and Mt-0 showing leaves before (A) and after (B) addition of larvae.

Discussion

Non-choice feeding experiment with *S. littoralis*

The first experiment showed a significant difference in Spodoptera larvae feeding preferences, reflected in larger body mass, favouring the *Ler-0* and *Mt-0* ecotypes over the *Can-0* and *Eh-0* ecotypes, respectively. These results support the first hypothesis since the *Can-0* and *Eh-0* plants apparently have some properties that deter the larvae from feeding. In fact, these properties were so strong that the larvae on the *Can-0* and *Eh-0* plants even died because of cannibalism or starvation (if they did not manage to escape but that is found to be unlikely) rather than feed on these plants. One possibility is that there are higher levels of secondary metabolites in the *Can-0* and *Eh-0* ecotypes. This suggests higher levels of glucosinolates because these larvae are chewing generalists that are supposed to be sensitive to glucosinolates and when they feed on the plant tissue the glucosinolate-myrosinase system is activated to thwart the attack (Schlaeppli et al. 2008). The JA and ET system might have contributed to regulate the glucosinolate levels but had probably minor interference since the plants usually need some time to activate the response and the larvae were fed with fresh plant material.

Another explanation is that the larvae that fed on *Ler-0* and *Mt-0* are the ones that were affected by the plant chemical defenses, because it is possible that secondary metabolites, e.g. antinutritional compounds, could make the larvae constipated and decrease nutrient uptake from the food making the larvae constantly hungry. However, this possibility seems unlikely since extensive amounts of frass were noticed and if the larvae had problems to satisfy the necessary needs of nutrients from the plants a higher mortality would be expected. Instead the big larvae observed seemed healthy and survived several days of storage at 4°C. This was in contrast to the small larvae observed on the *Can-0* and *Eh-0* ecotypes that did not survive the sudden environmental shift from 25°C to 4°C for three days.

Free choice feeding experiment

The free choice feeding experiments also showed that the Spodoptera larvae preferred the *Mt-0* and *Ler-0* ecotypes over the *Can-0* and *Eh-0* ecotypes. However, because of the small size of the experiment it needs to be repeated to make any firm conclusions.

Trichome analysis

The trichome analysis showed that *Can-0* and *Eh-0* plants had almost the same trichome density and that number was more than twice that of the *Ler-0* and *Mt-0* ecotypes, which had similar trichome densities. This is also supporting the results and conclusions from the non-choice feeding experiment because larval size should be and also were found to be negatively correlated with trichome density. Since trichome density was found to be positively correlated with glucosinolate levels in natural field populations of *A. lyrata* and *A. thaliana* (Mauricio & Rausher 1997, Clauss et al. 2006), there could be a double defensive effect for the *Can-0* and *Eh-0* ecotypes. These observations are supporting the first hypothesis.

Although the trichome density is reported to be positively correlated with glucosinolate levels, it would be preferable to analyze the glucosinolate levels in these particular ecotypes to be able to verify such a correlation on a more general basis. This kind of knowledge would also make it possible to compare the effect of the trichome and the chemical defenses.

Experiment with *Bacillus* for effects on Spodoptera feeding behavior

Unfortunately an unidentified error occurred during the *Bacillus* experiment and many larvae died. For example, seventeen larvae died on the *Can-0* ecotype while just one larva died in the first feeding experiment. One possible explanation is that the feeding experiment started too late so the plants had started to go into senescence with decreasing nutrient value. Another possibility is that the larvae were

not healthy. The results from the Mt-0 control group were not reliable because mold was present in two out of three petri dishes. Results from the Edi-0 ecotype showed that larvae that fed on untreated plants were bigger than those fed on Bacillus treated plants and this difference was statistically significant. Bacillus treatment may have had a priming effect so the inducible defenses were activated rapidly by larval feeding. It is also possible that there was no priming effect because accidentally too many larvae were put in two of the petri dishes and there is no exact information available if any larvae had perished during the experiment. Meanwhile the correct amounts of larvae were put in the other petri dishes. So it is possible that more larvae had died in the petri dishes with too many larvae than in the other petri dishes. The experiments need to be repeated to draw a firm conclusion.

In the second Bacillus experiment some interesting results were obtained. The larvae that fed on the Bacillus treated N13 ecotype were bigger than the control. Apparently the Bacillus treated plants became more susceptible. The reason for that might be that the Bacillus dose was too high for this specific ecotype and the plants somehow got weakened. But still the results from this ecotype are rejecting my second hypothesis because the plants whose induced defensive systems are supposed to be activated faster defended themselves worse than the control. The results from the Ta-0 and Shadara ecotypes are also rejecting the second hypothesis. It is not possible to rule out the possibility that some kind of priming reaction had occurred but that had no effect against the Spodoptera larvae.

Since the results from the Edi-0 and N13 plants are so contrasting it would be interesting to replicate the experiment to verify the results. If the results in the repeated experiment are similar to those already obtained it would be interesting to investigate the contrasting ecotypes more closely.

The comparison between the ecotypes and treatments for Shadara, Ta-0 and N13 indicated that Ta-0 is the most susceptible ecotype, while Shadara is the most resistant one and N13 is in between them. But in the primed state the N13 ecotype became equally susceptible as the Ta-0 ecotype. This comparison both supports the explanation that the Bacillus dose applied was too high on the N13 plants or/and that the N13 ecotype in primed state for some reason downregulates the defenses against generalist pests.

A suggestion for future experiments would be to inject the priming agent into the plant phloem and/or xylem to assure that the priming agent has had the possibility to affect the plant. This would rule out the possibility that the lack of priming effect observed for Can-0, Shadara and Ta-0 ecotypes could be due to failure to penetrate the cuticular layer.

Non-choice feeding experiment with *P. xylostella* on Arabidopsis

The *P. xylostella* feeding experiment showed that there were no differences between the larvae fed on the different ecotypes. But the experiment was limited in size so a future experiment with further samples and better sealed petri dishes (to avoid larvae to escape) are needed. Anyhow, if the results from this experiment should be interpreted they provide two different explanations:

1. The induced defensive systems might not have reached full power in any of the tested plants.
2. It does not matter how fast the induced defense systems are activated, rather the strength of the response matters.

The analysis of *LOX2* expressions showed that the inducible defensive systems were activated already after 6 hours. The plant materials in the feeding experiment were exposed to feeding for a much longer time span than 6 hours and the tested plants were exposed to more larvae. Accordingly the tested plant's defense systems had probably reached full power and the first explanation can be ruled out, but to be certain a similar feeding experiment could be conducted where the plant ecotypes are primed in advance so the inducible defensive systems are activated much faster.

I cannot rule out the second explanation because according to the analysis of *LOX2* gene expressions, the ecotypes have different defensive responses to the larvae and the larvae seem to respond equally to the different responses.

Future aspects and applications of the study

The results obtained together with the published knowledge about the levels of secondary metabolites could be used in the future for modifying crop plants relevant to agriculture. By identifying the genes responsible for production of glucosinolates it is possible to modify the levels. That would for example make oil seed rape more resistant against generalist insects. But there is a risk for overestimation of the power of these modifications since it may change resource allocation and result in a different energy distribution in the plant that can lead to e.g. lower yield or poorer overwintering ability. Also for quality reasons it is not always desirable to have high levels of secondary metabolites. For example, fodder containing rapeseed cake with high levels of glucosinolates causes digestion or health problems for animals (Hay 2006).

Priming of inducible defenses is an interesting strategy for integrated pest management and organic crop production. If the right bacteria could be found it would be possible to develop organic biopesticides that would make the plant able to defend itself better against insect pests and pathogens using its own resources. It might even be possible to develop a broad spectrum combined insecticide and fungicide action that has very low toxicity. But if such biocontrol pesticides would be developed they should only be used when needed, e.g. at high densities of specific insect pests, for example aphid years in cereals, high beetle infestations in oilseed rape production or for rainy years with high risk for pathogens. Frequent usage of priming agents as a precautionary measure could result in development of pests that somehow circumvent this defense resulting in increased damage.

Conclusions

The report has provided support for the following conclusions:

1. According to the experiments and the literature study, plants that are resistant against generalist insect pests show higher trichome counts that has been reported to correlate with secondary metabolite based defense.
2. According to the literature, in some cases plants have a higher resistance against plant pathogens if their inducible defenses are activated faster. Against insect pests, this limited study failed to prove that the activation speed of the inducible defenses had a positive effect for the plants.

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