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Do eggs of *Pieris rapae* induce indirect plant defense in Brussels sprouts?

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Abstract

Plant volatiles induced by herbivorous feeding play an important role in parasitoids host searching behaviour. In addition, plants can respond to herbivorous eggs attracting or arresting egg parasitoids. In this study we investigated this indirect plant defense mechanism in the tritrophic system: Brussels sprout plants - *P. rapae* - *Trichogramma* egg parasitoids. Little information is available on induction of plant synomones in Brussels sprouts by *P. rapae* eggs. Here, we studied the use of induced plant cues by the egg parasitoids *T. brassicae* and *T. evanescens* to localize *P. rapae* eggs. First, we tested whether female egg parasitoids respond to volatiles or contact synomones induced by *Pieris rapae* eggs. Our data showed that only *T. evanescens* respond to volatiles and contact cues of *P. rapae* egg-infested plants 72 h after egg deposition. *Trichogramma evanescens* used in this study are originated from *P. rapae* eggs, which may have an influence on the wasp' response. In contrast, *T. brassicae* did not respond to volatile or contact plant cues. Finally, we found an induction of *BoPR1* and *BoDEF* genes by egg deposition of *P. rapae* on Brussels sprout plants. We hypothesize that this induction could be related with the synomone production arresting *T. evanescens*. Nevertheless, further chemical, molecular and behavioural information are needed to get reliable conclusions about this tritrophic system.

Key words: volatiles, synomones, egg parasitoid, oviposition, host searching, anti-aphrodisiac, *Pieris rapae*, Trichogrammatidae.

1. Introduction

Plants constantly face several biotic and abiotic challenges in their natural habitats. To protect themselves plants have evolved an array of constitutive and induced defenses. Constitutive defenses are independent of damage while induced defenses depend on herbivore attack. The aim of these defenses is to affect the aggressor's growth and development and to trigger defenses for upcoming attacks. Constitutive defenses are represented by e.g. thick cell walls, poisonous compounds, trichomes (Karban *et al*., 1997). Induced defenses are grouped into indirect (e.g. production of volatiles odors to attract natural enemies) and direct responses (e.g. production of toxic secondary compounds, anti-digestive proteins and enzymes that affect herbivorous growth and development) (Dicke *et al*., 2003).

Infochemicals play an important role in multitrophic contexts among plant-carnivorous and plant-herbivore interactions (Vet and Dicke, 1992; Hilker and Meiners 2006; Dicke *et al*., 2004). Those infochemicals produced by plants and used by natural enemies to find infested plants are called synomones and can be produced systemically through the whole plant (Vet and Dicke, 1992; Price, 2002). Additionally, in the process of host location, natural enemies exploit chemical cues from their hosts, called kairomones (Nordlund, 1981 mentioned by Fatouros, 2006). Plant volatiles induced by herbivorous feeding may differ in quality and quantity from volatiles emitted by undamaged plants. Such herbivores-induced plant volatiles may alter plant interactions with their environment (Dicke and van Loon, 2000). Herbivorous secretions released at the moment of feeding can contain substances that elicit the herbivore-induced volatile production in plants (Turlings *et al*., 1990).

Recently, studies have demonstrated that plants can notice and respond to herbivorous egg depositions. Oviposition by herbivores has been reported to attract egg parasitoids by induce plant volatiles in three systems: the bean system (*Vicia faba* - *Nezara viridula* - *Trissolcus basalis*) (Colazza *et al*., 2004), the elm system (*Ulmus campestris* - *Xanthogaleruca luteola* - *Oomyzus gallerucae*) (Meiners and Hilker, 1997), and the pine system (*Pinus sylvestris* - *Diprion pini* - *Chrysonotomyia ruforum*) (Mumm *et al.,* 2005). Also, *Trichogramma* egg parasitoids have been shown to use oviposition-induced plant cues to locate their hosts. *Trichogramma brassicae* wasps were arrested to Brussels sprouts leaves infested with 3-day-old *Pieris*

brassicae eggs. It was assumed that the butterfly eggs induce changes in the cabbage leaf chemistry that arrest the wasps (Fatouros *et al*., 2005a).

The host selection behaviour of *Trichogramma* wasps has been intensively studied due to their widespread application as biological control agent of lepidopteran species (van Lenteren, 2000). Egg parasitoids such as *Trichogramma* spp. have to deal with the problem of searching for an immobile and inactive host stage. Therefore egg parasitoids may exploit several alternative ways to reach their host. Volatile infochemicals from the adult host stage (termed infochemical detour) (Vet and Dicke, 1992) e.g. sex and anti-sex pheromones from lepidopteran hosts and non-volatile kairomones, such as wing scales (Noldus, 1989a mentioned by Fatouros *et al*., 2005a) can have a kairomonal effect, increasing the chances to encounter the host eggs (Reddy *et al*., 2002 and Scholler & Prozell, 2002 mentioned by Fatouros *et al*., 2005a). Andersson *et al*. (2003) demonstrated that pierid males transfer a pheromone to the female at the moment of mating. These chemicals act as anti-aphrodisiacs making the females unattractive to other males. In the case of *P. brassicae,* the males transfer to the females benzyl cyanide (BC) whereas *P. rapae* males synthesize methyl salicylate and indole (MeS+I) (Fatouros *et al.,* 2005b). Recent studies conducted by Faouros *et al*. (2005b), determined that *T. brassicae* is using the anti-aphrodisiac benzyl cyanide from *P. brassicae* as a foraging cue.

Until now, Fatouros *et al*. (2005a) have shown that *T. brassicae* is arrested by Brussels sprouts leaves infested with eggs of the large white cabbage butterfly *P. brassicae* 3 days after oviposition (3 d.a.o). Fatouros *et al*. (2008) reported the first elicitor of the phytochemical changes induce by the oviposition. They found that accessory glands from mated *P. brassicae* females applied on the leaves did elicit a response in the egg parasitoids (3 d.a.o), while accessory glands from virgin females did not. Fatouros *et al*. (2008) detected the anti-aphrodisiac BC in the egg gland secretion of mated females. They observed that when BC was applied onto leaves *T. brassicae* wasps were arrested 3 days after application. Indeed this male-derived compound puts in danger the offspring by inducing an indirect plant defense mechanism.

Little *et al*., (2007) studied the expression profile of *Arabidopsis thaliana* leaves after egg deposition of *P. brassicae* and *P. rapae*. Both butterflies changed the expression of hundreds of genes (671 induced genes and 426 repressed genes 3 d.a.o).

The transcriptional response of *P. rapae* is weaker than *P. brassicae*, this might be due to the deposition of single eggs. However, the molecular knowledge involve in the changes by egg deposition of *Pieris* butterflies is still limited (Little *et al*., 2007).

Recently, molecular studies have provided the first evidence that males by the donation of anti-aphrodisiac pheromones to females induce changes in the plant at the moment of oviposition. Fatouros *et al.* (2008) studied the gene expression changes in response to *P. brassicae* eggs and application of BC on Brussels sprouts by using a whole-genome *Arabidopsis* microarrays analysis. They identified the induction of 42 genes and the repression of 32 genes in those plants carrying 72h-old *P. brassicae* eggs, while leaves treated with the anti-aphrodisiac BC induced 61 genes and repressed 103 genes 72h after application. Oviposition by *P. brassicae* triggers a transcriptional response in Brussels sprouts plants is well correlated with the BC response of the plant. Some of the genes expressed after oviposition by *P. brassicae* may be related with the arrestment of *T. brassicae.*

Induced defenses are mediated by three main signal-transduction pathways: i.e., jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Dicke and van Poecke, 2002). The expression of specific cabbage genes (e.g. *BoPR1*, *BoLox*, *BoMYC*, *BoMYR*, *BoDEF*) has been related with different transduction pathways. such as SA and JA involved in the production of plant synomones. For instance, *PR1* (pathogenesis-related-protein 1) is inducible by various plant pathogens in several plant species and its accumulation is related with SA signal (De Vos *et al*., 2006). Some studies have shown that the *LOX* gene is induced by wounding or herbivorous feeding in tomato and *Arabidopsis* plants (Bell *et al.*, 1995; Heitz *et al*., 1997). *LOX* can be induced by exogenous applications of JA in *B. oleracea*, increasing the expression of the gene (Zheng *et al*., 2007).

In the present study we investigated indirect plant defense in the tritrophic system: Brussels sprouts-*P. rapae eggs*-*Trichogramma* egg parasitoids. Little information is available on the induction of a plant synomone in Brussels sprouts by *P. rapae* oviposition. In this study we will investigate the use of induced plant cues by the egg parasitoids *T. brassicae* and *T. evanescens* to localize *P. rapae* eggs. We hypothesized that oviposition of *P. rapae* induces little or no chemical leaf surface modifications in Brussels sprouts that can arrest the egg parasitoid *T. brassicae* or *T.*

evanescens. This might be due to their oviposition strategy: compared to *P. brassicae* that lays egg clutches, *P. rapae* lays single eggs.

First, it was tested whether female egg parasitoids respond to a volatile or a contact synomone of Brussels sprouts (*Brassica oleracea* var. *gemmifera* cv. Cyrus) induced by *Pieris rapae* oviposition. Second, it was studied if the synomone is induced by the antiaphrodisiac methyl salicylate and indole of *P. rapae*. Third, by molecular analysis it was evaluated, which genes are involved in the production of Brussels sprouts synomones induced by eggs of both *Pieris* species and their antiaphrodiasiacs BC (*P. brassicae*) and MeS+I (*P. rapae*).

2. Material and Methods

Plants and herbivores

Brussels sprouts plants (*Brassica oleracea* L. var *gemmifera* cv. Cyrus) were reared under controlled conditions in a greenhouse (18 ± 5 °C, 50-70% RH, L16:D8 photoperiod). For the experiments plants with 6 – 8 leaves were used, for the rearing of *Pieris rapae* (small cabbage white) and *Pieris brassicae* (large cabbage white) Brussels sprouts plants 8 - 12 weeks old with 12 - 14 leaves were used and kept under controlled conditions (21 ± 2 ºC, 50-70% r.h., L16:D8 photoperiod).

Parasitoids

Trichogramma brassicae Bezdenko (strain Y175) and *T. evanescens* Westwood (strain GD011) wasps (originating from *P. rapae* eggs collected in cabbage fields in the surrounding of Wageningen, the Netherlands), were reared for several generations on *Ephestia kuehniella* eggs in small Perspex tubes (75 mm long, 15 mm diameter) covered with cotton wool on top in a climate chamber (24 ± 2 ºC 50-70% r.h., L16:D8 photoperiod). For bioassays, an oviposition experience was given to the wasps with 2-5 days old *Pieris rapae* eggs deposited on Brussels sprout leaves 18 hours before the experiment. Previous studies indicated that naïve females did not respond to plant cues (Fatouros *et al*, 2005a). All tested wasps were about 2-5 days old mated females, the sexing was made under a microscope. Prior to experiments they were provided with a honey solution.

Plant treatments

For the experiments, Brussels sprout plants (with 6-8 leaves) were placed into a cage (80 x 100 x 80 cm^3) with more than 100 adults from either *P. rapae* of *P. brassicae* butterflies to allow egg deposition in two different egg densities (for *P. rapae* 10-20 or 50-100 eggs per leaf and for *P. brassicae* 2-3 or 5 egg clutches per leaf). In each experiment the 4^{th} leaf from top was tested. After oviposition plants were kept in a climate chamber (21 ± 2 ºC, 50-70 % r.h., L16:D8). The egg-infested plants were tested at 24 and 72 hours after oviposition for bioassays and gene expression analysis. Control plants were never in contact with *Pieris* butterflies but were kept under the same conditions.

In order to test whether methyl salycilate and indole (MeS+I) induces a local plant response arresting the wasps 24 or 72 hours after application, a volume of 100µl of MeS+I (ratio 1:1) (1/100 in hexane, ca. 0.1 and 100 ng/leaf), two concentrations were tested: 0,01 or 1 ng/µl) was applied onto the edge of a Brussels sprouts leaf. In the middle between the tip and the base of the leaf, a stretch of about 2cm of the edge was treated on the lower leaf side. After treatment the plants were kept for 24 or 72 hours in a climate chamber (21±1 ˚C, 50-70% rh, L16:D8). Control plants were treated with 100 µl methanol only. Leaf squares from an untreated section of the test leaf (methyl salicylate + indole (0.01 and 1 ng/ul) 24 h and 72h after application vs clean leaf) were cut and tested against leaf squares from the untreated part of the control leaf in the two-choice bioassay.

Static two-chamber olfactometer tests

To test whether volatile cues of Brussels sprouts plants carrying *P. rapae* eggs are used by the egg parasitoids *T. brassicae* or *T. evanescens* for host location, olfactometer bioassays were conducted in a two-chamber static air-flow olfactometer (a modified version of the four-chamber olfactometer described by Steidle and Scholler, 1997). This static olfactometer is an acrylic glass cylinder (18 cm high x 12 cm diameter) where a vertical plate is placed in the middle, dividing the cylinder in two chambers. On top of the cylinder a removable walking arena (2 cm high x 9 cm diameter) made with a plastic rim and gauze (mesh 0.1 mm) is placed. The walking arena is covered with a glass plate. The experiments were carried out in the laboratory (21 ± 3 ºC) using lamps (Euromex coldlight illuminator EK-I, the Netherlands) above the olfactometer. At the beginning of the bioassays a parasitoid female wasp was placed in the middle of the arena into the olfactometer. The time the wasps spend walking on one of the two chambers was recorded within 5 minutes using stopwatches. The position of the cylinder was rotated 180° after 3 tested wasps to avoid light influence in wasp choice. Ten to fifteen experienced wasps were analyzed per day and plant. For each treatment 50 wasps were tested. Right before the bioassay, the leaf with eggs was excised and placed into a vial with water in one of the two chambers while in the other chamber the control leaf was placed (leaves were similar in size, structure and position as much as possible). Bioassays were conducted to examine whether *T. brassicae* or *T. evanescens* are able to differentiate between volatiles from *P. rapae* egg-carrying leaves (24 and 72h after egg deposition) at two

densities: 10-20 or 50-100 eggs/4th leaf against those from egg-free leaves of control plants.

Contact two-choice bioassays

To test whether leaf surface modifications in Brussels sprouts plants carrying *P. rapae* eggs arrest the egg parasitoids *T. brassicae* or *T. evanescens*, the same two-choice bioassays as in Fatouros *et al*. (2005a; 2007) were conducted in a small Petri dish. A single parasitoid was released into the centre of the Petri dish (5.5 cm diameter). The Petri dish was illuminated from above (Euromex coldlight illuminator EK-I). Leaf squares (2cm^2) were excised right before the bioassay. The wasps were simultaneously offered a leaf square taken from an egg-carrying leaf in the vicinity of the eggs against a leaf square of a control plant. The leaf squares were place upside-down at 1.5 cm from each other. Time spent on the leaf square (with antennal drumming) was observed for 300 seconds (5 min) using The Observer® software, version 5 (Noldus Information Technology, 1993©, Wageningen, The Netherlands). The time they spent in the area outside the leaf squares was scored as "no response". Non-responders were included in the analysis. The total number of tested wasps was 50 individuals per treatment. Leaf squares were changed and total set-up rotated 180˚ after every third wasp tested. Each wasp was used once and then discarded. Corresponding leaves in size and position were taken from the test and control plant to equal the conditions of the experiments as much as possible to avoid variation in the results due to differences in size of leaves.

RNA isolation

The induction of expression of selected genes cloned from the Brussels sprouts genome from (a) 24h and 72h-egg-laden plants of the 2 *Pieris* species compared to uninfested plants and (b) plants treated with the two different anti-aphrodisiacs (BC or MeS+I) at two concentrations (0,01 and 1ng/ul) and tested at two time points (24h and 72h after treatment) compared to control plants treated with the solvent methanol.

For RNA-isolation, leaf segment approximately 4 x 4 cm right next to egg batches or single eggs depending on *Pieris* species or close to the anti-aphrodisiac treated part were collected. An equal size of leaf material from clean plants or plants treated with the solvent was used. From each treatment 1 plant was sampled. It was repeated 2 times.

For RNA isolation the harvest leaf segments were frozen in liquid nitrogen and grind with a cooled mortar and pestle, about 100 mg of the grind powder leaf material was put in an Eppendorf tube and the RNA extraction was done using the EZNA Plant RNA mini kit (Omega Bio-tek). Briefly, 600 µl RPL Buffer with β-mercaptoethanol was added to each 100mg/powder sample and vortex vigorously. Then, 140 µl SP Buffer was added and the samples were vortex and centrifuged for 10min. After centrifugation, the supernatant was taken out without disturbing the pellet. An equal volume of 2-propanol was added. Then, Pre-heat RB Buffer 65 ˚C was added and the dried RNA pellets were dissolved by adding 50 ul of DNase / RNase-free DEPC-treated water. RNA concentration was measured using a NanoDrop®ND-100 Spectrophotometer.

cDNA Synthesis of cDNA from RNA by reverse transcription and RT-PCR

RNA samples are not suitable for PCR, so they were first reverse transcribed using Superscript® III RTS First-Strand cDNA Synthesis Kit (Invitrogen) and 100 ng of RNA. The resulting cDNA samples were stored at -80 ˚C.

A Real Time PCR using GoTaq® Flexi DNA Polymerase (Promega) was performed with the cDNA and 5 different primer pairs (*BoPR1, BoLOX, BoMYC, BoMYR, BoDEF)*, and as loading control glyceraldehydes-3-phosphade dehydrogenase (*GAPDH)* (housekeeping gene) was used as a reference to connect for differences in total RNA used for cDNA synthesis. Because *GAPDH* has been proved to be a good housekeeping gene in expression analysis (Carraro *et al*., 2005) and it is active in mammalian tissues. PCR reaction was performed in a total of 25 µl containing 1 µM primers, 0.2 mM dNTPs, 1.25 units of Go Taq® DNA polymerase (Invitrogen), 1× Go Taq® Flexi buffer (Invitrogen) and 4µl of first-strand cDNA Cycling parameters were: Initial denaturation at 95 ºC for 2 min, then 32 cycles of 95 ºC for 30 s, 56 ºC 30 s, 72 ºC 1 min and final extension 72 ºC 5 min. The PCR products were evaluated by agarose gel electrophoresis. Gene expression was considered to be induced by the treatments if the two replicates showed higher expression than the replicates from undamaged control plants.

Statistics

The Wilcoxon`s matched pairs signed rank test was used to analyze the data collected from both bioassays, the static olfactometer and the contact bioassays.

3. Results

Effect of volatiles of *P. rapae* egg-carrying leaves

The first objective was to determine whether *T. brassicae* and *T. evanescens* are able to respond to volatiles of Brussels sprout plants infested by *Pieris rapae* eggs. In a two-choice static olfactometer bioassay *T. brassicae* wasp did not respond to any odours, while *T. evanesces* wasps were significantly arrested to 72 hours egg-carrying leaves at low egg density (Figure 1; *P*=0.002, Wilcoxon`s matched pairs test signed rank test). However, in all other tests, *T. evanescens* wasps did not discriminate between odours of a leaf with host eggs against odours of a clean leaf (Figure 1).

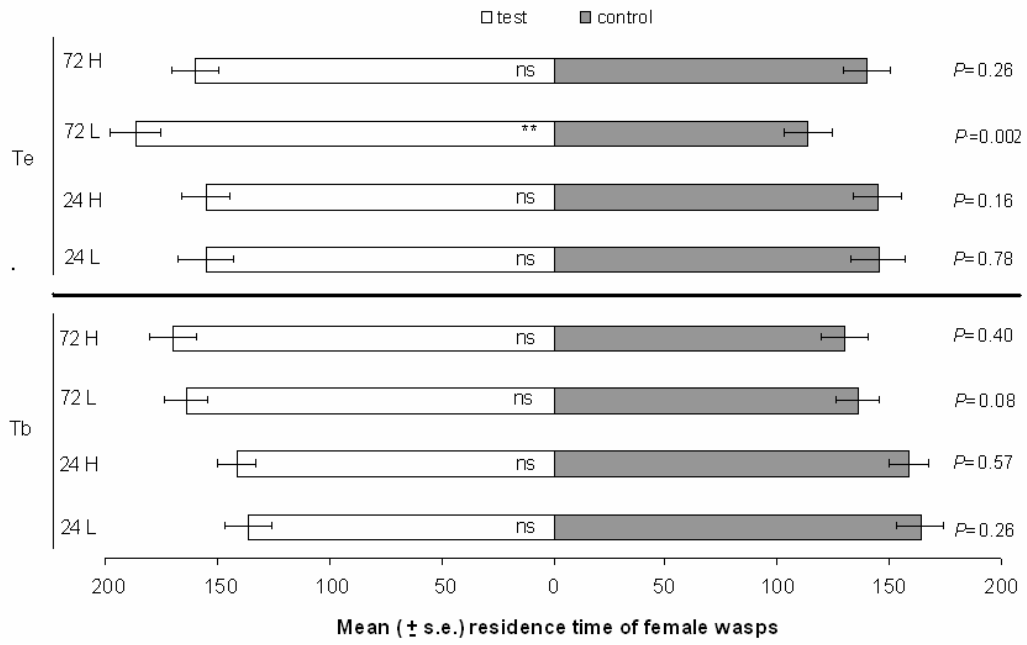

Figure 1. Response of *T. brassicae* (Tb) and *T. evanescens* (Te) wasps to odours from Brussels sprouts infested with eggs of *P. rapae* at two egg densities (L: low density, H: high density) and two different ages: 24 and 72 hours after oviposition. Mean residence time (± s.e.) in test and control field of a two-chamber olfactometer are given. N=50 tested female wasps per experiment. Asterisks indicate significant differences * P<0.05, ** *P*<0.01, ns., not significant (Wilcoxon`s matched pairs signed rank test).

Effect of close-range cues of *P. rapae* egg-carrying plants

In order to study whether modifications in plant surface chemicals close to egg deposition of *P. rapae* serve as cues that indicate the host presence, leaf squares were

excised right next to the eggs (test) and offered together with a leaf square from an egg-free leaf of a clean plant (control). Females of *T. brassicae* did not discriminate between test and control leaf squares in any of the tested combinations. *T. evanescens* wasps did not respond to leaf squares from a plant on which eggs were deposited at low density 24 hours prior to the bioassay (Figure 2, *P*=0.98, Wilcoxon's matched pairs test). However, leaf squares cut from an egg carrying leaf, on which eggs had been deposited at *high density* 24 hours prior to the bioassay significantly arrested *T. evanescens* females (Figure 2, *P*=0.03, Wilcoxon's matched pairs test). Additionally, *T. evanescens* wasps responded to leaf squares from plants with eggs in both densities deposited 72 hours prior to the bioassay (Figure 2, *P*= 0.01 and *P*= 0.004 respectively, Wilconxon's matched pairs test).

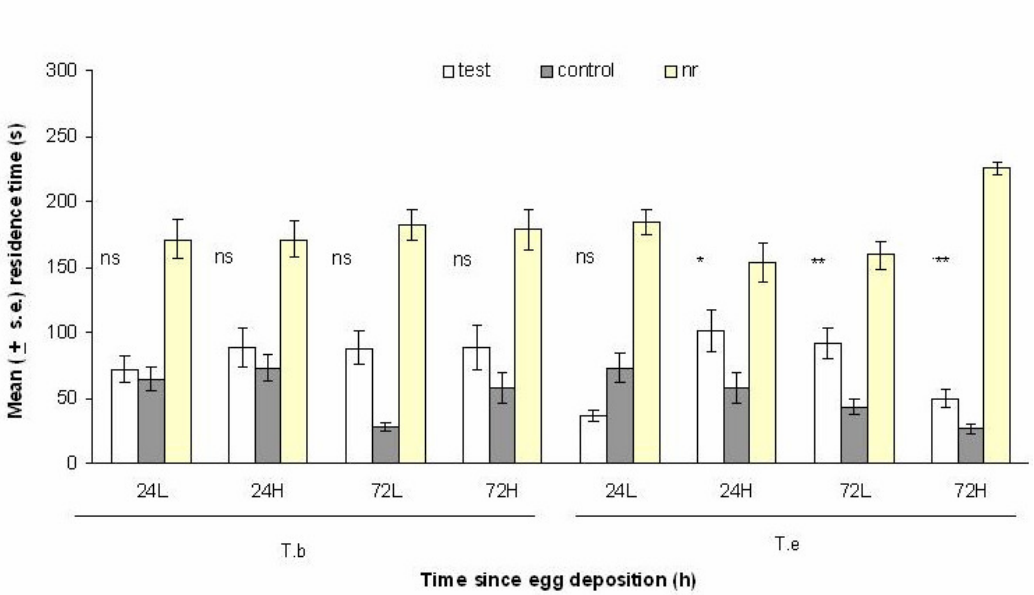

Figure 2. Contact 2-choice bioassay. Response of *T. brassicae* (Tb) and *T. evanescens* (Te) wasps to Brussels sprouts leaf squares excised right next to *P. rapae* eggs tested at two different egg densities (L: low density and H: high density) and two different ages: 24 and 72 hours after oviposition (white bars) and against control leaf squares of uninfected plants (grey bars). Nr: no response (yellow), time spent by wasps on bioassay surface other than leaves. Mean residence time (± s.e.) in test and control field are given. N=50 tested female wasps per experiment. Asterisks indicate significant differences between test and control within the same treatment *, *P*<0.05, **, *P*<0.01, ns. not significant (Wilcoxon`s matched pairs signed rank test).

Effect of methyl salicylate and indole on eliciting indirect defense

In order to study whether the application of the anti-aphrodisiac of *P. rapae* MeS+I induces leaf surface modifications arresting the egg parasitoids, test and control leaf squares were offered to both *Trichogramma* species. Neither females of *T. brassicae* nor of *T. evanescens* discriminated between test and control leaf squares in any of the tested concentrations and time points after application (Figure 3).

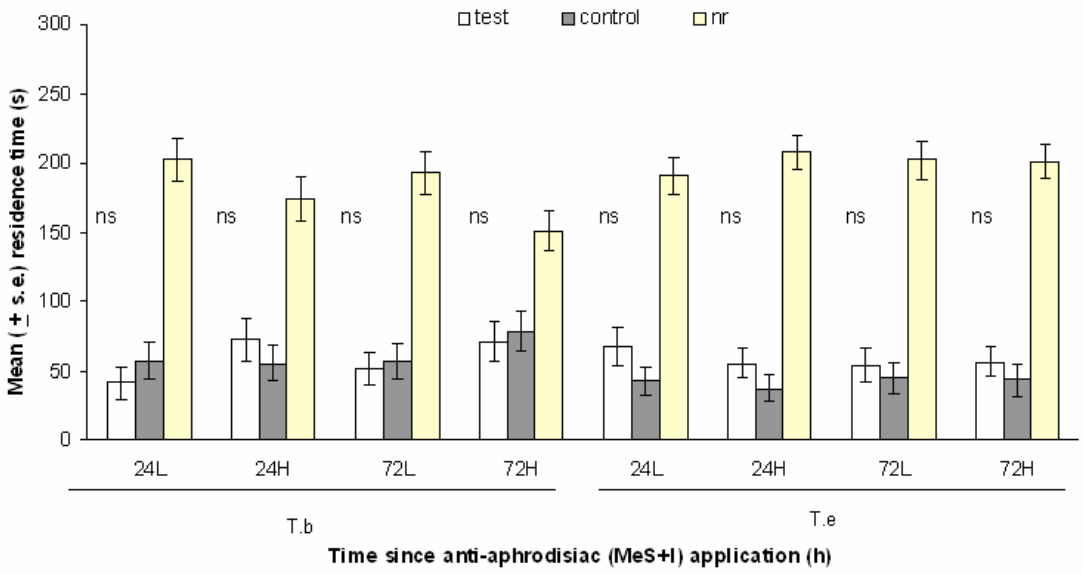

Figure 3. Contact 2-choice bioassay. Arrestment of *T. brassicae* (Tb) and *T. evanescens* (Te) wasps to leaves treated with the anti-aphrodisiac of *P. rapae* butterflies methyl salicylate and indole (MeS+I) (test) at two concentrations (L: low concentration and H: high concentration) and two different induction times (24 and 72 hours after application) tested against leaf squares of leaves treated with methanol only (control). Mean residence time (± s.e.) on test and control leaf square are given. N=50 tested female wasps per experiment, nr, non-responding females, ns, not significant (Wilcoxon`s matched pairs signed rank test).

Gene expression changes in response to *Pieris* eggs

Table 1 shows the expression of the specific cabbage genes (e.g. *BoPR1*, *BoLOX*, *BoMYC*, *BoMYR*, *BoDEF*) from different transduction pathways involved in the production of plant synomones. We studied the transcript levels in response to egg deposition by *P. rapae* and *P. brassicae* and their anti-aphrodisiac treatments (BC and MeS+I) (table 2).

All the genes were detected in most control plants as well as in egg-infested plant treatments (table 1). Brussels sprouts plants infested with *P. brassicae* eggs induced an up-regulation of *BoPR1* at high density after 24 hours of oviposition then the induction decreased 72h low egg density, but the treatment 72 h high egg density showed an up-regulation of the gene again. Furthermore, *P. rapae* egg deposition treatments showed the up-regultation of *BoPR1* 24 h low egg density and the decreased in the transcript levels occurred as in *P. brassicae* 72 h low egg density. *BoDEF*-gene expression was up-regulated by *P. brassicae* egg infested leaves at high density after 72 hours (Table 1). For *P. rapae*-egg deposition treatments the up-regulation was observed 24 hours at high density and 72 hours at low density. Our results did not show clear difference accumulation patterns of *BoLOX*, *BoMYC* or *BoMYR* transcript levels.

Table 1. Gene expression of *BoPR1, BoLox, BoMYC, BoMYR* and *BoDEF* in Brussels sprouts plants upon *P. brassiace* and *P. rapae* egg deposition at two different egg densities. RNA was extracted from egg-free plants (control) or plants where eggs had been deposited 24 or 72 hours before extraction. *GAPDH* was analyzed as the housekeeping gene, a constitutive control. "+" means a low expression pattern; "+++" means the highest expression pattern.

Gene	Control	***Pieris brassicae***				***Pieris rapae***			
		--- 24 Hours ---		--- 72 hours ---		--- 24 Hours ---		--- 72 Hours ---	
		Low	High	Low	High	Low	High	Low	High
GPDH	+++	++	+++	+++	+++	+++	+++	+++	+++
BoPR1	+++	+	+++	++	+++	+++	+++	++	+++
BoLOX	+	+	+	+	+	+	+	+	+
BoMYC	+	+	+	+	+	+	+	+	+
BoMYR	++	++	++	++	++	++	++	++	++
BoDEF	++	++	++	++	+++	+	+++	+++	+

Gene expression changes in response to anti-aphrodisiac application

We tested the expression of the genes induce by the application of the anti-aphrodisiacs benzyl cyanide (BC) or methyl salicylate and indole (MeS+I) on Brussels sprouts leaves. In addition, all genes were detected in most control plants as well as in the plant treatments (table 1). Plants treated by BC anti-aphrodisiac solutions had a higher *BoPR1* transcript levels and also faster accumulation (24h H) than those MeS+I treated plants (72h L) (table 2). Our results did not show clear difference accumulation patterns of *BoLOX*, *BoMYC, BoMYR* or *BoDEF* transcript levels. Quantified gene expression is needed in order to draw a solid conclusion.

Table 2. Gene expression of *BoPR1,BoLox, BoMYC, BoMYR* and *BoDZF* in Brussels sprouts plants after application of the anti-aphrodisiac benzyl cyanide or metile salycite indole at two concentrations. RNA was extracted from plants treated with MeOH (control) or plants were treatment was done 24 or 72 hours before extraction. RNA was extracted locally from anti-aphrodisiac treated leaves. *GAPDH* was analyzed as the housekeeping gene, a constitutive control. "+" means a low expression pattern; "+++" means the highest expression pattern.

Gene	Control	Benzyl cyanide				Metile salycite indole			
		--- 24 Hours ---		--- 72 hours ---		--- 24 Hours ---		--- 72 Hours ---	
		Low	High	Low	High	Low	High	Low	High
GPDH	+++	+++	+++	++	+++	+++	+++	+++	+++
BoPR1	+++	++	+++	+++	++	+	+	+++	+
BoLOX	++	+	+	+	+	+	+	+	+
BoMYC	++	+	+	+	+	++	++	++	++
BoMYR	++	++	++	++	++	++	++	++	++
BoDEF	+++	+++	+++	+++	+++	+++	+++	+++	+++

4. Discussion

Oviposition-induced responses

The results of our study suggest that egg depositions of *P. rapae* on Brussels sprout plants can arrest the egg parasitoid *T. evanescens* by the production of locally released plant synomones. In contrast, *T. brassicae* wasps did not show any response (Figure 1). Contact bioassays showed a time dependent effect: *Trichogramma evanescens* wasps were arrested to leaf areas nearby 72h-old eggs but they did not discriminate between different egg densities.

The response that *T. evanescens* showed to leaf parts close to *P rapae* eggs 24 h after oviposition needs further studies. Now, we assume that this arrestment could be due to: host residues cues (e.g wing scales) like it was shown for *P. brassicae*-system (Fatouros *et al.* 2005a); an early induction of plant synomones in contrast as in the *P. brassicae*-system; or other effects (e.g. egg substances that have diffused into the leaf surface).

Trichogramma brassicae did not respond to volatile or contact plant cues of Brussels sprouts infested with *P. rapae* eggs (Figure 2 and Table 3). Nevertheless, they do respond to the anti-aphrodisiac of mated *P. rapae* females (Huigens and Fatouros, unpublished data). *T. evanescens* wasps respond to Brussels sprout plant cues induced by *P. rapae* egg deposition. They respond to both, volatiles and to contact plant cues (Table 3). Further evaluation is needed to get a reliable conclusion about the respond.

There was not an egg density-dependent effect on the response of *T. evanescens* and the production of plant synomones. Thus, it seems that the different oviposition strategy of *P. rapae* by laying single eggs comparing to *P. brassicae* that lays egg masses does not inhibit indirect plant defense in Brussels sprouts.

Our olfactometer bioassays showed that *T. brassicae* did not discriminate between odours of *P. rapae*-eggs infested leaves 1-3 days after egg deposition and untreated leaves. Previous studies carried out by Noldus & Lenteren (1983) showed that *T. evanescens* was not attracted by volatiles from cabbage leaves with eggs of *P. brassicae* 24 h after oviposition. This corresponds with our study: *T. evanescens* wasps did not respond to volatiles from Brussels sprout leaves infested with *P. rapae* eggs 24 h after oviposition. However, in our study they showed a significant response

to volatiles from leaves infested with *P. rapae* eggs 3 days after oviposition (Figure 1). Here, plant volatiles could be induced by the butterfly eggs. Usually, such an oviposition-inudced plant response needs a three day time lapse as in the case of the Scots pine system (Hilker *et al*., 2002) and the bean system (Colazza *et al*., 2004), even in the elm system it can already starts after a few hours (Meiners and Hilker, 2000). However, in those tritrophic systems egg depositions by herbivores involve plant damage. *Pieris* butterflies do not cause damage to the plant while deposit the eggs. So far, in the table 3 a summary of the response of Trichogramma egg parasitoids to contact and volatiles infochemicals cues of Brussel sprouts plants infested with *Pieris* eggs is given.

Table 3. Summary of contact and olfactometer bioassays about the response of the *Trichogramma* egg-parasitoids to Brussels sprouts plants volatiles or contact cues after *Pieris* egg deposition (h).

		T. brassicae		***T. evanescens***	
Infochemical	**Time (h)**	*P. brassicae*	*P. rapae*	*P. brassicae*	*P.rapae*
Volatile	24	-	-	n.t.	-
	72	-	-	n.t.	+
Contact	0	+	n.t	+	n.t
	24	+	-	+	+
	48	-	n.t	-	n.t
	72	+	-	-	+
	96	+	n.t	-	n.t

+ arrestment; - no discrimination; n.t no tested

The application of the anti-aphrodisiac pheromone of *P. rapae*; methyl salicylate and indole on Brussels sprout leaves did not cause a response in *Trichogramma* wasps. Thus, in the tested concentrations the pheromone did not elicit an induction of plant synomones as it was observed with the anti-aphrodisiac of *P. brassicae*-system: benzyl cyanide. Here, an arrestment of *T. brassicae* wasps was observed at the dose of 1 ng (Fatouros *et al*., 2008). So far, we did not chemically analyze the accessory reproductive gland (ARG) of mated *P. rapae* females to see whether they contain traces of the anti-aphrodisiac, which were identified in ARG's of mated *P. brassicae* females (Fatouros *et al*., 2008).

Trichogramma spp are known to be generalist egg parasitoids. Nevertheless, the present study showed differences between *T. brassicae* and *T. evanescens* wasps

in their response to *P. rapae* eggs. Romeis *et al*. (2005), mentioned that *Trichogramma* spp wasps are more prevalent on specific plants and certain habitats. In our study, we used *T. evanescens* wasps originating from *P. rapae* eggs, which may have influenced the behaviour of the wasps. However, the original host of *T. brassicae* wasps used in this study is unknown.

Fatouros *et al*. (2005a; 2007) also found differences in the response of the two *Trichogramma* species to plant cues induced by *P. brassicae* eggs. Fatouros *et al.* (2005b) reported an arrestment to *P. brassicae* deposits for both wasps' species 24h after egg deposition and 72h after only *T. brassicae* was arrested by plant cues.

The behavioural response of *T. evanescens* may change in a complex and variable field conditions where females of *P. rapae* usually lay single eggs. This study was done in lab conditions where *P. rapae* egg density was higher than it could be in the field.

Role of oviposition-induced genes in plant resistance

In this study, we showed that Brussels sprouts are able to detect the presence of pierid butterflies eggs and application of anti-aphrodisiac pheromone (BC and MeS+I). From the tested genes *BoPR1* and *BoDEF* were the more up-regulated. They are induced during the defense response in plants. Thomma *et al*. (1998), mention that *PR1*-gene has been related to the induction of systemic acquired resistance (SAR) in and an Arabidopsis defensin gene (*PDF1.2*) protects plant against fungal pathogens (Mitter *et al*., 1998). An early recognition and activation of anti-egg mechanisms could provide an efficient defense response since eggs represent a future threat as larvae hatching from the egg will feed on the plant (Bruessow and Reymond, 2007).

It has been documented direct and indirect plant defenses towards oviposition. However the knowledge on the molecular changes triggered by egg deposition is limited (Hilker and Meiners, 2006). Recent studies carried out by Bruessow and Reymond (2007) analyzed the expression profile of *Arabidopsis thaliana* leaves after oviposition of two *Pieris* butterflies. The oviposition by the large cabbage white butterfly (*P. brassicae*) modified the expression of hundred genes including *PR1,* which was highly induced 72 hours after oviposition. Deposition of single eggs of *P. rapae* modified the expression of hundred genes as well but in a much weaker transcriptional response (Little *et al*., 2007). Little *et al*. (2007) provides molecular evidence about the detection of egg deposition by Arabidopsis plants and proposes

that oviposition transcription signature was similar to the one observed by hypersensitive response.

Reproduction and survival of egg parasitoids depend highly on their ability to locate host eggs. The molecular work we conducted show that *BoPR1* and *BoDEF* genes are induced in Brussels sprouts under infestation by *Pieris* eggs, while *BoPR1* was the only gene up regulated with the anti-aphrodisiac treatments. The expression of these genes provide molecular evidence about the detection of egg deposition by Brussels sprout plants and suggest that oviposition may cause a localize response. Oviposition might thus trigger the release volatiles to attract Trichogramma wasps and contribute to defense in the early stages of the herbivore previous to egg hatching (Little *et al*., 2007). However, the activation process of oviposition plant defenses is scarce of molecular knowledge. It is not well known which plant cells are able to perceive and respond to components from the information that an insect egg has been laid; and the role the elicitors have that activate oviposition-induced plant response (Hilker and Meiners, 2006). Fatouros *et al*. (2005a), state that probably when eggs components of *P. brassicae* touch the plant surface (wax layer), they need to move across the wax layer to reach the epidermal cell wall for eliciting an oviposition-induced plant response.

5. Conclusions

Egg depositions of *P. rapae* on Brussels sprouts leaves arrested *T. evanescens* females 72 h after oviposition. In contrast, *T. brassicae* did not respond to such plant cues.

The two generalist egg parasitoids have shown different responses to infochemicals of Brussels sprout plants infested by *P. rapae* eggs. However, these results were obtained under artificial conditions with higher host egg densities than they are present in the field.

We found an induction of *BoPR1* and *BoDEF* genes by egg deposition of *P. rapae* on Brussels sprout plants. We hypothesize that this induction could be related to the synomone production arresting *T. evanescens* wasps. To test this hypothesis further chemical, molecular and behavioural information are needed.

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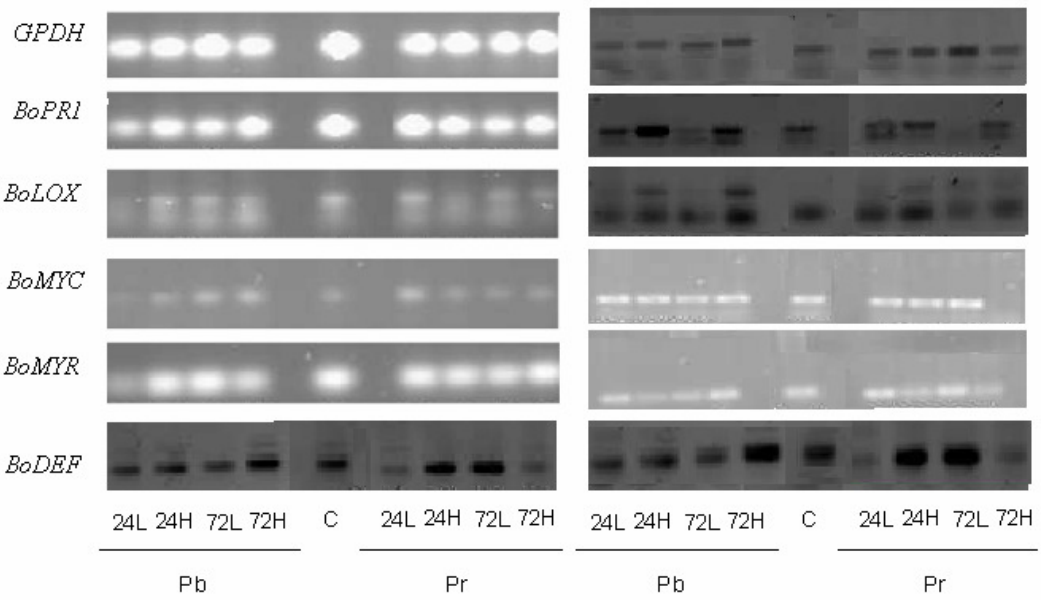
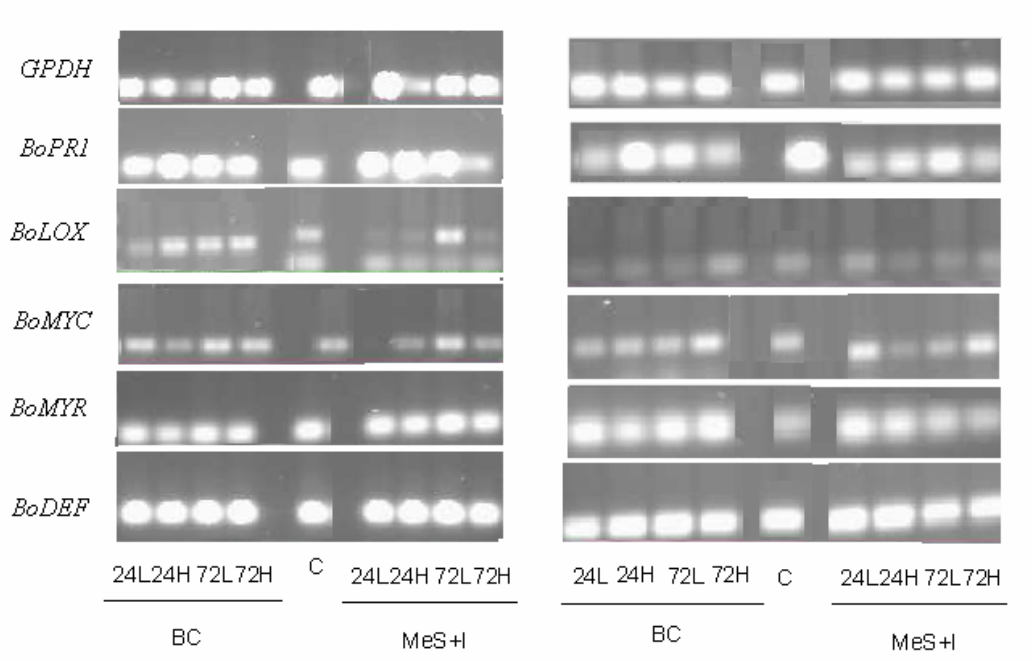
8. Appendix

Appendix A



Appendix A. Gene expression patterns of *BoPR1*, *BoLOX, BoMYC, BoMYR* and *BoDEF* in Brussels sprouts plants upon *P. brassiace* (Pb) and *P. rapae* (Pr) egg deposition at two different egg densities (L=low 20-50 eggs/4th leaf, H=high 50-100 eggs/4th leaf) RNA was extracted from egg-free plants (C=control) or plants where eggs had been deposited 24 or 72 hours before extraction. *GAPDH* was analyzed as the housekeeping gene, a constitutive control.

Appendix B



Appendix B. Gene expression patterns of *BoPR1*, *BoLOX, BoMYC, BoMYR* and *BoDEF* in Brussels sprouts plants after application of the anti-aphrodisiac benzyl cyanide (BC) or methyl salicylate and indole (MeS+I) at two concentrations (L=low 0,001 ng/µl, H=high 1ng/ µl). RNA was extracted from plants treated with MeOH (control) or plants treated 24 or 72 hours before extraction. RNA was extracted locally from anti-aphrodisiac treated leaves. *GAPDH* was analyzed as the housekeeping gene, a constitutive control.