Effect of aliphatic glucosinolate concentrations in *Arabidopsis thaliana* on multi-trophic interactions

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Abstract

The cabbage aphid Brevicoryne brassicae sequesters glucosinolates from its host plant. Endogenously present myrosinase enzymes in the aphid body can hydrolyze the accumulated glucosinolates upon attack by natural enemies. The aim of experiment was to unravel the impact of aliphatic glucosinolates on multitrophic interactions including Arabidopsis thaliana, B. brassicae and two natural enemies. A generalist predator Episyrphus balteatus and an aphid parasitoid Diaeretiella rapae were reared on B. brassicae that were reared on different wild accessions of A. thaliana (Col-0-WT, Cvi-WT, Eri-WT) that differ naturally in their glucosinolate content, and one transgenic line with over-expression of aliphatic glucosinolates. Aphids reared on the different accessions differed in their glucosinolate concentrations, as found in previous experiments. Performance of both natural enemies, when fed with or developing inside B. brassicae reared on the different plant accessions, was tested. Simultaneously, preference of E. balteatus and D. rapae for an accession was tested. Survival rate of E. balteatus was low (17-40%), survival rate of D. rapae was high (56-76%). Slower development of E. balteatus was observed when larvae were fed aphids which were reared on Cvi-WT and contained the highest glucosinolate concentration. Thus, glucosinolate concentrations in the prey seemed to have a negative effect on the development of E. balteatus. However, no differences in other parameters (survival, sex ratio and adult dry weight) among the host-plant accessions were observed.

There were no differences in survival or development time among D. rapae wasps developing in hosts reared on the different A. thaliana accessions. A higher adult dry weight of D. rapae was observed when its host aphid was reared on Cvi-WT. Aphids were larger, but contained higher glucosinolate concentrations on this plant accession. Performance of the wasp was therefore depending more on aphid performance (=size) than glucosinolate concentrations in the prey.

Due to a low number of replications of the preference tests and a lack of information on volatile blends, we cannot make strong conclusions on the preference of natural enemies for one of the accessions.

Based on our results, sequestration of glucosinolate by B. brassicae seems only effective against generalist predators, and not against specialist parasitoids.

Key words: Brevicoryne brassicae, parasitoid, predator, performance, preference, sequestering
Introduction

Plant resistance against herbivores

Plants are suffering from many attackers that chew on their tissues, suck up their cell contents or damage their cells otherwise. To overcome these problems plants have constitutive as well as induced resistance mechanisms. In constitutive resistance, morphological structures and chemical compounds are involved. Trichomes and epicuticular waxes could directly hinder the activity of herbivores and are categorised as morphological resistance. Moreover, plants contain hundreds of different chemicals compounds. Some of these play significant roles in resistance against their herbivores. It is believed that chemical compounds like phenols, glucosinolates and cyanogenic glycosides negatively affect the physiology of herbivores (Schoonhoven et al., 2005). In addition to this direct resistance that directly targets the herbivores, plants have indirect resistance against herbivores, in which they recruit natural enemies (predators and parasitoids) of herbivores not only by providing domatia for shelter and extra floral nectaries for food (Schoonhoven et al., 2005) but also by emitting volatiles cues (Dicke & Baldwin, 2010).

The plant family Brassicaceae contains many important crop species such as Brussels sprouts, cabbage and cauliflower that are a main food source worldwide. Besides being important crop plants, Brassica plants are used as a trap crop to reduce the main crop's infestation by insect pests as much as possible (Ahuja et al., 2010). According to Cook et al., (2006) turnip rape showed good potential as a trap crop for oilseed rapes pest, especially for pollen beetles. Furthermore, because of their glucosinolates (specific secondary metabolites) content Brassica plants are used in bio-fumigation. In bio-fumigation, the crop residues are incorporated in the soil and breakdown product of the glucosinolates, principally isothiocyanates affect the soil inhabiting organisms, such as fungal pathogens (Angus et al., 1994).

Glucosinolates

Plants belonging to the Brassicaceae have unique secondary metabolites called glucosinolates. The basic structure of all glucosinolates consists of β-thioglucose moiety, a sulfonated oxime moiety and a variable side chain. Depending upon the amino acid precursor of the side chain there are three main structural groups of glucosinolates; the aliphatic glucosinolate (derived from methionine), indole glucosinolate (derived from tryptophan) and aromatic glucosinolate (derived from phenylalanine or tyrosine) (Hopkins et al., 2009). Glucosinolates may confer resistance to insect herbivores (Kim and Jander, 2007) which is enhanced after hydrolysis by myrosinase enzymes. Myrosinases are hydrolysing enzymes which are stored in myrosin cell (Rask et al., 2000). Normally, glucosinolates and myrosinase are stored in separate compartment of the plant cell. When plant tissues are damaged by herbivores the glucosinolates and myrosinase come into contact. Consequently, myrosinase hydrolyses the glucosinolates and several toxic compounds such as nitriles and isothiocyanates are produced. Glucosinolates are in general toxic to generalist herbivores but not to specialist herbivores that have evolved mechanisms to overcome the glucosinolates-myrosinase system. For example, the cabbage specialist Pieris rapae has nitriles-specifying proteins that have ability to form nitriles (less toxic) than isothiocyanates (Wittstock et al., 2004). Furthermore, glucosinolates breakdown products are utilized as oviposition and feeding stimulants by these specialists herbivores (Kliebenstein et al., 2001 a).
*Brassica* populations have different concentrations of glucosinolates, which influence the development time and adult body mass of the specialist parasitoid *Diadegma semiclausum* (Gols et al., 2008). However, about the effect of the glucosinolate-myrosinase system on parasitoids is not much known (Polaszek, 1986; Kazana et al., 2007).

Some specialist herbivores even sequester glucosinolates from their host plants and store these in their own body. These stored products might be toxic to the natural enemies of these herbivores upon attack (Kazana et al., 2007). The cabbage aphid *Brevicoryne brassicae* is one of these glucosinolate-sequestering herbivores. It was found that when fed *B. brassicae* reared on host plants containing high levels of glucosinolates, larvae of the polyphagous ladybird *Adalia bipunctata* failed to complete development (Francis et al., 2001).

This study

Glucosinolates are recognized as a natural pesticide since they exhibit toxic or repellent effects against pest and diseases (Mithen, 1992). Furthermore, the concentration of glucosinolates increases in response to herbivore feeding. Nowadays, we can genetically modify plants to produce higher concentrations of glucosinolates, potentially leading to stronger resistance against herbivores. The higher level of glucosinolates can affect both generalist and specialist herbivores, but their natural enemies as well. Thus, in this study we test the effect of plants with higher aliphatic glucosinolate concentrations on an insect herbivore and its natural enemies. To test these effects, we use different wild accessions of the model plant *Arabidopsis thaliana* that naturally differ in their aliphatic glucosinolate concentrations, and one transgenic line of *A. thaliana* that has been genetically engineered to over-express aliphatic glucosinolates. We observe the performance and preference of the specialist parasitoid *Diaeretiella rapae* and the generalist predator *Episyrphus balteatus* feeding upon the *Brassica* specialist aphid *B. brassicae* reared on these different *A. thaliana* accessions.

*Arabidopsis thaliana* L. Brassicales : Brassicaceae

*Arabidopsis thaliana* is a small, annual winter plant found in most parts of the world and is gaining popularity among biological researches as a model plant. This plant germinates in autumn and flowers in spring. *Arabidopsis thaliana* is a member of the Brassicaceae family and contains glucosinolates. Between ecotypes (or accessions), there are large differences in glucosinolate profiles. For instance, leaves of accessions Landsberg erecta (Ler) contain primarily 3-hydroxypropyle and 8-methyl sulfinoctxyl glucosinolates whereas Cape Verdi Islands (Cvi) leaves dominantly contain allyl and 3-butenyl glucosinolates (Kliebenstein et al., 2001 b). If these compounds vary from plant to plants then ultimately the degradation products also differ. For example, accessions Wassilewskija (WS) and Columbia (Col; Lambricx et al., 2001) produce isothiocyanates, whereas Cvi and Ler produce epithionitrites and nitriles respectively (Kusnierczyk, 2007). Furthermore, young leaves and reproductive tissues such as siliquea and seeds contain higher concentrations than senescent leaves. Organs like roots, stems and leaves contain intermediate level of glucosinolate (Halkier & Gershenzon, 2006).

To gain plants with higher glucosinolate concentrations, we use the gene MYB28. In *Arabidopsis*, the gene MYB28 is already presents and responsible for regulation of the glucosinolate pathway. The expression of most genes involved in the aliphatic glucosinolate biosynthesis is regulated by MYB28 (Hirai et al., 2007). After transformation of MYB28 to *A.
*Arabidopsis thaliana*, plants have two sets of this gene, causing over-expression of the gene and higher production of glucosinolate than wild type plants.

**The cabbage aphid (Brevicoryne brassicae L. Hemiptera : Aphididae)**

Almost 4700 species of aphids have been identified throughout the world (Blackman and Eastop, 2007). They are categorised as one of the important pests in crops. Due to the unique nature of their reproduction they can multiply sexually as well as asexually and individuals are either winged (alatea) or wingless (apterae) depending upon the situation (Dixon, 1977). This feature allows aphids to switch to another host plant, to escape from over crowded areas and to avoid parasitisation (Dixon, 1977).

Aphids have piercing-sucking mouth-parts with a long slender stylet. The aphid stylet penetrates the plant epidermis to find phloem and aphids can suck up phloem sap from sieve elements for a long period of time (de Vos et al., 2007). They secrete saliva to prevent stylet and phloem clogging. Due to excessive aphid feeding plants can show chlorosis, necrosis and leaf curling (Goggin, 2007). Aphid species that feed on many different host plants are called generalists, such as the green peach aphid *Myzus persicae*. Species that only feed on a certain plant family are called specialists, such as the cabbage aphid *B. brassicae* that feeds only on Brassicaceae plants and forms large colonies on plants with high glucosinolate concentrations (Cole, 1997; de Vos et al., 2007).

Although glucosinolates are considered as defensive compounds against various kinds of attackers in Brassicaceae plants, *B. brassicae* exploits these compounds for its own resistance against its natural enemies (Kazana et al., 2007). *Brevicoryne brassicae* sequesters glucosinolates from the host plant and stores the compounds in its haemolymph. The concentration of glucosinolates in the aphid body was found to be 15-20 times higher than the concentration in the leaf tissue (Hopkins et al., 2009). Furthermore, this aphid has an endogenous myrosinase enzyme that is spatially separated from the sequestered glucosinolates. After damage by natural enemies, the glucosinolates come into contact with the myrosinase, and toxic breakdown products are formed (Kazana et al., 2007). *Brevicoryne brassicae* was found to sequester glucosinolates selectively from the host plant’s phloem: aliphatic glucosinolates were sequestered more than indole glucosinolates, which were almost not sequestered by the aphid (Kos et al., 2010).

**The marmalade hoverfly (Episyrphus balteatus De Geer, Diptera: Syrphidae)**

The hoverfly *E. balteatus* is a solitary predator that is found in meadows, heath land and moorland. In Central Europe, it is considered as the most abundant natural enemy in agro-ecosystems and natural habitats (Tenhumberg and Poehling, 1995). Generally, adult hoverflies feed on flower nectar and pollen whereas larvae feed on aphids. Hoverfly larvae have been used as effective biological control agents of aphids. Hoverfly larvae are voracious feeders in which a single larva can consume more than two hundred aphids in its larval period (Tinkeu and Hance, 1998). Furthermore, *E. balteatus* larvae have sclerites which are inserted into the cuticle of the prey and suck up the haemolymph completely (Tinkeu and Hance, 1998).

Due to limited mobility of hoverfly larvae adult females have to make sure to lay eggs on those plants which have an assured supply of food for their offspring. Gravid female prefer to lay eggs on plants having a large number of aphid colonies (Scholz and Poehling, 2000; Almohamad et al., 2007).
As discussed before, the aphid *B. brassicae* sequesters glucosinolates from its host plant and stores these glucosinolates in its body (Kazana et al., 2007). The fitness and reproduction rate of hoverflies was more strongly affected when fed with *B. brassicae* reared on plants that contain high glucosinolate concentrations (e.g. *Sinapis alba*) than when fed with aphids reared on plants that contain low glucosinolate concentrations (e.g. *B. napus*) (Vanhaelen et al., 2002). Similarly, performance of *E. balteatus* was significantly higher when fed on non-sequestering *M. persicae* than fed *B. brassicae* and this was probably due to the high glucosinolate content of *B. brassicae* (Kos et al., 2010).

The aphid parasitoid (*Diaeretiella rapae* McIntosh, Hymenoptera: Braconidae)

The solitary parasitoid *D. rapae* can parasitize many aphid hosts but it is predominantly specialized on the Brassicaceae specialist herbivores *B. brassicae* and *Lipaphis erysimi* (Blande et al., 2007) and is seen as an effective bio-control agent for Brassicaceae aphids (Zhang and Hassan, 2003). *Diaeretiella rapae* lays its eggs into aphids and after hatching the larvae feed on the aphid from the inside. After the larvae consumed the aphid only the aphids’ integument is remaining: this hard shell is called a mummy and contains the parasitoid pupae. The longevity of adults is influenced by the environmental condition. According to Bernal and Gonzalez (1997) the longevity of adult *D. rapae* at 21.1 °C was 11.50 days for females and 8.64 days for males.

*Diaeretiella rapae* is able to locate its host with the help of compounds emitted by damaged plants and aphids themselves. Kairomones released from the cuticle, cornicle secretion and aphids’ honeydew have been shown to be important cues for locating hosts by *D. rapae* (Bradburne and Mithen, 2000). Furthermore, the electroantennogram responses indicated that *D. rapae* was significantly responding towards the volatile compounds isothiocyanates and to, although less strongly, nitriles and epithionitriles (Pope et al., 2008).

Recent research revealed that *D. rapae* performance traits such as egg to mummy development time, adult longevity and sex ratio were not influenced by glucosinolates concentrations in the host, *B. brassicae*. This was found in a study which *B. brassicae* was reared on different *Brassica* species: *B. napus*, *B. oleracea*, *B. nigra* and *S. arvensis* (Le Guigo et al., 2010). Interestingly, glucosinolate concentrations in *B. nigra* and *S. arvensis* were substantially higher than the concentrations in *B. napus* and *B. oleracea*, and *D. rapae* therefore seemed not to be affected by glucosinolate concentrations of its host. (Le Guigo et al., 2010).

Research questions

We have formulated the following research questions and hypotheses to test the effect of aliphatic glucosinolates on a tritrophic system including *A. thaliana*, *B. brassicae* and two natural enemies.

1) Does the difference in aliphatic glucosinolate concentrations between aphids reared on different *Arabidopsis* accessions lead to a difference in performance of the aphid parasitoid *D. rapae* and the aphid predator *E. balteatus*?

Hypothesis: The performance of the parasitoid *D. rapae* is not affected by the different concentrations of aliphatic glucosinolates in the host aphid, because they are specialist parasitoids. In contrast, *E. balteatus* is a generalist predator and we expect that its performance is negatively correlated with higher levels of aliphatic glucosinolates in the prey aphid.
2) Can female *D. rapae* and *E. balteatus* discriminate between *Arabidopsis* accessions with different concentrations of aliphatic glucosinolates and do they prefer the plants on which their offspring performs best?

Hypothesis: Females of *D. rapae* are attracted towards plants with high aliphatic glucosinolate concentrations, whereas *E. balteatus* lays more eggs on plants containing low aliphatic glucosinolate concentrations. You did not answer your second question about if they prefer plants on which their offspring performs best.

As we discussed before, *B. brassicae* accumulates glucosinolates from its host plant. Previous studies have provided us with data on glucosinolates concentrations in *B. brassicae* (Figure 1) and the average body weight of *B. brassicae* (Figure 2) that were reared on different accessions of *A. thaliana* (Wietsma, 2010). Glucosinolate concentrations in *B. brassicae* that were reared on Col-0-MYB plants is still being analyze at the moment but that we expect that it is about twice higher than in Col-0-WT.

![Figure 1: Glucosinolate concentrations in *B. brassicae* that were reared on different accessions of *A. thaliana*. Bars (mean ± SE) within each glucosinolate group (indole, aliphatic or total), bars with the same letters are not significantly different between the accessions at *P* = 0.05.](image1)

![Figure 2: Weight of *B. brassicae* adults that were reared on different accessions of *A. thaliana*. Bars (mean ± SE) with the same letters are not significantly different between the accessions at *P* = 0.05.](image2)
Experimental set-up

Plant material

Three wild accessions of *Arabidopsis thaliana* (L.) and one genetically modified line were used for experiments. The wild accessions were Cape Verdi Islands (Cvi-WT), Eriengsboda (Eri-WT) and Columbia (Col-0-WT) and the genetically modified line had a Col-0-WT background. Genetic modification of Col-0-WT was performed by Benyamin Houshyani (Plant Physiology, Wageningen University). For making the transgenic line, the gene MYB28 was transferred in Col-0-WT plants. In *Arabidopsis*, the gene MYB28 is predominantly involved in regulating aliphatic glucosinolates production. Furthermore, to allow for selection of transgenic plant, we inserted a gene for resistance against the antibiotic kanamycin. A pathogenic bacterium, *Agrobacterium tumefaciens* was used for plant transformation. The *Agrobacterium* was disarmed so only the desired part of bacterium was transferred, but not pathogenic material. In the disarmed *Agrobacterium*, there were in this case two plasmids: one helper that contains genes for transfer of the material and one expression plasmid that contains the gene of interest. By use of electric shock, the desired gene (MYB28) was inserted into *Agrobacterium*. Thereafter, it was tested by PCR if the bacterium contained both plasmids. If positive, *Arabidopsis* plants were modified by dipping the young flowers in the *Agrobacterium* medium. The *Agrobacterium* then transferred the relevant part of the plasmids to the young embryo in the flowers, thus creating genetically modified embryos. The plants with the transgenic embryos were grown until seed production. Seeds of the transgenic line were sown on an agar medium supplied with antibiotics (kanamycin) to ensure that only transgenic plants could grow. Wild-type seeds of each of the three accessions were grown on the same medium, but without kanamycin. Two-week-old seedlings of each accession were transplanted to a plastic pot (5.5 cm height, 7 cm and 5 cm diameter on top and bottom respectively) filled with Lentse Potgrond and placed in a climate chamber at 21 ± 2 °C, RH 60% and 8:16 Light: Dark (L:D) photoperiod. Plants were watered three times a week and entomopathogenic nematodes (Koppert Biological Systems, Berkel en Rodenrijs) were added to the soil once a week to remove any larvae of sciarid flies that might be present. Six-week-old plants were used in experiments.

Insect rearing

Three insect species, the specialist cabbage aphid *B. brassicae*, the aphid parasitoid *D. rapae* and generalist predator *E. balteatus* were used for experiments. *Brevicoryne brassicae* was maintained on Brussels sprout (*Brassica oleracea* var. *gemmifera* cv. *Cyrus*). *Diaeretiella rapae* was reared on Brussels sprouts containing *B. brassicae* colonies. Honey and water were provided regularly in the rearing cage.

Pupae of *E. balteatus* were obtained from Koppert Biological System and reared in net cages (67×50×75 cm). Adults emerging from the pupae were supplied with bee-collected pollen (Koppert Biological System), organic sugar, water and a Brussels sprout plant infested by *B. brassicae*.

All insects species were maintained separately either in a greenhouse compartment or a climate room at 21 ± 2 °C, RH 60% and 16:8 (L: D) photoperiod.
Performance test of *Diaeretiella rapae*

Approximately 20 adult aphids were released on six-week-old plants of four *Arabidopsis* accessions (Col-0-WT, Col-0-MYB28, Eri-WT, and Cvi-WT) and allowed to larviposit. After 24 hours, all adults were removed from the plants. The newborn nymphs were allowed to grow until the second instar (three days after removal of adults). At that moment, individual nymphs were collected and native female *D. rapae* of three days old were allowed to parasitize these nymphs individually. To ensure oviposition in each nymph each wasp was observed very keenly. Three parasitized nymphs were transferred to 22 six-week-old plants of each accession. Each plant was placed into a cylindrical plastic pot (13 cm length and 11 cm diameter) covered by a lid containing a gauze mesh and were kept in a climate chamber at 21 ± 2 °C, RH 60% and 16:8 (L:D) photoperiod. Plants were watered when needed. Upon formation of mummies (parasitoid pupae in the host integument), individual mummies were collected from each plant and transferred into a glass vial with a cotton plug. Upon emergence of the adult wasps, mummies were checked every two hours for adult emergence. Once the adult emerged, it was sexed and placed in a freezer (-18 ºC). Wasps were dried in stove (80 ºC) for 72 h and the dry weight was measured on microbalance (Sartorius Gem\textsuperscript{Plus} Model CP2P). Survival rate until the adult stage, sex, egg-to-adult development time and adult dry weight were measured.

Performance test of *Episyrphus balteatus*

Six-week-old plants the four *Arabidopsis* accessions (Col-0-WT, Col-0-MYB28, Cvi-WT, and Eri-WT) were used for the hoverfly performance test. One week before introduction of the predatory larvae, 35 plants of each accession were infested by 10 adult *B. brassicae* and transferred to a climate cell (21 ± 2 °C, 60% RH and 8:16 L:D photoperiod). After one week plants were transferred to long-day conditions (16:8 L:D photoperiod) and one neonate hoverfly larva was introduced to each aphid-infested plant. Survival of larvae was monitored and new aphids from the stock rearing were supplied if necessary. Upon pupa formation, the fresh weight of one-day-old pupae was measured on a microbalance and pupae were transferred to a petridish containing a small piece of Brussels sprouts leaf. Adult emergence was checked daily. Once the adult emerged it was sexed and placed in a freezer (-18 ºC). Adults were dried in a stove (80 ºC) for 72 h and dry weight was measured on a microbalance. Survival rate until the adult stage, sex, larva-to-adult development time, pupal fresh weight and adult dry weight were measured.

Preference test of *Diaeretiella rapae*

For testing the preference of *D. rapae* a Y-tube olfactometer was used (Figure 3). We tested whether 2-day-old female *D. rapae* made a choice between the different accessions. Bioassays were conducted in a dynamic air-flow Y-tube at 21 ± 2 °C using a fiber optic light source (32 Watt, Phillips) above the olfactometer. Pressurized air was filtered through a charcoal filter divided into equal two parts and each sub-flow was led through a glass container (5 liters) containing an odor source (four six-week-old plants of the specific accession each infested for three days by 100 nymphs of mixed ages). Equal volume (2 L min\textsuperscript{-1}) of air flow in each glass jar was regulated by flow meters. Subsequently, the odor flow was led to each of two arms of a glass
Y-tube olfactometer (diameter 3.5 cm). In order to detect the choice of wasp the olfactometer was divided into three different parts (Figure 3). The first part was marked 6 cm from the opening of Y-tube, called wasp released line. Seven centimeter onward from the wasp release point a virtual line was made on each arm of tube called first choice line. The third part was the decision line which was 4 cm beyond the first choice line. All glass parts were cleaned with hot tap water, rinsed with 70% ethanol and dried in an oven at 100 ºC for at least one hour before bioassays were performed. Plant pots were covered by aluminum foil before the experiment.

Parasitoids that were used in the bioassay were removed from the rearing cage as mummified aphids with the help of a vacuum pump and placed in a new cage containing water and honey. Only two-day-old, mated female adults were used in the experiments. Female parasitoids were given an oviposition experience one hour before the bioassay by transferring them to an aphid-infested Arabidopsis plant. For each plant combination that was tested, half of the wasps received their experience on the first plant accession and other half on the other plant accession.

Experienced female parasitoids were released individually into the Y-tube and their behavior was observed. We observed which arm of the Y-tube was chosen as a first choice (crossing the first choice line) and as a decision line (crossing the decision line and staying between the final and first choice line for at least 15 seconds (see Figure 3)). Wasps that did not cross the first choice line within 10 minutes or the decision line within 15 minutes were recorded as making ‘no choice’. The glass pots containing the odor source were switched after testing half of the selected number of parasitoids to eliminate any bias for one side of the set-up. Due to a lack of a sufficient number of plants, Col-0-MYB28 was excluded from the experiment.

Treatments for D. rapae preference test:
1) Col-0-WT vs Cvi-WT
2) Col-0-WT vs Eri-WT
3) Eri-WT vs Cvi-WT

Figure 3: Y-tube olfactometer set up.
Oviposition preference of *Episyrphus balteatus*

For oviposition preference of female *E. balteatus*, two choice tests were performed. Two-to-three-week old females were released in a net cage (30×30×30 cm) in a greenhouse compartment (21 ± 2 °C, 60% RH and 16:8 L:D photoperiod). Each net cage contained one six-week-old *Arabidopsis* plant of two different accessions. Plants were infested with approximately 100 *B. brassicae* of mixed nymphal instars three days prior to the experiment. Individual hoverfly females were allowed to lay eggs for 24 hours. Water and organic sugar were supplied to the adults. After 24 hours, the numbers of eggs on each plant was recorded.

Treatments for oviposition preference of hoverfly:
1) Col-0-WT vs Cvi-WT
2) Col-0-WT vs Eri-WT
3) Eri-WT vs Cvi-WT
4) Col-0-WT vs Col-0-MYB28
5) Cvi-WT vs Col-0-MYB28

Data analysis

Survival and sex-ratio of both natural enemies were analyzed by Generalized Linear Models in Genstat (binomial distribution, logit link function, dispersion fixed). Development time and weight were analyzed by ANOVA in SPSS 17.0 and mean separation was done by Tukey (multiple comparison-test) if the result was significant.

Data collected from the preference test of *D. rapae* were analyzed by using chi-square tests. Parasitoids that were recorded as ‘no choice’ were removed from the data set.

The number of eggs laid by *E. balteatus* from the preference experiments were analyzed by Wilcoxon signed rank matched pairs test in SPSS 17.0. Females that laid no eggs at all plant were removed from the data set.

For all statistical analyses the significance level was measured at $P = 0.05$. 

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Results

Episyrphus balteatus performance

Survival of Episyrphus balteatus

Episyrphus balteatus larvae were exposed to aphids (B. brassicae) that were reared on different accessions of A. thaliana. The survival of E. balteatus until the adult stage was not significantly affected (Logistic regression, deviance ratio = 1.44, P = 0.233; Figure 4) by the plant accession on which the aphids were reared. Nevertheless, survival until adult stage varied from 17 to 40% (Figure 4). Mortality of hoverflies was mainly observed in the larval stage, not in the pupal stage.

![Figure 4: Survival of E. balteatus from larval to adult stage, when fed B. brassicae that were reared on different accessions of A. thaliana. Bars (mean ± SE) with the same letter are not significantly different from each other at P = 0.05. Numbers on bar indicate number of adults.](image)

Development time, pupal weight and adult weight

Larval-to-adult development time of E. balteatus was significantly affected by the plant accessions on which the prey was reared, by sex of the adults and by the interaction between plant accessions and sex (Table 1). In general, males showed a longer development time than females, except on Eri-WT (Figure 5). In general, males and females developed slowest on the host-plant Cvi-WT, and fastest on the host-plant Eri-WT and Col-0-MYB28 (Figure 5).

![Figure 5: Larval-to-adult development time of E. balteatus males (grey bars) and females (white bars), when fed aphids (B. brassicae) that were reared on different accessions of A. thaliana. Bars (mean ± SE) with the same letter are not significantly different at P = 0.05 (Tukey multiple comparison test among means). Numbers on bar indicate number of adults.](image)
The fresh weight of *E. balteatus* pupa was not significantly affected by the plant accessions on which their prey was reared (Table 1, Figure 6). Similarly, adult dry weight was not affected by plant accessions (Table 1, Figure 7). Furthermore, males were heavier than females, irrespective of the prey host-plant (Figure 8; Table 1).

![Figure 6: Pupal fresh weight of *E. balteatus* when fed *B. brassicae* that were reared on different accessions of *A. thaliana*. Bars (mean ± SE) with the same letter are not significantly different from each other at *P* = 0.05 (Tukey multiple comparison test among means). Numbers on bars indicate number of pupae.](image)

![Figure 7: Adult dry mass of *E. balteatus* when fed *B. brassicae* that were reared on different accessions of *A. thaliana*. Bars (mean ± SE) with same letters are not significantly different from each other at *P* = 0.05 (Tukey multiple comparison test among means). Numbers on bars indicate total number of adults.](image)

The sex-ratio of adult *E. balteatus* was equal irrespective of the plant accession (Logistic regression, deviance ratio = 0.48, *P* = 0.69) where total females percentage was 58.33.

Table 1. Effect of plant accessions on performance traits of *Episyrphus balteatus*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Accession (d. f. = 3)</th>
<th>Sex (d. f. = 1)</th>
<th>Accession * Sex (d. f. = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>F</em></td>
<td><em>P</em></td>
<td><em>F</em></td>
</tr>
<tr>
<td>Development time (days)</td>
<td>19.838</td>
<td><em>&lt;0.001</em></td>
<td>8.006</td>
</tr>
<tr>
<td>Pupal fresh weight (mg)</td>
<td>0.677</td>
<td>0.573</td>
<td>nm</td>
</tr>
<tr>
<td>Adult dry weight (mg)</td>
<td>1.193</td>
<td>0.330</td>
<td>8.787</td>
</tr>
</tbody>
</table>

*a* *F* = ratio of mean sum of square and error mean sum of square.

*b* *P* < 0.05 shows significant effect (ANOVA) and significant *P* values are in bold case.

*c* nm = not measured because sex of the pupae could not be determined.


**Diaeretiella rapae** performance

Survival of *Diaeretiella rapae*

*Diaeretiella rapae* were allowed to parasitize *B. brassicae* aphids that were reared on different accessions of *A. thaliana*. Survival rate of the wasps from egg to adult stage was not significantly affected (Logistic regression, deviance ratio = 2.06, $P = 0.112$; Figure 9) by the type of host-plant on which its host was reared.

![Figure 8: Adult dry weight of male and female of *E. balteatus* when fed *B. brassicae* that were reared on different accessions of *A. thaliana*. Bars (mean ± SE) with different letters are significantly different from each other at $P = 0.05$. Numbers on bars indicate total number of adults.](image1)

![Figure 9: Percentage survival of *D. rapae*, when reared from the host *B. brassicae* fed on different accessions of *A. thaliana*. Bars (mean ± SE) with same letters are not significantly different from each other at $P = 0.05$. Numbers on bars indicate total number of adults.](image2)
Egg-to-adult development time and adult dry weight

Egg-to-adult development time of *D. rapae* was not significantly different among host-plant accessions (Table 2; Figure 10 A). However, host-plant accession had a significant impact on adult dry weight (Table 2; Figure 10 B). The heaviest adults were recorded on accession Cvi-WT.

Figure 10: Egg-to-adult development time (A) and adult dry weight (B) of *D. rapae* reared from the host *B. brassicae* that were reared on different accessions of *A. thaliana*. Bars (mean ± SE) with same lower case letters are not significantly different from each other at *P* = 0.05 (Tukey multiple comparison test among means). Numbers on bars indicate number of adults.

A higher number of males (n = 142) emerged than females (n = 90) (Logistic regression, deviance ratio = 4.68, *P* = 0.005). The percentage of females was significantly influenced by the *B. brassicae* host plant (Figure 11).

Figure 11: Percentage of female *D. rapae* reared from the host *B. brassicae* that were reared on different accessions of *A. thaliana*. Bars with different lower case letters are significantly different from each other at *P* = 0.05. Numbers inside bars represent the total number of females.
Adult dry weight and egg-to-adult development time (Table 2; Figure 12 A and B) were not significantly different between males and females. Moreover, there was no interaction between plant accessions and sex of adults for adult dry weight or development time of *D. rapae* (Table 2).

![Figure 12: Egg-to-adult development time (A) adult dry weight (B) of (male and female) *D. rapae* reared from host *B. brassicae* that were reared on different accessions of *A. thaliana*. Bars (mean ± SE) with same lower case letters are not significantly different from each other at *P* = 0.05. Numbers inside bars represent the total number of adults.](image)

**Table 2. Effect of plant accession on performance traits of *Diaeretiella rapae***

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Accession (d. f. = 3)</th>
<th>Sex (d. f. = 1)</th>
<th>Accession * sex (d. f. = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>F</em>²</td>
<td><em>P</em></td>
<td><em>F</em></td>
</tr>
<tr>
<td>Development time (days)</td>
<td>1.309</td>
<td>0.272</td>
<td>3.181</td>
</tr>
<tr>
<td>Adult dry weight (mg)</td>
<td>10.694</td>
<td><strong>0.001</strong></td>
<td>2.405</td>
</tr>
</tbody>
</table>

*a* *F* = ratio of mean sum of square and error mean sum of square.

*b* *P* < 0.05 shows significant effect (ANOVA) and significant *P* values are in bold case.
**Episyrphus balteatus** oviposition preference

Female *E. balteatus* displayed no preference, as measured in the number of eggs laid on a plant, for a certain *A. thaliana* accessions (Figure 13).

![Figure 13: Oviposition preference of *E. balteatus* to different combinations of *A. thaliana* accessions. Each pair of *A. thaliana* accessions represents one treatment set. Comparisons were made only within each treatment set (Wilcoxon Signed Ranks Test), while "n" represents the number of replicates for each combination. Plant combinations without eggs were excluded from the data analysis.](image)

**Diaeretiella rapae** preference

Significantly more female *D. rapae* selected the odor source from *B. brassicae* infested Col-0-WT than from Cvi-WT (*P = 0.02*; Figure 14). However, responses to other odor source combinations (either Col-0-WT vs Eri-WT [*P = 0.19*] or Cvi-WT vs Eri-WT [*P = 0.41*]) were not significantly different (Figure 14).

![Figure 14: Attraction of female *D. rapae* towards different odor combinations from *A. thaliana* infested by *B. brassicae*. * indicates significant difference and "ns" indicates no significant difference at *P = 0.05* (Binomial distribution probability test) within a combination. Numbers on bars indicate number of wasps selecting this odor source. Numbers of non-responding wasp are indicated on the right-side.](image)
Discussion

Effect of aliphatic glucosinolates on the generalist predator Episyrphus balteatus

Performance of Episyrphus balteatus

The survival rate of the generalist predator *E. balteatus* until the adult stage was not significantly affected by the plant accessions on which its prey was reared. However, survival varied from 17-40%, and was lowest on the accessions on which aphids contained the highest concentrations of glucosinolates (Cvi-WT, Figure 1). When we compare the survival of *E. balteatus* from this study with the survival of this species when fed *B. brassicae* reared on *B. oleracea* cultivars (on average 50%, Kos et al., 2010), the survival observed in our study is low. This could be due to the glucosinolate concentration in aphids reared on *A. thaliana* that was several times higher than in aphids reared on *B. oleracea* (Kos et al., 2010; Wietsma, 2010).

The concentration of glucosinolate in *B. brassicae* was more than 10 times higher than in the leaves of its host plant, and several hundreds of times higher than in the phloem sap of its host plant (Wietsma, 2010), in agreement with other studies (Hopkins et al., 2009; Kos et al., 2010). This is the result of sequestration of glucosinolate by the aphid. Myrosinases are also present in the aphid body, where they hydrolyze glucosinolates upon damage by carnivores to produce compounds like isothiocyanates which are toxic to predators (Kazana, 2007; Francis et al., 2002; Jones et al., 2002). The aphids contained mainly aliphatic glucosinolates (Figure 1), such as glucoraphanin, gluconapin and glucoiberin (Wietsma, 2010).

Survival of *Adalia bipunctata* larvae reared on *B. brassicae* was also negatively correlated with glucosinolates concentration in the host plant (Francis, et al., 2000). Moreover, Kos et al., (2010) also reported higher larval mortality of *E. balteatus* fed with the glucosinolate sequestering aphid (*B. brassicae*) as compared to the non-sequestering one (*M. persicae*) on *B. oleracea* plants. Similarly, in one experiment all *E. balteatus* larval mortality was observed that were exposed to high concentrations of allyl-isothiocyanates, survived (Vanhaelen et al., 2001).

Larval-to-adult development time of *E. balteatus* was longer when prey was reared on Cvi-WT, on which aphids contained substantially higher concentrations of aliphatic glucosinolates than on the other plant accessions (Figure 1). Although hoverflies contain the glutathione-s-transferase (GST) enzyme, which can detoxify products of the glucosinolate-myrosinase system, the activity of this enzyme is relatively low in second and third larval stages as compared to pupal and adult stages (Vanhaelen et al., 2001). Therefore, we believe that larval-to-adult development time of *E. balteatus* was higher on prey reared on Cvi-WT because of the higher concentrations of glucosinolate in the prey that was reared on this host plant.

Fitness parameters such as adult dry weight and sex ratio of *E. balteatus* were not influenced by different plant accessions on which the prey was reared. Performance, in terms of survival (although not significantly different) and development time, was lowest on Cvi-WT, on which aphids contained highest glucosinolate concentrations. We therefore believe, in agreement with our hypothesis, that higher aliphatic glucosinolate concentrations negatively affected hoverfly performance. However, performance of *E. balteatus* did not correlate completely with aphid glucosinolate concentrations. Hoverfly performance was higher on Eri-WT than on Col-0-WT, whereas aphids contained higher glucosinolate concentrations on Eri-WT than on Col-0-WT. This observation could be due to other plant or aphid traits. In addition to the glucosinolate
concentrations in the host-plant and the prey body, other physical factors like trichomes on plant leaves might have an effect on performance of *E. balteatus*. Wietsma (2010) found that Cvi-WT contained higher trichome density than Eri-WT. Perhaps trichomes on leaves of plants affected the movement of *E. balteatus* larvae, and this caused a higher performance on Eri-WT (less trichomes) than on Col-0-WT (more trichomes). Furthermore, aphids were larger on Eri-WT than on Col-0-WT, (although not significantly different, Figure 2). Smaller size of aphids on Col-0-WT may have increased the time and energy used by *E. balteatus* larvae to handle these aphids, and this might have reduced performance.

We did not observe differences in hoverfly performance on Col-0-WT and its equivalent transgenic line. Although glucosinolate concentrations were twice as high in the transgenic line than in the wildtype line, aphid performance and trichome density were not different between the two plant accessions (Wietsma, 2010). Perhaps differences in glucosinolate concentrations between these two accessions were not large enough to cause differences in hoverfly performance, and a lack of difference in aphid performance and trichome density have caused a lack in difference in predator performance. We did, however, not analyze other aphid quality traits, and do therefore not know whether the aphids also differed in other quality traits, potentially affecting predator performance.

Preference of *Episyrphus balteatus*

We did not observe a preference of *E. balteatus* for any of the host accessions, probably because of the low number of replicates. However, oviposition of a female is influenced by various internal factors and external factors such as age of female, host plant, size of aphid colonies, semiochemicals (from prey or host plant) and presence or absence of feeding competitors (Reviewed by Almohamad et al., 2009). Due to limited mobility of its larval stage, selection of a good host-plant is a crucial task for a female.

According to Sadeghi et al., (2000) trichomes play a significant role in acceptance of host-plants for oviposition. In agreement with this, we found a lower number of eggs on Cvi (although differences were not significant), that contains a higher trichome density (Wietsma, 2010). Furthermore, oviposition in *E. balteatus* is induced by green leaf volatiles (Verheggen et al., 2008). We did, however, not analyze volatile blends of the tested accessions. In the future, it would be better to trap volatiles from each aphid-infested plant to correlate volatile emission with predator oviposition. A higher number of replications are necessary to obtain reliable results.

Effect of aliphatic glucosinolates on the specialist parasitoid *Diaeretiella rapae*

Performance of *Diaeretiella rapae*

Survival and egg-to-adult development time of *D. rapae* were not significantly different among different aphid host-plants. However, if we look at the results, survival and speed of development were best on Cvi-WT, the accession on which aphids had the highest glucosinolate concentrations (Figure 1). Furthermore, dry weight of *D. rapae* adults was significantly higher when aphids were reared on Cvi-WT than on the other accessions. The size of the host and *D. rapae* were correlated, as they were both highest on Cvi-WT. Similar results, namely a larger
size of the host and its parasitoid *D. rapae*, were observed in another study (Le Guigo et al., 2010). Furthermore, host and parasitoid size are often positively correlated (Bukovinszky et al., 2008), and in general, larger parasitoids have a higher fitness (Godfray, 1994; cited by Clark, 2010).

In agreement with our hypothesis, glucosinolates do not seem to affect performance of *D. rapae* negatively, as parasitoid size was highest when developing in hosts containing the highest glucosinolate concentrations.

We do, however, not know if and how *D. rapae* can cope with glucosinolate in its host. This depends on the feeding nature of the larva. *Diaeretiella rapae* is a koinobiont parasitoid, which means that its host continues to grow during parasitoid development. The parasitoid consumes only haemolymph at the beginning of its development, were glucosinolates are stored by the aphid, but not the myrosinase, which is stored in the non-flight muscle. Eventually, larvae consume the vital organs of the aphid and cause death of the host (Godfray, 1994; cited by Clark, 2010). This could be a possible reason for the absence of negative effects of glucosinolate on *D. rapae*.Possibly, they have the capacity to detoxify glucosinolates that were sequestered in its host; however, this requires more studies. As *D. rapae* develops inside its host, and does not encounter plant morphological traits during its development, trichomes do not affect this parasitoid.

We recorded a higher number of *D. rapae* males than females on Cvi-WT and Eri-WT, but not on Col-0-WT and Col-0-MYB. Most hymenopteran parasitoids are able to select the sex of their offspring during egg deposition. When fertilized, eggs develop into females, and when unfertilized, eggs develop into males (Godfray, 1994; cited by Clark, 2010). Sex allocation in parasitoids reflects host quality and primary nutrient sources in which females have been observed to emerge from larger hosts (Bukovinszky et al., 2008). Our results are contrasting because the percentage of females was lowest on Eri-WT and Cvi-WT. Aphids reared on Cvi-WT were larger (Figure 2) than on the other accessions and therefore aphids on Cvi-WT were comparatively better hosts for the parasitoids. Indeed, *D. rapae* wasps were larger when developing in aphids that were reared on Cvi-WT. However, as mentioned, the percentage of females was low in this accession, in conflict with expectations based on the size of the hosts (Figure 2).

We did not observe differences in parasitoid performance on Col-0-WT and its equivalent transgenic line, similar to effects on the hoverfly. This was expected, because the two accessions only differed in glucosinolate concentrations, and we did not expect glucosinolate concentrations to affect parasitoid performance.

Preference of *Diaeretiella rapae*

For *D. rapae*, we did observe a preference for one of the accessions: Col-0-WT was more attractive than Cvi-WT. The other tested combinations did not result in a significant preference. *Diaeretiella rapae* is a primary parasitoid of *B. brassicae* (Bukovinszky et al., 2008) and is strongly attracted to volatiles produced by *Brassica* plants infested by *B. brassicae* (Bradburne and Mithen, 2000). Pope et al., (2008) suggested that naïve *D. rapae* respond very well to different isothiocyanates but not to nitriles and epithionitriles. Additionally, *D. rapae* parasitized a relatively larger numbers of *B. brassicae* that were reared on plants containing higher concentrations of glucosinolates (*B. nigra*) than *B. brassicae* that were reared on plants containing lower concentrations of glucosinolates (Le Guigo et al., 2010). Likewise, aphid
parasitoids identify their host by means of chemical cues released from the host insect, for example kairomones form the cuticle and cornicle secretions (Powell et al., 1998) and specific volatiles released from the insect’s host plant (Bradburne and Mithen, 2000). However, we did not measure volatiles emitted by our tested *A. thaliana* accessions, so that it is very difficult to come to a conclusion from this experiment. As for *E. balteatus* preference, a higher number of replications are necessary to obtain reliable results, and it would be better to trap volatiles from each aphid-infested plant to correlate volatile emission with predator oviposition.
Conclusion

The aphid *B. brassicae* sequestered glucosinolates from its host plant, *A. thaliana*. Survival of the generalist predator *E. balteatus* was low when fed with these aphids, compared to other studies in which aphids contained lower glucosinolate concentrations. Performance of *E. balteatus*, in terms of survival rate, adult dry weight and sex ratio were not significantly different according to *A. thaliana* accessions on which their prey was reared. However, larval-to-adult development was slower when the prey was reared on Cvi-WT, the accession on which the aphids contained highest glucosinolate concentrations. Thus, glucosinolate concentrations in the prey seem to have a negative effect on the development of *E. balteatus*.

Survival of *D. rapae* was high on *B. brassicae* reared on *A. thaliana*. There were no differences in survival or development time among wasps developing in hosts reared on the different accessions. A higher adult dry weight of *D. rapae* was observed when its host aphid was reared on Cvi-WT. Here, performance of the wasp was correlated with the size of the aphid, so that performance of *D. rapae* was depending more on aphid performance (=size) than glucosinolate concentrations in the host, which were highest on Cvi-WT. Sequestration of glucosinolates by *B. brassicae* therefore seems only effective against generalist predators, and not against specialist parasitoids.

Due to a low number of replications of the preference tests and a lack of information on volatile blends, we cannot make conclusions on preference of natural enemies.

Glucosinolates are a group of resistance secondary metabolites in *Brassica* that have a potential use in insect pest management. Presently, we can manipulate the glucosinolate concentrations and profiles in *Brassica* plants by genetic engineering. For example, MYB28 and MYB29 transcription factors are responsible for producing glucosinolates in *A. thaliana* and could be genetically engineered into plants (Beekwilder et al., 2008). These transgenic plants have a capacity to produce high concentrations of glucosinolates that could provide resistance against damage by generalist herbivores. Specialist herbivores, however, are adapted to feeding on glucosinolate containing plants and use glucosinolates as feeding and oviposition stimulants. Engineering plants to enhance glucosinolate production might therefore not lead to higher resistance against specialist herbivores. Furthermore, some specialist herbivores, such as the cabbage aphid *B. brassicae*, sequester glucosinolates from their host plant and contain endogenously present myrosinase enzymes that upon damage by natural enemies come together and lead to toxic hydrolysis products. We show that generalist predators can be negatively affected by glucosinolates from the prey host-plant. Therefore, direct plant resistance mechanisms may limit the abundance of natural enemies or their efficiency to reduce herbivore populations, which could ultimately render a negative effect on the ecological balance in agroecosystems. Hence, biological control and host plant resistance are less synergistic than previously expected. There is a challenge in developing a crop that is enhanced in both direct and indirect resistance against herbivores. Perhaps the only solution is to either to develop a crop with enhanced direct resistance (e.g. with high concentrations of toxins) or a crop with indirect (predator mediated) resistance, but effects on higher trophic levels should always be considered.
Future work

In the future, it would be interesting to look at extra fitness traits of the natural enemies we tested, like fecundity rate and performance of offspring. We had only one transgenic line (Col-0-MYB28), and more transgenic lines, e.g. of the accessions Eri-WT and Cvi-WT, would be beneficial to study effects of aliphatic glucosinolates on multitrophic interactions. Furthermore, insects reared on different concentrations of pure glucosinolates compounds in artificial diets, offered in combination with the enzyme myrosinase, would provide interesting methods to test effects of glucosinolates on natural enemies of herbivores in more detail.
References


