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Abstract

In two feeding experiments *Bacillus* induced priming of plant defense was tested for its effect against the generalist insect pest *Spodoptera littoralis*. The main hypothesis for the study was that *Bacillus* induced priming would enable the plant *Arabidopsis thaliana* to defend itself better against this insect herbivore since this priming seems to involve jasmonic acid, known to be important for plant defense to insects. A secondary objective of the study was to examine if any differences in a primed defense capability could be seen between different natural genetic variants (ecotypes) of *A. thaliana* reflecting habitat differences in pest pressure.

The methods used for plant cultivation and *Bacillus* inoculation were aimed at reflecting conditions that can be expected in an agricultural cropping system. This meant that all plants were cultivated in soil, and the feeding experiments were conducted with the plants growing intact in the soil system. Inoculation of plants was done with the *Bacillus* bacteria in its stress resistant spore form. A literature survey was performed to gather knowledge involving some of the more important concepts in plant defense against herbivores. The glucosinolate/myrosinase system as well as defense activation was studied in more detail.

The study concluded that although *Bacillus* mediated priming was found to have an effect on some induced defenses in *A. thaliana,* no enhanced resistance against an insect herbivore was observed within the experiments conducted.

Sammanfattning

I två olika matningsförsök testades om växters försvarsförmåga mot den herbivora insekten *Spodoptera littoralis* påverkades av priming med *Bacillus* bakterier. Hypotesen för studien var att *Bacillus* inducerad priming skulle stärka försvaret mot herbivora insekter hos *Arabidopsis thaliana.* Hypotesen grundade sig på information som indikerade att denna priming involverade Jasmonsyra systemet som visat sig vara en viktig del i växters försvar mot insekter. Ett sekundärt mål för studien var att undersöka eventuella skillnader i *Bacillus* primad försvarsförmåga mellan olika ekotyper av *A. thaliana* som skulle kunna bero på skillnader i skadegörartryck mellan olika habitat.

Metoderna som användes vid odling av växter och inokulering med *Bacillus* syftade till att likna förhållanden som kan förväntas i agrara odlingssystem. Detta innebar att alla växter som användes i försöken odlades i jord från groning till avslutat experiment samt att inokuleringen gjordes med *Bacillus* i sin tåliga sporform. En litteraturstudie gjordes för att samla kunskap om de viktigare koncepten i växtförsvar mot herbivora insekter. Glukosinolat/myrosinas systemet samt aktiveringen av försvarssystem studerades mer ingående.

Studien kunde inte påvisa någon ökad motståndskraft mot en herbivor insekt till följd av *Bacillus* priming. Detta trots att denna form av priming konstaterades ha en effekt på några delar av det inducerbara försvaret i *A. thaliana*.

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Introduction

The practice of agriculture has evolved throughout history, however many of the challenges faced by farmers have remained the same. Among these is the constant competition with insects that share our interest in crops. This battle has been fought in different ways over the years, but has during the last century been largely driven by rapid and extensive implementation of chemical insecticides on a global basis. Major concerns have lately been raised regarding insect pest resistance towards many of these pesticides and damage inflicted by them on non target organisms, as well as environmental pollution and food safety issues (Gerhardson, 2002). This calls for alternate methods to manage insect pests in agricultural cropping systems. With a broad spectrum of available methods to combat insect pests, resistance problems are less likely to become a problem. The use of beneficial bacteria for pest control has been proven successful for pathogen management but may also have potential for insect pest management. Although there are a number of bacterium based biocontrol products on the market today, these are mainly intended for use against pathogens, with a few exceptions e.g. the toxin forming *Bacillus thuringiensis* (Gerhardson, 2002). This report focuses on the use of the rhizobacteria *Bacillus amyloliquefaciens* to enhance the plants own resistance to insect pests through the interaction called priming.

Background

The innate immunity system of plants is composed of several defensive barriers. These defenses differ in nature, and range from physical defense structures like thorns and hairs (trichomes) to chemical substances with repelling or toxic effects. The defenses may be either pre-formed like trichomes or inducible e.g. production of pathogenesis-related (PR) proteins, and are thus activated after infection. The glucosinolate-myrosinase system in Brassicales has elements both of basal and inducible defense. The components are preformed but sequestered in the normal healthy plant. However, the system will become activated and produce a blend of repelling chemical compounds after mechanical damage such as chewing damage caused by insect herbivores. The inducible defenses have been found to be triggered by plant hormones, including jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA). These hormones may interact in different ways and thus shape the response expression (Pieterse, 2009). For the purpose of visualization, the defenses of the plant may be pictured as structures that have been commonly used in warfare (Fig. 1).

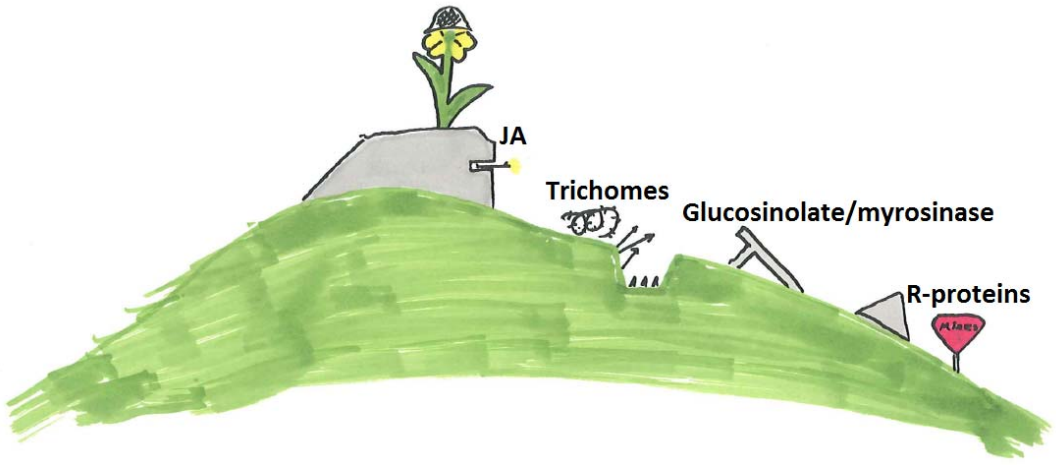

Fig. 1. Plant defenses can be preformed (trichomes) or induced (JA) upon attack. Activation of induced defenses can be accomplished in different ways; one of them is by perception of pest emitted cues by receptors (R-proteins).

Glucosinolates

Glucosinolates are amongst the most thoroughly studied herbivory defense chemicals present in plants. They constitute a large group of secondary metabolites that are rich in sulfur and nitrogen which can be mostly found in the Brassicaceae family. Glucosinolates do not possess biologic activity in themselves, but the products of their hydrolysis can participate in a wide range of biological interactions (Halkier & Gershenzon, 2006). The hydrolysis of glucosinolates is catalyzed by enzymes called myrosinases. Myrosinases are normally kept separate from the glucosinolates, but brought together upon wounding of the tissue.
The compartmentalization of the glucosinolate/myrosinase is subject for some dispute. One theory being that they are stored in separate cells, S-cells (glucosinolates) and myrosin cells (myrosinases) (Andréasson, 2000). Another idea is that glucosinolates and myrosinases are both present within the same type of cells, but stored in different subcellular compartments (Koroleva & Cramer, 2011). The final outcome (type and amount of breakdown products produced) when glucosinolates are activated by myrosinases has been shown to be dependent

upon several factors including plant age, tissue type, planting density and plant genotype (Wentzell & Kliebenstein, 2008)

Glucosinolates have been found to be unevenly distributed throughout plants. Through the use of mass spectrometric imaging, glucosinolate abundance throughout *A. thaliana* leaves has been mapped. Glucosinolates appear to be more abundant in the midvein and the edges of the leaves than in the inner lamina. Furthermore a difference in the proportions of major glucosinolates appear in different parts of the leaf (Shroff *et al.*, 2008). A similar study performed on flowers, buds, sepals and siliques of *A. thaliana* also found evidence for a non-uniform glucosinolate distribution (Sarsby *et al.*, 2012).

Although the glucosinolate/myrosinase system offers protection towards many insect herbivores, some generalist and specialist herbivores have managed to overcome this defense. This can be accomplished in several ways, for example by converting glucosinolates into inactive compounds or by rapidly ingesting the glucosinolates while still intact (Winde & Wittstock, 2011).

Glucosinolates are of great interest in plant breeding, since glucosinolates and their hydrolysis products have different properties when ingested in food. Some are toxic to mammals, while others have positive dietary effects (Tripathi & Mishra, 2007). Certain products are health promoting, being anti inflammatory or even possess anticarcinogenic properties (Hayes *et al.*, 2008).

Activation of inducible defenses in response to herbivory

The formation of defenses are metabolically costly for plants since resources have to be allocated from plant growth (Karban & Baldwin, 1997). This is why inducible defenses are not activated until the plant perceives some signal indicating that it is under attack. For this purpose plants have specific receptors (R-proteins) that recognize herbivore associated molecular patterns (HAMPs) (Mithöfer & Boland, 2008). These act similar to the receptors for pathogen/microbial associated molecular patterns (PAMPs/MAMPs) that have received far more attention (Wu & Baldwin, 2010). These elictors may be present in the oral secretions (OS) and in the oviposition (egglaying) fluid of insects (Alborn *et al.*, 1997; Howe & Jander, 2008). Furthermore, the plant may also release its own elictors from damaged cells, e.g., cell wall fragments, as a self damage recognition (Heil, 2009; Yamaguchi & Huffaker, 2011). Yet another possible defense activation route could be the recognition of certain feeding patterns expressed by the herbivore (Mithöfer *et al.*, 2005).

When the plant has recognized that it is under attack, an array of defensive countermeasures are activated (Howe & Jander, 2008). This activation needs signaling though, which is mediated by a range of different molecules. Volatile substances have been found to play important roles in the activation of inducible defenses as well as for attracting natural enemies to indirectly combat invading insect herbivores (Paré & Tumlinson, 1999; Wu & Baldwin, 2010). Arguably the most important signaling molecule when it comes to herbivore defense is JA. It triggers an array of defense responses systemically throughout the plant and has been shown to be critical for insect herbivore resistance (Kessler *et al.*, 2004; Bodenhausen & Reymond, 2007). A methylated form of JA, (MJ) emitted from a plant has been shown to effect other plants in its vicinity in a form of interplant communication (Farmer & Ryan,

1990). Perception of MJ from a nearby plant will then activate the systemic signaling in the receiving plant to prepare for an expected attack in the near future (Matthes *et al.*, 2011).

Pest countermeasures to plant defenses

Some pathogens can interfere with the host plant defense response and thus become virulent causing a systemic induced susceptibility (SIS) by compromising pathogen triggered immunity (PTI) of plants. One example of such a pathogen is the bacterium *Pseudomonas syringae*, which injects different virulence effector proteins into plant cells (Pieterse, 2009). *Pseudomonas* can cause SIS in host plants by production of coronatine (COR), which mimics JA. Since JA and SA have an antagonistic relationship, SA signaling drops and consequently defenses are not mounted against the pathogen (Brooks *et al.*, 2005; Cui *et al.*, 2005). SIS has also been shown to be induced by certain insect herbivores (Consales *et al.*, 2011). OS have in some insects been proven to contain defense suppressors (Ahmad *et al.*, 2011). These compounds counteract the effect of the defense genes activated by the elictors in the herbivores' OS to some extent (Consales *et al.*, 2011).

Priming

Inducible defenses need a certain time to reach full effect when activated from an unprepared state. Priming affects the defense capability without triggering a full scale response, but rather prepares the system for what is to come. The exact mechanisms of priming are poorly understood but are thought to be based on up-regulation of defense related genes and secondary metabolites (Frost *et al.*, 2008). Changes in chromatin structure that give an amplified expression of defense related genes upon activation might also play an important part in priming (Jaskiewicz *et al.*, 2011). The priming can be seen as a perception of signals that indicates imminent threat and as a result enhances the defense capability without large fitness costs (van Hulten *et al.*, 2006). This improved but latent capacity for defense referred to as priming gives a faster and/or stronger defense gene expression upon attack (Conrath *et al.*, 2006). Priming does not generally originate from harmful organisms. Certain non-pathogenic rhizobacteria, including varieties of *Pseudomonas spp.* and *Bacillus spp*. strains, can trigger the priming of inducible defenses (Ryu *et al.*, 2004). This primed state can also be achieved by perception of herbivory induced plant volatiles (HIPVs)(Frost *et al.*, 2008) and by low levels of chemicals including ß-amino butyric acid (BABA) (Jakab *et al.*, 2001).

Benificial bacteria

Certain bacteria, for example strains of *Bacillus* spp. and *Pseudomonas* spp. can act as growth promoters for plants (Lucy *et al.*, 2004). These bacteria are commonly referred to as plant growth-promoting rhizobacteria (PGPR) or more generally plant growth-promoting bacteria (PGPB). PGPR have different ways to achieve this effect, either by direct action such as facilitating nutrient uptake or by helping the plant in the suppression of pathogens (Lucy *et al.*, 2004).

When used for crop protection these beneficial bacteria are called biocontrol agents or biopesticides. The concept of biocontrol revolves around the use of one organism to control another organism (Gerhardson, 2002). Bacteria have the potential for protecting the crops in

different ways. One way is to form a impenetrable layer (biofilm) on the roots of the plant, which makes it harder for pathogens to penetrate the same (Bais *et al.*, 2004).
Another feature of bacteria is that they produce antimicrobial substances, such as surfactin, that counteract pathogens (Whipps, 2001). The positive aspects of using beneficial bacteria can ideally be thought of as twofold, as plants can experience both enhanced growth and defense towards pests (Babalola, 2010).

Bacillus amyloliquefaciens

Bacillus amyloliquefaciens is a rod shaped gram-positive bacterium. This particular species of *Bacillus* has been found to have many qualities making it suitable for use in biocontrol (Reva et al. 2004). Among these are its production of antimicrobial compounds, rapid colonization of the rhizosphere and formation of endospores (Compant *et al.*, 2005).
Bacillus endospores are highly resistant to external stress in comparison to other potential biocontrol bacteria. This is a favorable property that makes it possible to use conventional farming equipment for application in field (Fravel, 2005). *Bacillus* has shown its ability to provide protection of oilseed rape towards fungal pathogens (Danielsson *et al.*, 2007).
Strains of *B. amyloliquefaciens* are being used in commercial products but mainly for its role as a PGBR, in which it has proved its value (Adesemoye *et al.*, 2009)

Spodoptera littoralis

Spodoptera littoralis or Egyptian cotton leaf worm is a moth from the family Noctuiadae. The adult moth is night active and spans approximately 4 cm between wingtips. The larvae of *S. littoralis* are highly polyphagous and can use host plants from 44 different plant families. Many economically important food crops are among the potential hosts. Adult females lay in excess of 1000 eggs over the course of just a few days. Eggs are positioned in clusters of a few hundred on the backside of host plant leaves. The larvae normally have six instars during their development to adults. Older larvae feed only during night, and seek cover in the ground during daytime. The larvae also pupate in the soil when developing in to adult moths. Generation time as well as development time for *S. littoralis* is highly temperature and/or humidity dependent. The generation time might range from 19 to 144 days. The natural habitat for *S. littoralis* is semi-arid subtropical regions in pre-Saharan Africa (Bayer crop science, 2012; EPPO, 2012). The color of *S. littoralis* larvae has been shown to be correlated to population density. Crowded larvae have darker color than larvae kept isolated. Crowded larvae are also generally more active (Altstein *et al.*, 1994).

Pseudomonas syringae

P. syringae is a species of rod shaped aerobic gram negative bacteria. They are motile and propel themselves using one or more polar flagella. *P. syringae* is a plant pathogen but can also survive as an epiphyte under some conditions. Among the hosts are a number of legume species, notably common bean (*Phaseolus vulgaris*) and the majority of species in the tribe Phaseoleae. The disease symptoms of *P. syringae* infection appear in the form of water soaked lesions and/or chlorosis in susceptible plants. These may appear on the leaves, pod and stem of the plant. In resistant plants the infected cells will undergo a hypersensitivity reaction (HR) and become necrotic. Infection routes are through wounds in the plant, or through the

stomata (Bender *et al.*, 1999; Arnold *et al.*, 2011). *P. syringae pv* tomato strain DC3000 is a race that is often used in pathogenic studies since it can infect *A. thaliana.* The susceptibility of *A. thaliana* to DC3000 is due to inability of the R-proteins to recognize the avirulence factors (AVR).

Arabidopsis thaliana

A. thaliana is a plant that is commonly used in research as a model organism especially for dicot plants and it has a number of features that makes it well suited for this purpose. It has a short generation time, produce an ample amount of seeds and has a small genome compared to many other plants (Ankeny & Leonelli, 2011). Another neat feature is that many genetic variants and other tools are available. Different ecotypes have been shown to contain different types of glucosinolates (Kroymann *et al.*, 2003) and possess different levels of resistance to insect herbivory (Ahmad *et al.*, 2011).

Aims

The first aim was to test the effect of *Bacillus* mediated priming on the plant *A. thaliana* in its ability to defend itself against the generalist herbivore *S. littoralis.*

The second aim was to study the possible difference in defense capability between two genetic variants of *A.thaliana* when treated with *Bacillus* spores.

Experimental procedures

Non-choice feeding experiment

The plant growth conditions for this experiment were 16 h day (22°C) and 8 h night (18°C). The fluorescent light intensity was 60-139 µmol $m^{-2}s^{-2}$(during plant cultivation) depending on plant position relative to light source. During the feeding experiment, light intensity was 200-220 µmol $m^{-2}s^{-2}$. Relative humidity was kept at 70% . Growth conditions with controlled environment were assured by using growth chambers.

In preparation of the experiment more than 30 seeds from each of the *A. thaliana* ecotypes Can-0 and L*er*-0 were planted in autoclaved soil. The seeds were not chemically disinfected before planting, but had previously been heat treated. An excess of seeds were planted to compensate for germination losses.

When 10 days had elapsed from the date of planting, the seedlings were transplanted to medium sized pots filled with autoclaved soil. Two plants of the same ecotype were planted in each pot, giving a total of 20 pots or ten pots of each ecotype. These were then moved to a different growth chamber.

After 12 days from transplantation, five pots (ten plants) from each ecotype were primed by inoculation with *B.amyloliquefaciens UCM B5113* spores. The spores were suspended in

water at a concentration of 10^7 cfu/ml. For every plant in the primed group 1 ml of spore suspension was applied to the soil, right next to the stem base. The five remaining pots from each ecotype were treated in the same manner as the inoculated pots, but using water instead of spore suspension. This was the control group (Fig. 2).

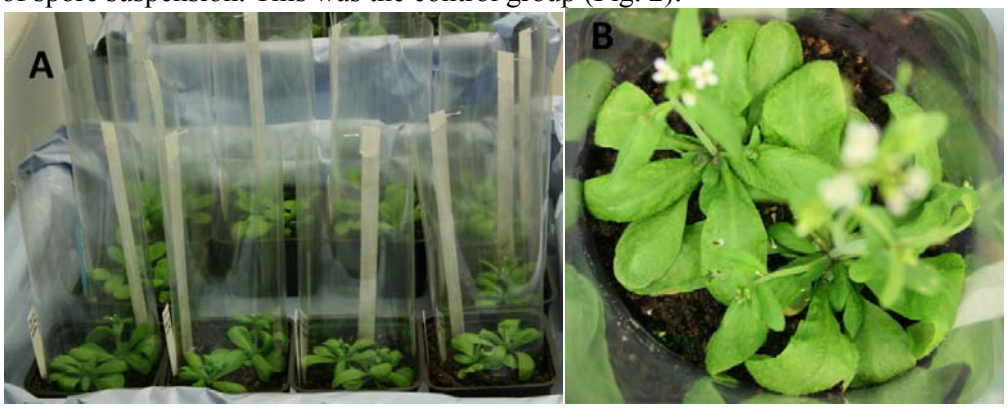

Fig. 2. (A) The picture illustrates the experimental setup for the control group. Plants were surrounded by transparent cages and pots were standing in a water containing tray. The tray containing inoculated plants can be seen in the background. (B) L*er*-0 control plants depicted inside their cage.

Three days after inoculation the actual feeding experiment was initiated. The two plants in each pot were surrounded by a cage, made of overhead plastic film. The cage was sunk roughly 1 cm into the soil.

S. littoralis eggs had been allowed to hatch in a Petri-dish during three days preceding the experiment. During this time they had access to a tomato leaf as food to avoid host plant adaptation.

The *S. littoralis* larvae were inserted to the cages by letting them cling on to an artist's brush and gently dropped onto the plants. Two first instar larvae were placed on each plant. When larvae were transferred it was noticed that they differed in color, ranging from light green to blue. This was, however, not taken into account during the transfer, which was made in a random fashion. The larvae were then allowed to feed freely on the plants for the next 8 days. After this feeding period, the larvae were collected and their color was noted. The larva were then placed in separate Petri-dishes, one for each cage and stored in a cold-room at 4°C. The plants were photographed and stored in a freezer at -20°C. The larvae were weighed 24 h later using a laboratory scale with a draft shield installed. Photographs of the larvae were taken after they had spent 6 days in the cold room.

The experiment was repeated once using methods that were identical in most ways, however some changes were made. The transplanting in to bigger pots was made 8 days after planting. The inoculation with *Bacillus* spores commenced 3 days before start of experiment. Cages were installed at the time of inoculation. *S. littoralis* larvae were one day younger at transfer than during the first experiment and had been kept on artificial substrate. The experiment was terminated 9 days after insertion of *S. littoralis*. The larvae were kept in cold room for two days before weighing.

When both replicates of this experiment were completed, measurements of light conditions in different parts of the growth chambers were conducted, using a photometer.

Multiple choice experiment

The experiment was designed as a multiple choice experiment with four plants of the same ecotype contained in the same cage. Two plants were inoculated with *Bacillus* suspension, and two plants were inoculated with water. The plants were planted in separate pots suspended in a larger pot. The idea was to give the feeding larvae free access to all plants while maintaining the soil separate for each plant (Fig. 3). Due to the plants being suspended, excess water from plants drained in to the bigger pot without contacting the soil of the other plants.

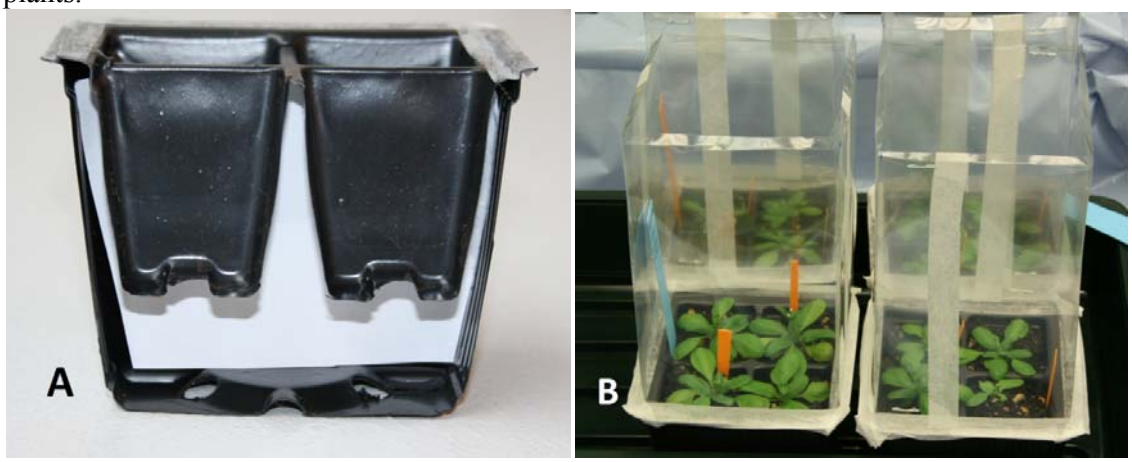

Fig. 3. (A) Arrangement of smaller pots suspended in a bigger pot, aimed at restricting the flow of water between plants. white background is for clarification only. (B) Experiment setup showing L*er*-0 plants surrounded by rectangular cages, featuring an inward sloping upper rim to prevent larval escape.

The system of containing the plants did not incorporate any measures for containment or control of the volatile substances emitted from plants and/or soil. In total 15 L*er*-0 plants and 16 Can-0 plants were used for the actual feeding experiment.

S. littoralis first instar larvae were inserted in the middle of the plant arrangement. They were placed on the plastic brim forming a divider between the small pots. The larva was pointing in random directions upon insertion. One larva was inserted for each plant in the cages, in order to keep the feeding demand equal.

The plants were watered by the use of a syringe equipped with needle. The water was injected in to the soil in order to minimize the risk of disturbing the feeding of the larvae. Separate syringes were used for watering inoculated and control plants. The plants were photographed every day to create a time-lapse, showing the feeding pattern of the larvae. Four days after insertion, the larvae were collected and their position was noted. The stems of the L*er*-0 plants were then cut to improve overhead visibility and the leaf rosettes were photographed. The pictures were later used for visual damage assessment and analysis of damaged area using ImageJ (Image Processing and Analysis in Java). Images were converted to 8-bit grayscale and thresholds were set by manually outlining the damaged leaf tissue, which was then analyzed with particle analyzing tool. The damaged area was analyzed separately for each plant and the scale was calibrated between each picture. The cross sectional distance of the outer pot was used for calibration.

Pseudomonas infection experiment

The plant growth conditions for this experiment were 16 h day (22°C) and 8 h night (18°C). The fluorescent light intensity was 200 µmol $m^{-2}s^{-2}$. Seeds from the ecotype L*er*-0 were germinated in autoclaved soil. Two weeks after germination 40 of these plants were transplanted into a single trough containing 40 minor pots (4x4cm) that had been filled with autoclaved soil. One week after transplant the 40 pot trough was cut in two pieces which were then suspended in two separate trays. The pots were suspended by a number of wooden sticks in such a manner that the pots were not touching the bottom or the sides of the tray (Fig. 4).

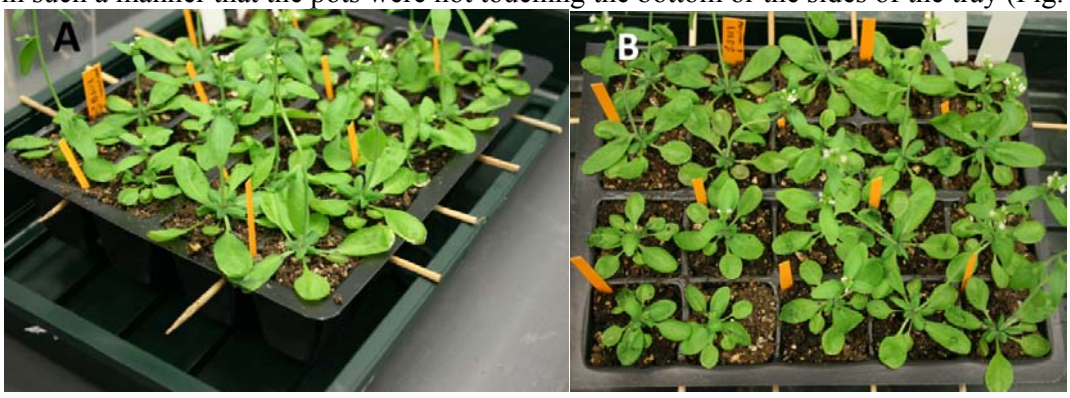

Fig. 4. (A) Suspended tray of 20 minor pots, containing L*er*-0 plants. (B) Inoculation pattern marked with orange tags. Each orange marked plant is inoculated with 1 ml of 5113 spore suspension (10^7 cfu/ml) through soil drenching.

Ten plants in each tray were then inoculated with 1 ml of 5113 spore solution (10^7 cfu/ml) in a checkered pattern. The remaining plants were treated with 1 ml of sterilized tap water. Three days after inoculation with *Bacillus*, all plants were inoculated with *P. syringae* ($5x10^5$ cfu/ml). The inoculation was made by injecting 10µl bacterial suspension into the vascular tracts of two opposite leaves in each plant using pressure infiltration.

Four days after infection with *P. syringae* the experiment was terminated. Inoculated leaves were cut, photographed and pooled together according to treatment and tray. Plant material was stored in a freezer at -20°C for three days. The plant material was then crushed and homogenized in liquid nitrogen. Equal amounts of homogenized tissue from each sample were used for DNA extraction using a GeneMole automated nucleic acid extractor (Mole Genetics) according to the manufacturer's instructions. The concentration of the resulting DNA solution was then measured using a microvolume spectrophotometer (Thermo Scientific) and thereafter concentrated by evaporating roughly 50 % of the water content in a SpeedVac concentrator (Thermo Scientific). A PCR plate was then prepared using the protocol supplied with Maxima SYBR Green/ROX qPCR Master mix kit (Fermentas). Slightly different volumes were used however: SYBR Green 10 µl, forward primer 1.2 µl, reverse primer 1.2 µl (Table 1), nuclease-free water 2.6 µl. The real-time PCR analysis (qPCR) was performed according to the two-step cycling protocol supplied with the kit using an ABI PRISM7700 thermo cycler. No technical replicates were made. A positive control was used for *Pseudomonas*, which generated a standard curve that was used for quantification of the 16S rRNA gene (Fig. 5). The quantification of this pseudomonas specific gene was made in order to compare the abundance of *P. syringae* in *Bacillus* treated plants and untreated plants. This was then viewed as a measurement of *Bacillus* mediated defense.

Table 1 *P. syringae* primers used in the qPCR analysis.

Forward primer: ´5-CAGCTCGTGTCGTGAGATGT-3´

Reverse primer: ´5-CACCGGCAGTCTCCTTAGAG-3`

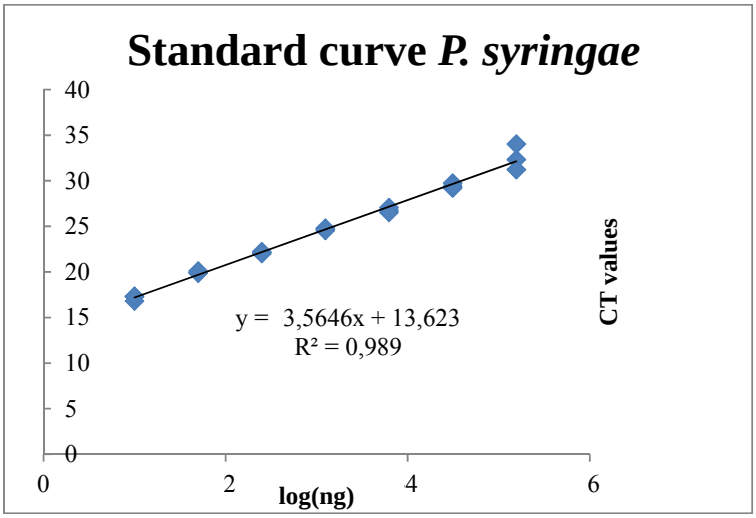

Fig. 5. qPCR Standard curve for the *P. syringae* positive control.

Pictures of inoculated leaves were analyzed, using the ImageJ software. The images were converted to 8-bit grayscale and made into binary form. Thresholds were set to include only discolored leaf tissue, which was then analyzed with the particle analyzing tool. The area of the discolored tissue was then compared with the total area of the leaves, which were obtained at a later stage by adjusting the thresholds. A filter setting for removing particles smaller than 0.5 mm^2 was used during area analysis in order to reduce image noise.

Soil samples were gathered at the end of the experiment. Pots were randomly selected and sample soil material from the two treatments was pooled together for each tray. Soil from five plants in each treatment was put in a bag and mixed thoroughly. Two samples of 0.25 g were taken from each bag and put in separate eppendorf tubes, giving a total of 8 samples. These were then soaked with 1 ml of LB medium and vortexed. The suspension was allowed to sit for 10 minutes. The soil suspensions (1 ml) were then transferred from each tube, and diluted 50 times in LB. An aliquot of 50 µl from each of these suspensions was then spread on two agar plates which were incubated in darkness at 28°C for two days. The plates were inspected regularly to detect microbial growth.

Results

Non-choice experiment

A clear difference in larval body size after feeding on the two ecotypes was apparent (Fig. 6). The results from the weighing of the larvae were interpreted by the use of two sample t-statistics in Minitab (Minitab inc.). The analysis showed no statistically significant difference between larvae that had been feeding on *Bacillus* treated plants and control plants, within either the ecotypes Can-0 or L*er*-0 (P>0.05). The mean larval weight was however significantly higher for those *S. littoralis* larvae that had fed on the L*er*-0 ecotypes in comparison to those that had fed on Can-0 (P<0.05) (Fig. 7). A total of 2 larvae were missing in L*er*-0 and 3 in Can-0 by the end of the first replicate. In the second replicate experiment 1 larva was missing from L*er*-0 and 7 from Can-0. These were regarded as having zero weight.

The color of the larvae differed significantly between L*er*-0 and Can-0 in all groups (Table 2). The larvae that had been feeding on L*er*-0 had a darker color than those that fed on Can-0. There was also a significant color difference among larva that had been feeding on *Bacillus* inoculated L*er*-0 plants and L*er*-0 control in one of the replicates (Table 3).

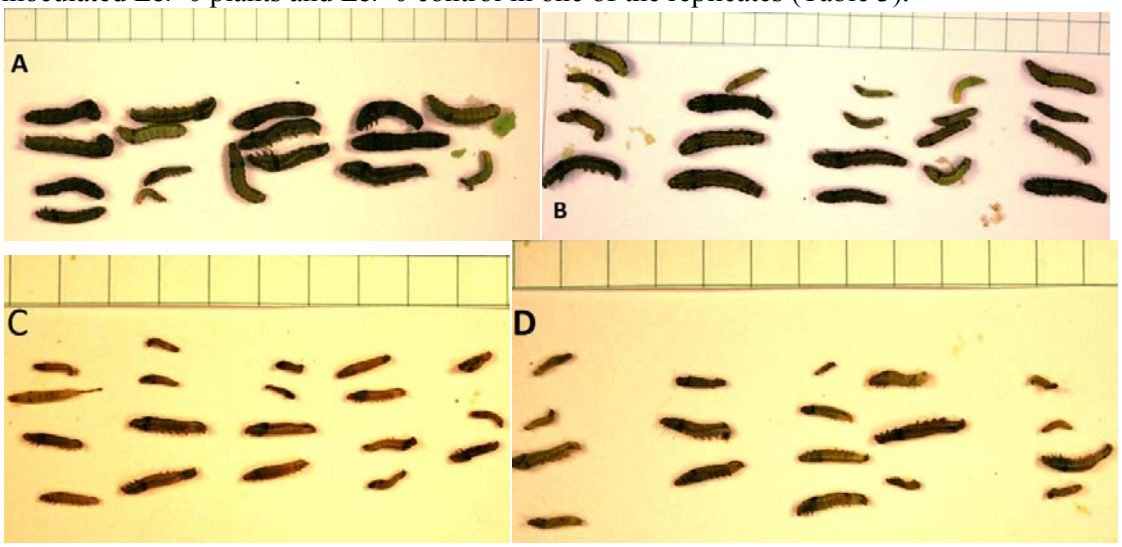

Fig. 6. (A) *S. littoralis* larvae after 8 days of feeding on L*er*-0 control plants, (B) L*er*-0 inoculated, (C) Can-0 control, (D) Can-0 inoculated plants. The distance between each line in the top scale represents 5 mm.

Table 2. Fisher method grouping of values representing larval color. (1=light green, 2= medium green, 3= dark green/black). No significant color differences in larval color between treatments.

Treatment	n	Mean color value	Std. deviation	Grouping
L*er*-0 control	18	2.39	0.78	A
L*er*-0 inoculated	20	2.25	0.72	A
Can-0 control	19	1.68	0.48	B

Can-0 inoculated	18	1.56	0.51	B

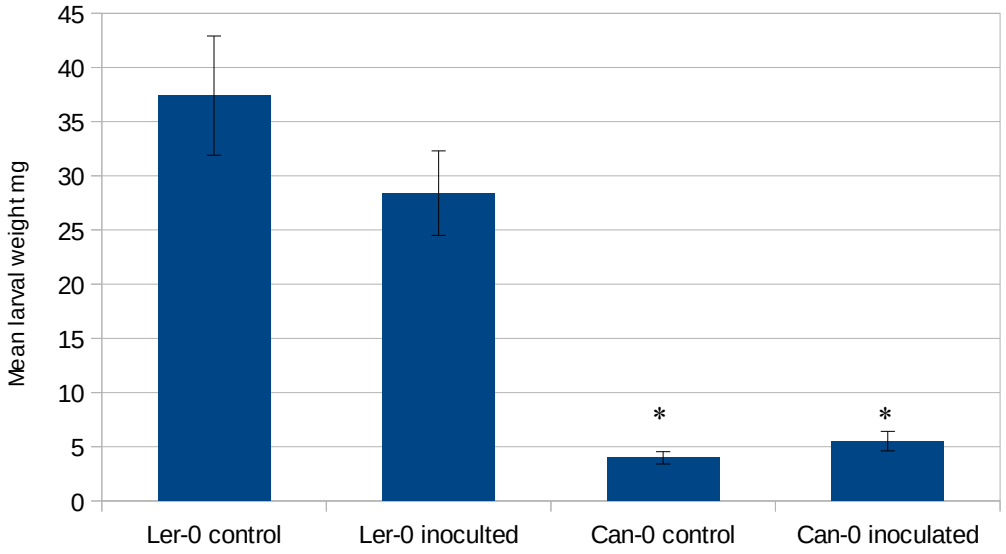

Fig. 7. Mean larval weight for *S. littoralis* that had been feeding for 8 days on L*er*-0 control, L*er*-0 5113 inoculated, Can-0 control or Can-0 5113 inoculated plants of *A . thaliana*. Welch´s t-test : L*er*-0 control > L*er*-0 inoculated; P= 0.095, no significance, Can-0 control > Can-0 inoculated; P= 0.92, no significance. Asterisks indicate statistically significant lower mean larval weight in comparison to corresponding L*er*-0 treatment, P<0.05.

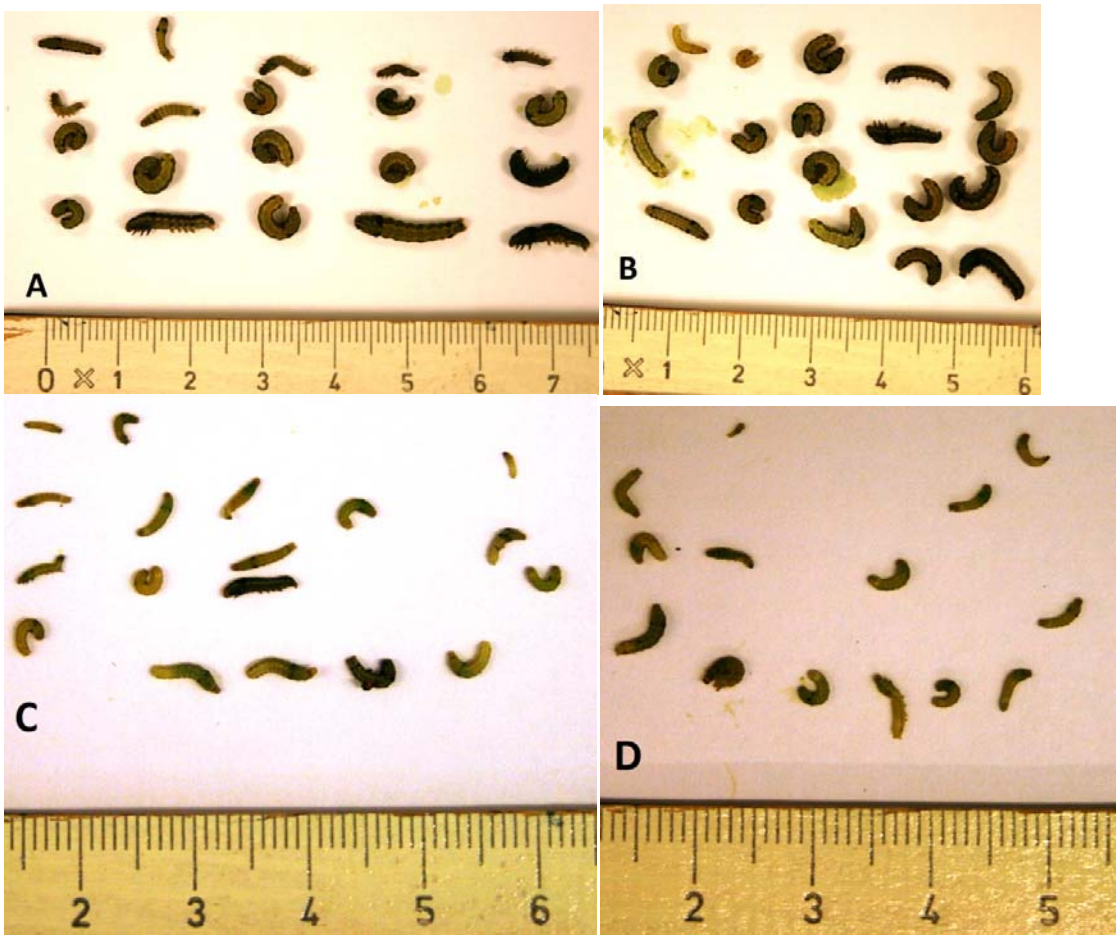

Fig. 8. ***S. littoralis*** **larvae after 9 days of feeding on L*er*-0 control plants (A), L*er*-0 inoculated (B), Can-0 control (C), Can-0 inoculated (D).**

Table 3. Fisher method grouping of values representing larval color. (1=light green, 2= medium green, 3= dark green/black). Significant color difference between larvae fed on L*er*-0 control and L*er*-0 5113 inoculated.

Treatment	n	Mean color value	Std. deviation	Grouping
L*er*-0 control	20	2.35	0.49	A
L*er*-0 inoculated	19	2.00	0.47	B
Can-0 inoculated	15	1.40	0.63	C
Can-0 control	18	1.33	0.59	C

The results from the two replicates of the non-choice feeding experiment were identical in most respects. The same difference in larval weight between the Ler-0 and Can-0 ecotypes could be seen in both the first and the second replicate (Fig. 9). Larval color also differed between larvae that had been feeding on Ler-0 in comparison to those that had been feeding on Can-0 as was the case in the first replicate (Fig. 8). A difference in color among the larvae that were retrieved from the two different treatments of Ler-0 could however only be seen in the second replicate (Table 3).

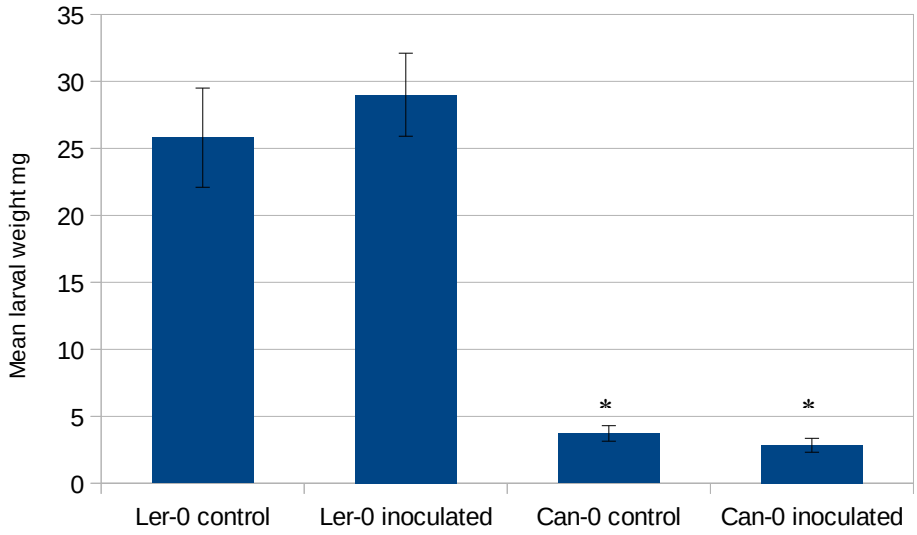

Fig. 9. Mean larval weight for *S. littoralis* that have been feeding for 9 days on L*er*-0 control, L*er*-0 5113 inoculated and Can-0 control, Can-0, B5113 inoculated plants of *A. thaliana*. Welch´s t-test: L*er*-0 control > L*er*-0 inoculated; P= 0.75, no significance, Can-0 control > Can-0 inoculated; P= 0.13, no significance. Asterisks indicate statistically significant lower mean larval weight in comparison to corresponding Ler-0 treatment, P<0.05.

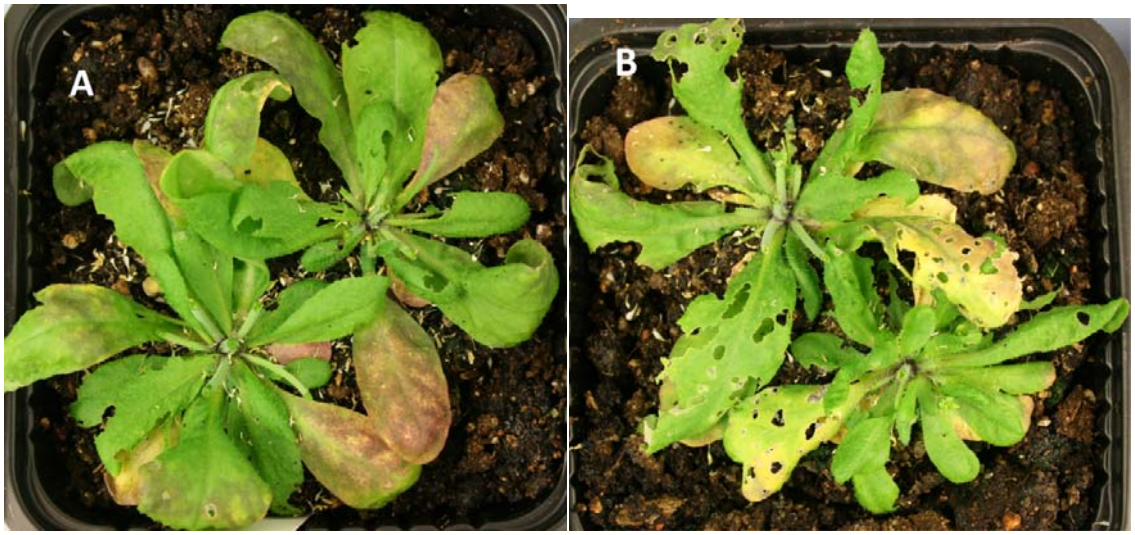

Fig. 10. (A) Picture showing L*er*-0 *Bacillus* 5113 inoculated plants after 9 days of feeding by *S. littoralis*. Note the color of the L*er*-0 plants in the same experiment which have not been treated with Bacillus 5113 (B).

In the second replicate of the no-choice experiment it was observed that some of the older leaves on the *Bacillus* inoculated L*er*-0 plants attained a lilac color, while on the control plants only very slight lilac coloration was noted. Some of the L*er*-0 plants demonstrated feeding damage patterns that resembled test bites, but no large area of feeding. Soil-borne Acari (*Tetranychus urticae)* were found in some of the L*er*-0 pots in the first replicate of the experiment, however, no feeding symptoms could be observed.

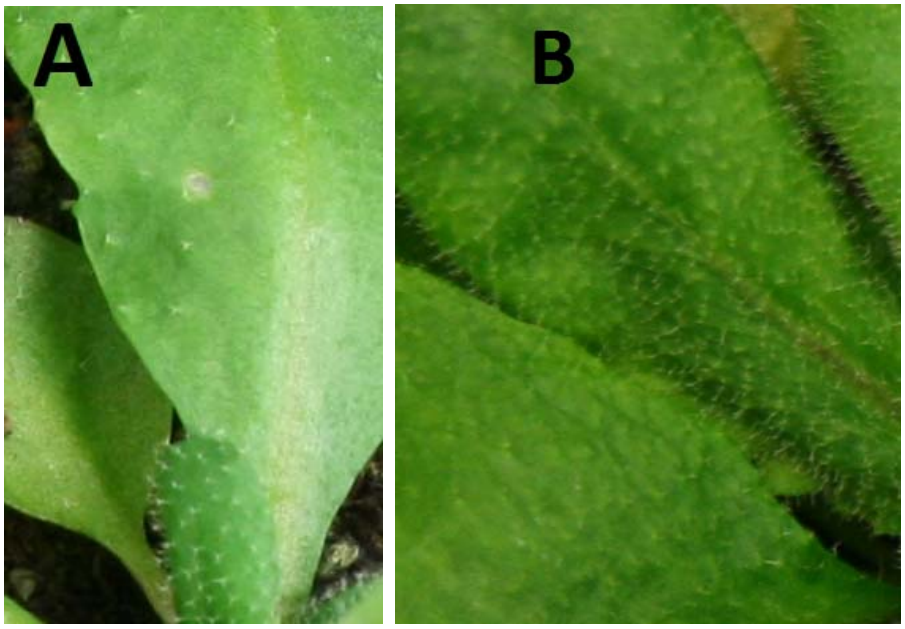

Fig. 11. Trichomes on leaves of different ages (A) L*er*-0 control, (B) Can-0 control.

Trichrome density between leaves of different ages appeared to vary greatly in both L*er*-0 and Can-0 ecotypes (Fig.11). This was however not confirmed by any quantitative methods.

Multiple choice experiment

More larvae were found on L*er*-0 control-plants than on L*er*-0 inoculated plants when the feeding experiment was terminated after 4 days of feeding. An average of 0.6 *S. littoralis* larvae was located on each *Bacillus* inoculated L*er*-0 plant, while an average of 1.3 larvae were located on each control plant. The experiment contained too few plants to statistically confirm this tendency. Older leaves appeared to have suffered more extensive feeding damage in Can-0 plants (Fig. 12, A)

The image analysis showed that the control plants lost a combined area of 108 mm^2 to larval feeding and the inoculated plants lost 78 mm^2. However, neither of these results could be proven statistically significant while using a Welch's t-test by using the individual values for all plants ($P>0.05$) (Fig.13).

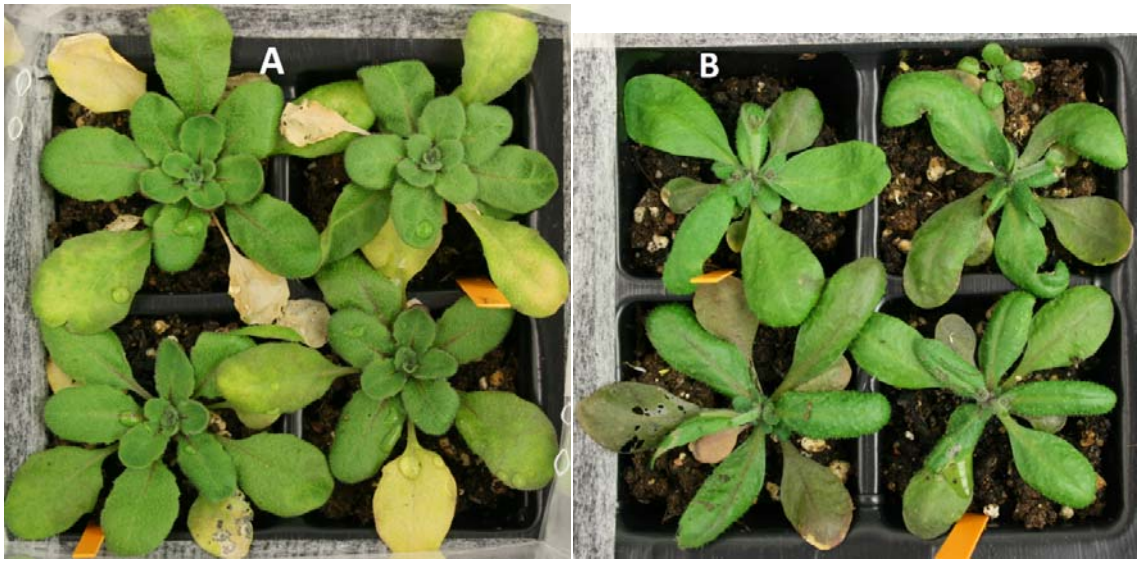

Fig. 12. Pictures are illustrating plants after removal of larvae from the multiple choice feeding experiment. Plants tagged with orange sticks were inoculated with 5113 spores. Can-0 (A), Ler-0 (B).

Several Can-0 plants died of drought two days after inoculation with 5113, probably due to intense wind stream from a ventilation duct. The air duct was re-directed which resolved the problem but left the surviving plants highly stressed. When collecting the larvae from the Can-0 plants, almost half of them could not be accounted for compromising the interpretation.

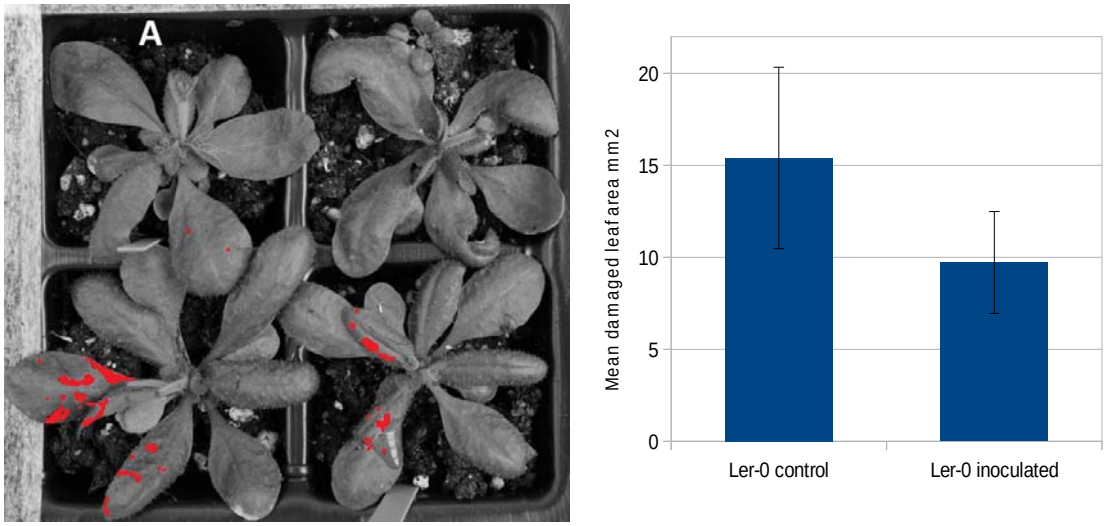

Fig. 13. (A) Image converted to 8-bit showing threshold for particle area analysis using ImageJ. The damaged area was manually outlined in ImageJ as to include areas where only epidermis remained. The unprocessed image can be seen in Fig. 12, B. The bar chart depicts the total results from damaged area analysis. L*er*-0 control, n=7, x=15.4 SE= 4.9. L*er*-0 inoculated, n=8, x=9.7 SE=2.8. L*er*-0 control > L*er*-0 inoculated, P>0.05, not significant.

Pseudomonas infection experiment

Image analysis of the pseudomonas infected leaves performed using ImageJ revealed that *Bacillus* inoculated plants appeared to have suffered a less extensive chlorosis than control plants (Fig. 14). In the first replicate the un-inoculated plants had 7 % larger chlorosis area than the control. In the second replicate the corresponding figure was 10 % (Table 4). Important to note is that a necrotic or shrunk leaf might give a lower chlorotic portion due to measurement of total leaf area being underestimated.

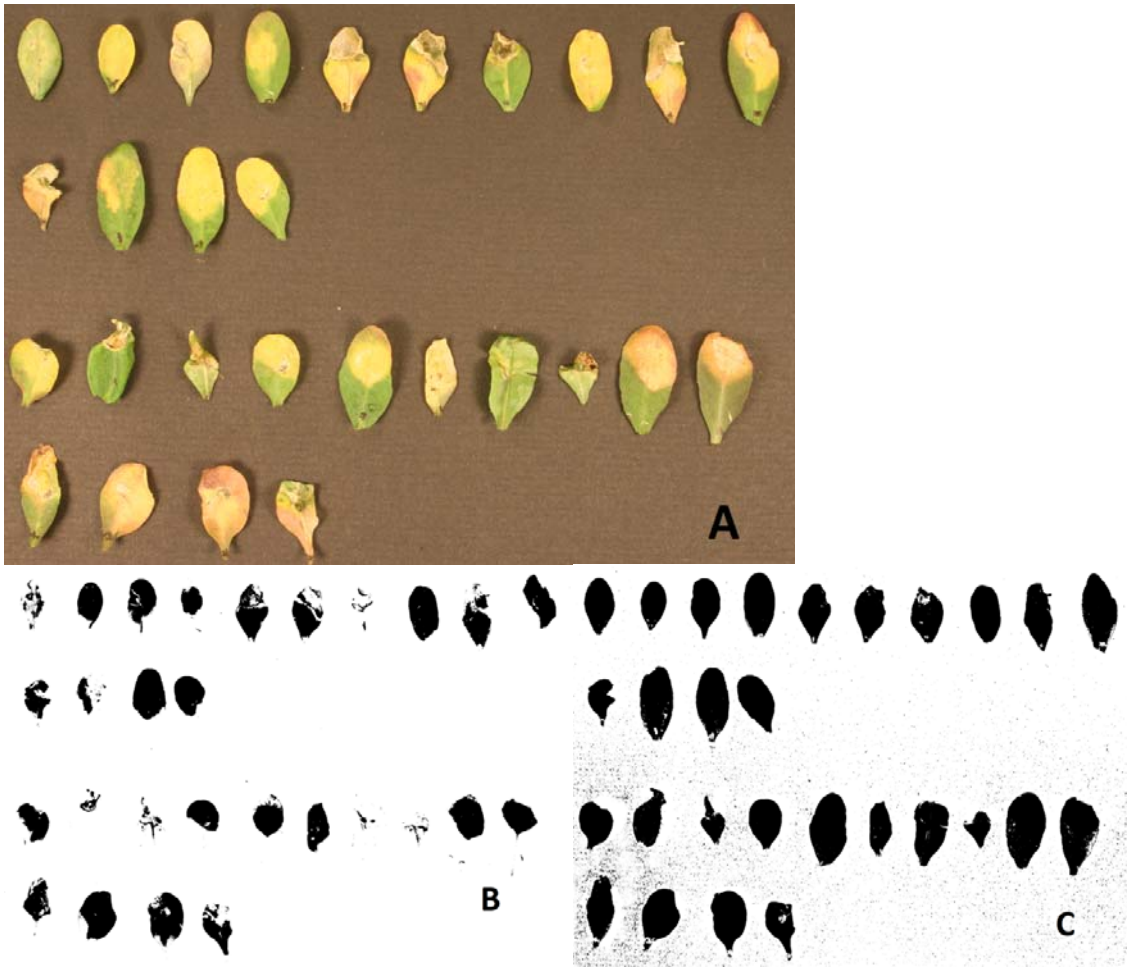

Fig. 14. (A) RGB color image illustrating L*er*-0 leaves 5 days after inoculation with *P. syringae.* The upper two rows of leaves are from control plants, while the lower are from *Bacillus* inoculated plants. (B) Threshold of image A, adjusted to include only chlorotic tissue. (C) Threshold adjusted to include whole leaf area.

Table 4. ImageJ analysis of chlorosis area.

Tray	Treatment	Chlorosis (mm^2)	Total leaf area (mm^2)	Chlorosis (%)
1	control	1312	2058	64
	inoculated	1182	2073	57
2	control	1705	2930	58
	inoculated	1123	2350	48

All LB plates inoculated with soil sample dilutions showed formation of white colonies after 24 h of incubation. Orange colonies, indicating *Bacillus* growth was spotted on all LB plates with samples from inoculated plants after 48 h of incubation. Three out of eight plates incubated with samples from control plants showed such colonies. *Bacillus* colonies were back calculated to the original concentration in the soil- samples (Fig. 15). Analysis of *Pseudomonas* levels in inoculated plants by qPCR showed highest abundance in one replicate of the control (Fig. 16).

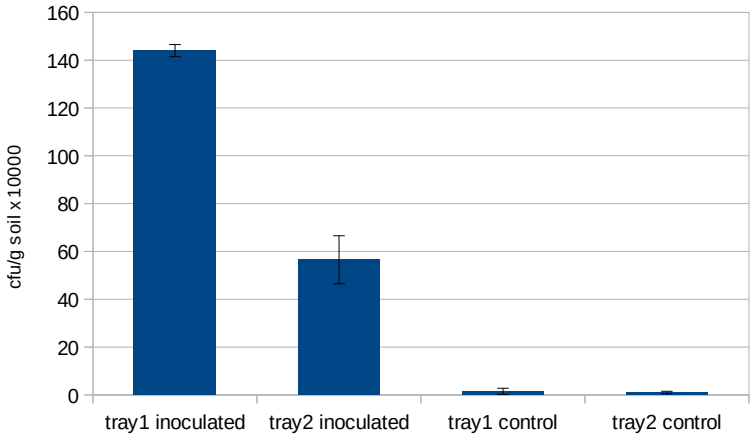

Fig. 15. Concentration of *Bacillus* colony forming units in the soil samples from the two trays of the pseudomonas experiments. Each group is the result of two soil samples, with each sample being incubated on two Petri dishes.

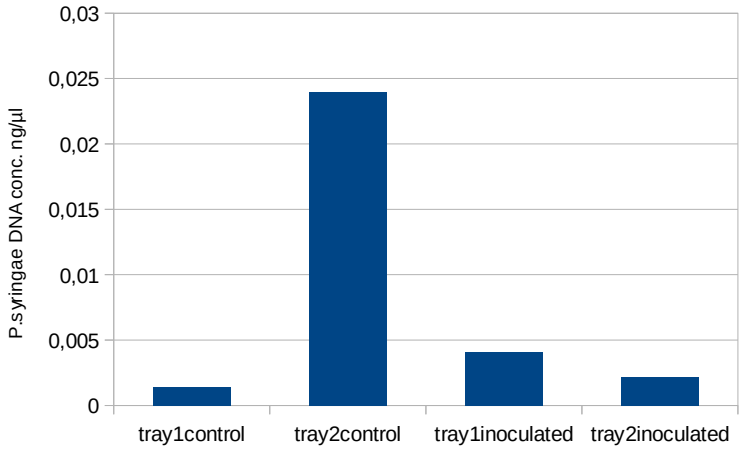

Fig. 16. Concentration of *P. syringae* DNA in sample extractions from infected L*er*-0 leaves. Values are for comparison between groups only.

Discussion

Non-choice experiment

No significant difference in larval weight could be detected between *Bacillus* inoculated and control plants of either L*er*-0 or Can-0 ecotypes. Based on this result, the main hypothesis could not be proven accurate if larval weight is assumed to correspond to plant damage. Backed by the visual damage assessment, larval weight seems to correlate well to at least plant defoliation.

Both non-choice experiments yielded a significantly higher larval weight of those *S. littoralis* larvae that had been feeding on the L*er*-0 ecotype in comparison to those who fed on Can-0, ($P>0.05$). This confirms results from a previous study (Markgren, 2012). With these two results in mind it becomes evident how important genetic traits are for successful plant defense against a generalist insect herbivore. The relative success of Can-0 in comparison to L*er*-0 in this particular experiment might derive from differences in composition of secondary metabolites such as glucosinolates between the two ecotypes. Difference in trichome density (Fig. 11) might also have contributed to this difference in susceptibility.

Although the results do indicate that there is no effect from the *B. amyloliquefaciens* mediated priming in defense capability towards the generalist herbivore *S. littoralis,* the results should be interpreted with caution. There are many factors that might have affected the outcome of the experiment. First and foremost the deviation in weight of larvae retrieved from the same cages suggests that not all *S. littoralis* have the same feeding preferences or rate. However this effect on the result could be addressed by using a large number of repeats.

A major concern for producing significant results in this experiment with the methodology used, was that any larva that was not retrieved for the weighing process was accounted for as having zero weight. This was done as missing larvae could be assumed being dead or escapees. Both of these scenarios would indicate an aversion to the substrate. Every missing larva would therefore make a great contribution to the deviation of the sample, rendering the test insignificant. If the missing larvae would have been excluded from the statistical analysis the result in the first replicate would have been significant for lower mean larval weight in *Bacillus* inoculated plants.

Another aspect to take into account is the possibility that the larvae may have been affected by the crowding induced by the limited space within the cages. According to a study performed using *Spodoptera exempta*, crowded larva show a more efficient feeding behavior than those kept in less dense populations (Simmonds & Blaney, 1986). A possible route for controlling such influences on the experiment could be to use big cages in an effort to keep population density below the critical level, or use small very small cages to ensure that all larvae enter this crowded feeding behavior.

The uneven light conditions experienced in the period between germination of the seeds until the relocation of plants to the experiment chamber might also have affected the result. The fluorescent light intensity could be twofold higher for a plant growing underneath the middle of the lamp armature, compared to one growing at the far edge of the same. As studies have shown that light intensity affects defense signaling (Karpinski *et al.*, 2003) chances are that this light effect may alter the defense signaling that might have been able to override the

priming effect. However, light conditions appear to affect mainly the SA pathway and not the JA pathway and hence the defenses against microbes might be more affected than the insect herbivory defense (Zeier *et al.*, 2004). The possible impact of uneven light could be addressed by shifting the plants positions regularly or by distributing plants that have been exposed to different light intensities evenly between experiment groups.

Yet another important circumstance to consider was that primed and unprimed plants were kept in trays next to each other in the same growth chamber, this makes it possible that volatile signaling substances from inoculated plants or the bacteria might affect unprimed adjacent plants.

Given that, some interesting observations were made. In the second non-choice feeding experiment, inoculated L*er*-0 plant was observed to flower earlier than control plants. This effect might be attributed to the growth promoting characteristics of *B. amyloliquefaciens* or a result of the light conditions. Observation of feeding patterns in Can-0 indicated that *S. littoralis* larvae prefer to feed from the tip of the oldest leaves. A preference for stressed leaves showing signs of chlorosis were seen. The preference for older leaves in the Can-0 ecotype would seem a bit perplexing since plants generally allocate recourses from older leaves to younger leaves. As a result one would expect lower nutritional quality in the older leaves. With that in mind, the feeding choice might be a result of lower trichrome density in comparison to younger leaves (Fig. 11) or have to do with glucosinolate levels and localization (Shroff *et al.*, 2008). In L*er*-0 no specific preference could be observed, other than initial avoidance of leaf edges and middle nerve. This coincides with discoveries suggesting that glucosinolates are more abundant in these structures (Shroff *et al.*, 2008)

Multiple choice experiment

This was a pilot experiment and was not constructed to produce significant data. The intent was rather to examine the possibility of detecting feeding preferences of *S. littoralis* when presented with both *Bacillus* treated and control plants, growing in soil. Under natural conditions generalist herbivores are likely to have alternate food sources to use if the current one does not suit their preferences. This theory was the main reason for conducting this experiment.

Studies have pointed out the importance of volatile substances in defense priming signaling pathways (Ryu *et al.*, 2004). This means that ideally a multiple choice experiment with primed and unprimed plants would have to control both gas exchange and root water exchange between plants, while still granting larvae unrestricted movement between plants. Such a setup would probably require some effort to complete, but a possible setup is depicted in Figure 17.

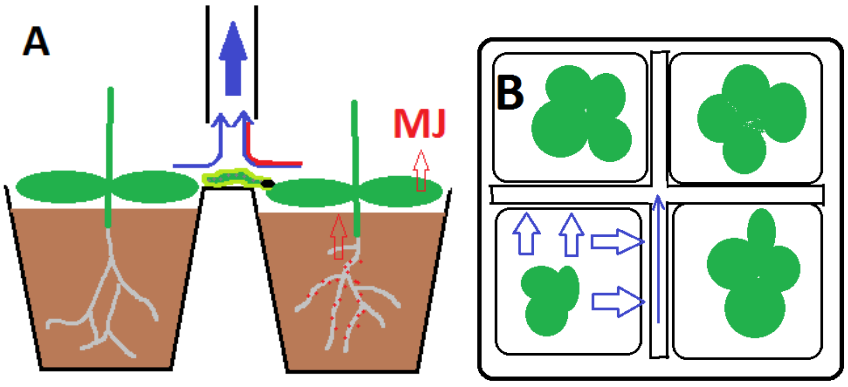

Fig. 17. (A) A possible experimental setup that could control the flow of volatile substances and contain soil bacteria, while still allowing for larval freedom of feeding choice. (B) Overhead view of the same.

As previously stated the plants in this experiment were suspended to restrict transfer of *Bacillus* between pots, and as a consequence the soil dried out quickly and had to be watered often. A solution to reduce this burden would be to use drip irrigation, which if properly used should not compromise the isolation of the bacteria.

Pseudomonas syringae infection experiment

The experiment was originally designed as an upscaled version of the multiple choice *Spodoptera* feeding experiment. However, after a failure in the shipment of *S. littoralis* eggs, the experiment was rapidly re-designed. As a *P. syringae* infection experiment the possible differences between primed and unprimed defenses was still tested. Visual damage assessment revealed that L*er*-0 was very susceptible to the DC3000 strain of *P. syringae.* The symptoms appeared severe for both treatments of L*er*-0 (Fig. 14) The image analysis indicated a very slight difference in chlorosis affected leaf area (Table 4), *Bacillus* inoculated plants having the lowest affected area. A qPCR analysis of *P. syringae* DNA in the inoculated leaves indicated that *P. syringae* was more abundant in the control plants than in the inoculated plants. This was however only the case in one of the experiment replicates (Fig. 16). These results would however have to be cautiously interpreted since there is no guaranty that the infiltration pattern and inoculum quantity were identical for all plants. A slight dilution error in the positive control is also thought to have occurred, which would render the quantification of DNA inaccurate.

The results from the soil samples revealed that the containment of the *Bacillus* bacteria to only the inoculated plants worked reasonably well (Fig. 15). Only three out of eight agar plates incubated for the control samples showed traces of *Bacillus* contamination. To facilitate containment further, an even more careful watering strategy could be used, and or the soil could be covered by fabric to prevent accidental transfer of soil between the pots. Another interesting finding was that the *Bacillus* was very slow to colonize the agar plates. This might indicate that they had formed spores. The formation of spores was probably a result of the soil being dry at times between the manual watering. None the less this raises the question if the spores are still capable of inducing a priming effect.

Future studies

Although the experiments in this report could not find evidence for the usefulness of *Bacillus* mediated priming in plant herbivory defense against this particular herbivore, some possibilities for a renewed approach to the topic was found. Alternative pathways for defense could be examined, since the *Bacillus* initiated JA pathway did not seem to produce an adequate end result in the generalist insect herbivore context. Furthermore some ideas for an alternative experiment setup that can be used to further explore the topic of *Bacillus* mediated priming was derived from the experiments.

References

Adesemoye, A., Torbert, H. & Kloepper, J. (2009). Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. *Microbial Ecology* 58, 921–929.

Ahmad, S., Hulten, M. van Martin, J., Pieterse, C. M. J., Wees, S. C. M. & van Ton, J. (2011). Genetic dissection of basal defence responsiveness in accessions of *Arabidopsis thaliana*. *Plant, Cell and Environment* 34, 1191–1206.

Alborn, H. T., Turlings, T. C. J., Jones, T. H., Stenhagen, G., Loughrin, J. H. & Tumlinson, J. H. (1997). An elicitor of plant volatiles from beet armyworm oral secretion. *Science* 276, 945–949.

Altstein, M., Ben-Aziz, O. & Gazit, Y. (1994). Pheromone biosynthesis activating neuropeptide (PBAN) and colour polymorphism: an immunochemical study in *Spodoptera littoralis*. *Journal of Insect Physiology* 40, 303–309.

Andréasson, E. (2000). Structural and functional studies of the myrosinase-glucosinolate system in *Arabidopsis thaliana* and *Brassica napus*. *Acta Universitatis Agriculturae Sueciae. Agraria* 208.

Ankeny, R. A. & Leonelli, S. (2011). What's so special about model organisms? *Studies In History and Philosophy of Science Part A* 42, 313–323.

Arnold, D. L., Lovell, H. C., Jackson, R. W. & Mansfield, J. W. (2011). *Pseudomonas syringae pv. phaseolicola*: from "has bean" to supermodel. *Molecular Plant Pathology* 12, 617–627.

Babalola, O. O. (2010). Beneficial bacteria of agricultural importance. *Biotechnology Letters* 32, 1559–1570.

Bais, H. P., Fall, R. & Vivanco, J. M. (2004). Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. *Plant Physiology* 134, 307–319.

Bayer crop science (2012). Crop compendium *Spodoptera littoralis*. Bayer cropscience. Available from: http://compendium.bayercropscience.com/BAYER/CropScience/CropCompendium/BCSCropComp.nsf/id/EN_Spodoptera_littoralis?open&ccm=200010. [Accessed 2012-05-06].

Bender, C. L., Alarcón-Chaidez, F. & Gross, D. C. (1999). *Pseudomonas syringae* Phytotoxins: Mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiology and Molecular Biology Reviews* 63, 266–292.

Bodenhausen, N. & Reymond, P. (2007). Signaling pathways controlling induced resistance to insect herbivores in *Arabidopsis*. *Molecular Plant-Microbe Interactions* 20, 1406–1420.

Brooks, D. M., Bender, C. L. & Kunkel, B. N. (2005). The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. *Molecular Plant Pathology* 6, 629–639.

Compant, S., Duffy, B., Nowak, J., Clément, C. & Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology* 71, 4951–4959.

Conrath, U., Beckers, G. J. M., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., Newman, M.-A., Pieterse, C. M. J., Poinssot, B., Pozo, M. J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L. & Mauch-Mani, B. (2006). Priming: getting ready for battle. *Molecular Plant-Microbe Interactions* 19, 1062–1071.

Consales, F., Schweizer, F., Erb, M., Gouhier-Darimont, C., Bodenhausen, N., Bruessow, F., Sobhy, I. & Reymond, P. (2011). Insect oral secretions suppress wound-induced responses in *Arabidopsis*. *Journal of Experimental Botany* 63, 727-737.

Cui, J., Bahrami, A. K., Pringle, E. G., Hernandez-Guzman, G., Bender, C. L., Pierce, N. E. & Ausubel, F. M. (2005). *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores. *Proceedings of the National Academy of Sciences of the United States of America* 102, 1791–1796.

Danielsson, J., Reva, O. & Meijer, J. (2007). Protection of oilseed rape (*Brassica napus*) toward fungal pathogens by strains of plant-associated *Bacillus amyloliquefaciens*. *Microbial Ecology* 54, 134–140.

EPPO (2012). Data Sheets on Quarantine Pests *Spodoptera littoralis* and *Spodoptera litura*. European and Mediterranean Plant Protection Organization. Available from: http://www.eppo.int/QUARANTINE/insects/Spodoptera_litura/PRODLI_ds.pdf. [Accessed 2012-05-06].

Farmer, E. E. & Ryan, C. A. (1990). Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proceedings of the National Academy of Sciences of the United States of America* 87, 7713–7716.

Fravel, D. R. (2005). Commercialization and implementation of biocontrol 1. *Annual Review of Phytopathology* 43, 337–359.

Frost, C. J., Mescher, M. C., Carlson, J. E., Moraes, D. & M, C. (2008). Plant defense priming against herbivores: getting ready for a different battle. *Plant Physiology* 146, 818–824.

Gerhardson, B. (2002). Biological substitutes for pesticides. *Trends in Biotechnology* 20, 338–343.

Halkier, B. A. & Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. *Annual Review of Plant Biology* 57, 303–333.

Hayes, J., Kelleher, M. & Eggleston, I. (2008). The cancer chemopreventive actions of phytochemicals derived from glucosinolates. *European Journal of Nutrition* 47, 73–88.

Heil, M. (2009). Damaged-self recognition in plant herbivore defence. *Trends in Plant Science* 14, 356–363.

Howe, G. A. & Jander, G. (2008). Plant immunity to insect herbivores. *Annual Review of Plant Biology* 59, 41–66.

van Hulten, M., Pelser, M., Loon, V., C, L., Pieterse, C. M. J. & Ton, J. (2006). Costs and benefits of priming for defense in *Arabidopsis*. *Proceedings of the National Academy of Sciences* 103, 5602–5607.

Jakab, G., Cottier, V., Toquin, V., Rigoli, G., Zimmerli, L., Métraux, J.-P. & Mauch-Mani, B. (2001). β-Aminobutyric acid-induced resistance in plants. *European Journal of Plant Pathology* 107, 29–37.

Jaskiewicz, M., Conrath, U. & Peterhänsel, C. (2011). Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Reports* 12, 50–55.

Karban, R. & Baldwin, I. T. (1997). Induced Responses to Herbivory. University of Chicago Press. Chicago, pp. 326.

Karpinski, S., Gabrys, H., Mateo, A., Karpinska, B. & Mullineaux, P. M. (2003). Light perception in plant disease defence signalling. *Current Opinion in Plant Biology* 6, 390–396.

Kessler, A., Halitschke, R. & Baldwin, I. T. (2004). Silencing the jasmonate cascade: induced plant defenses and insect populations. *Science* 305, 665–668.

Koroleva, O. A. & Cramer, R. (2011). Single-cell proteomic analysis of glucosinolate-rich S-cells in *Arabidopsis thaliana*. *Methods* 54, 413–423.

Kroymann, J., Donnerhacke, S., Schnabelrauch, D. & Mitchell-Olds, T. (2003). Evolutionary dynamics of an *Arabidopsis* insect resistance quantitative trait locus. *Proceedings of the National Academy of Sciences of the United States of America* 100(Suppl 2), 14587–14592.

Lucy, M., Reed, E. & Glick, B. R. (2004). Applications of free living plant growth-promoting rhizobacteria. *Antonie Van Leeuwenhoek* 86, 1–25.

Markgren, J. (2012). Plant defensive mechanisms against insect pests. Swedish university of agricultural sciences, faculty of natural resources and agricultural sciences, department of plant biology and forest genetics. ISSN: 1651-5196.

Matthes, M., Bruce, T., Chamberlain, K., Pickett, J. & Napier, J. (2011). Emerging roles in plant defense for cis-jasmone-induced cytochrome P450 CYP81D11. *Plant Signaling & Behavior* 6, 563–565.

Mithöfer, A. & Boland, W. (2008). Recognition of herbivory-associated molecular patterns. *Plant Physiology* 146(3), 825–831.

Mithöfer, A., Wanner, G. & Boland, W. (2005). Effects of feeding *Spodoptera Littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiology* 137, 1160–1168.

Paré, P. W. & Tumlinson, J. H. (1999). Plant volatiles as a defense against insect herbivores. *Plant Physiology* 121, 325–332.

Pieterse, C. M. (2009). Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology* 5, 308–316.

Ryu, C.-M., Farag, M. A., Hu, C.-H., Reddy, M. S., Kloepper, J. W. & Paré, P. W. (2004). Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiology* 134, 1017–1026.

Sarsby, J., Towers, M. W., Stain, C., Cramer, R. & Koroleva, O. A. (2012). Mass spectrometry imaging of glucosinolates in *Arabidopsis* flowers and siliques. *Phytochemistry* 77, 110–118.

Shroff, R., Vergara, F., Muck, A., Svatoš, A. & Gershenzon, J. (2008). Nonuniform distribution of glucosinolates in *Arabidopsis thaliana* leaves has important consequences for plant defense. *Proceedings of the National Academy of Sciences* 105, 6196–6201.

Simmonds, M. S. J. & Blaney, W. M. (1986). Effects of rearing density on development and feeding behaviour in larvae of *Spodoptera exempta*. *Journal of Insect Physiology* 32, 1043–1053.

Tripathi, M. K. & Mishra, A. S. (2007). Glucosinolates in animal nutrition: A review. *Animal Feed Science and Technology* 132, 1–27.
Wentzell, A. M. & Kliebenstein, D. J. (2008). Genotype, age, tissue, and environment regulate the structural outcome of glucosinolate activation. *Plant Physiology* 147, 415–428.
Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany* 52, 487–511.
Winde, I. & Wittstock, U. (2011). Insect herbivore counteradaptations to the plant glucosinolate-myrosinase system. *Phytochemistry* 72, 1566–1575.
Wu, J. & Baldwin, I. T. (2010). New insights into plant responses to the attack from insect herbivores. *Annual Review of Genetics* 44, 1–24.
Yamaguchi, Y. & Huffaker, A. (2011). Endogenous peptide elicitors in higher plants. *Current Opinion in Plant Biology* 14, 351–357.
Zeier, J., Pink, B., Mueller, M. & Berger, S. (2004). Light conditions influence specific defence responses in incompatible plant pathogen interactions: uncoupling systemic resistance from salicylic acid and PR-1 accumulation. *Planta* 219. 673-683.

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