Attraction of *Trichogramma* wasps to *Brassica nigra* plants induced by lepidopteran eggs

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Abstract

Plants possess a broad spectrum of defense mechanisms against herbivore attack. The black mustard *Brassica nigra*, is able to display early defense mechanism against egg infestation by pierid butterflies. This plant shows induced direct defense through hypersensitive response (HR), which kills the eggs as well as indirect defense by the emission of egg-induced plant volatiles that attract egg parasitoids such as *Trichogramma* wasp. In this study, I investigate whether *B. nigra* plants infested by the small cabbage white butterfly (*Pieris rapae*) or the cabbage moth (*Mamestra brassicae)* express both kind of defense strategies, and whether plants expressing HR still attract *Trichgramma* wasps in the laboratory and in nature. Tests in the y-tube olfactometer showed that volatiles of plants infested with *P. rapae* eggs 24h after egg deposition were attractive to the egg parasitoid *Trichogramma brassicae* when tested against volatiles of uninfested plants. All tested *P. rapae*-infested plants expressed HR 24h after oviposition. In contrast, plants infested with *M. brassicae* eggs did not express HR. Volatiles of *M. brassicae* egg-infested plants were attractive to *T. brassicae* only when tested against clean air but not when tested against volatiles of uninfested plants. In nature, 77% of the *P. rapae* eggs collected from HR+ *B. nigra* plants died, whereby 37% because of *Trichogramma* parasitism. Eggs collected on HR- *B. nigra* plants had a significantly lower mortality rate (45 %), and only 15 % died because of *Trichogramma* parasitism. No *M. brassicae* eggs were found in the field on *B. nigra* plants. My results from the laboratory tests and the field collection indicate that *B. nigra* plants successfully defend *P. rapae* eggs by combining direct and indirect defenses. Hypersensitivity response against pierid egg deposition seems to work in concert with egg parasitoid recruitment instead of a being trade-off in terms of energy costs (for the plant) or reduction in offspring (for the parasitoid). Furthermore, it seems that the plant's defense response is specific against *Pieris* eggs whereas the moth eggs were not (directly) defended.

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Introduction

Plants use several strategies to defend themselves when they are attacked by herbivores, which can be divided into direct and indirect defenses (Schoonhoven *et al.* 2005). Direct defenses can consist of physical barriers in the cuticle such as trichomes, spines or the induced production of herbivore deterrents. Indirect defense are those features that indirectly protect the plant by enhancing the probability of attracting the herbivores' natural enemies (Dicke and van Loon, 2000).

Direct defense includes the induced production of secondary metabolites like protease inhibitor (antifeedant), alkaloids, glucosinolates, terpenoids or phenolics (bad taste), give the plant the ability of counter-attack the destructive activity of the herbivore by becoming unsuitable for ingestion or being unattractive (Schoonhoven *et al.* 2005). The indirect plant defense repertoire is mostly represented by the emission and spread of volatile organic compounds involved in interaction between other organisms, which might be exploited by members of higher trophic levels (carnivores) to locate their food sources (Schoonhoven *et al.* 2005). These plant-released compounds are known as herbivore-induced plant volatiles (HIPV) or the ability of plants to "cry for help" (Dicke and van Loon, 2000).Herbivore-induced plant volatiles can be produced after feeding by phytophagous insects, at the site of attack as well as systemically by undamaged plant parts. They are key components of indirect plant defenses (Dicke and van Loon, 2000). Carnivorous such as predators and parasitoids are attracted by HIPV and use them during location of their herbivorous prey or host (Dicke and van Loon, 2000). The volatile production and emission by an induced plant under herbivore attack may be very specific and have the feature of being very detectable and relatively reliable cues that can be exploited by the attacker's natural enemies. The accuracy of this signal of plants that "cry for help" are so effective, that carnivores which are attracted or arrested by them can often discriminate between HIPV induced by different species. Thus, HIPV can be herbivore species-specific (Dicke, 2009).

The plants' defensive response is not only restricted to tissue damage by herbivorous feeding, but they may respond also to oviposition. Because they do not "want" to be damaged by the hatching herbivores, some were shown to respond toward the eggs avoiding the first step of herbivore attack (Hilker and Meiners 2002; 2006). Oviposition-induced defenses can also be divided in induced direct and indirect responses. Direct defenses are related to (1) the formation of neoplasm under the site of oviposition (Doss *et al.* 2000), (2) the necrotic tissue formation short after the oviposition or hypersensitivity response (Shapiro and DeVay, 1987, Balbyshev and Lorenzen 1997) and (3) the production of oviposition deterrents (Blaakmeer *et al.* 1994a). Direct reactions are addressed to kill the eggs or barely-hatched larvae, whereas indirect defenses are related with the emission of plant volatiles that attract egg parasitoids (Hilker and Meiners 2002; 2006).

Egg parasitoids developed several host location strategies in order to find their host eggs to solve the problem of the eggs' low detectability (Fatouros *et al.* 2008b). These minute insects are able to exploit infochemicals released/emitted either by their adult

host or by plants induced by herbivore oviposition, which guide the parasitoid to their target host – the eggs (Hilker, 2006). Host cues emitted by the eggs are reliable cues for the egg parasitoids, but difficult to find because they emit only short range cues and their small size (Fatouros *et al.* 2008b). Some eggs parasitoids are able to exploit (anti-) sex pheromones, like the egg parasitoid *Trichogramma* spp., a very tiny wasp species, which was reported to exploit host pheromones emitted by mated *Pieris* females butterflies (Fatouros *et al.* 2005a; Huigens *et al.* 2009), the so-called anti-aphrodisiacs. Anti-aphrodisiacs are male-derived pheromones transferred from males to females during mating in order to render the females less attractive to other males (Andersson *et al.* 2003). This anti-sex pheromone lures the parasitoid to a mated butterfly allowing the wasp to hitch-hike to the host plants in order parasitize the butterfly's freshly laid eggs (Fatouros *et al.* 2005a).

Host location by egg parasitoids using oviposition induced plant volatiles, seems to be more useful for them because these plant cues can be specific by indicating the host presence and address the parasitoid to locate suitable eggs. However, only in few plants, insect egg deposition have been shown to induce an egg-parasitoid attracting synomone (Fatouros *et al.* 2008b). In the field elm system (*Ulmus minor*) Meiners and Hilker (1997, 2000) described the attraction of the egg parasitoid *Oomyzus gallerucae* to induced-volatiles by oviposition of the elm leaf beetle *Xanthogaleruca lueola*. The Scots pine (*Pinus sylvestris*) also release egg-induced volatiles in response to oviposition of the sawfly *Diprion pini*, and those were attractive for the egg parasitoid *Chrysonotomyia rofurum* (Hilker *et al.* 2002). A combination of feeding-damage and egg-oviposition by the pentatomid adult *Nezara viridula* produce volatiles by bean plants, which attract the egg parasitoid *Trissolcus basalis* (Collaza, *et al.* 2004a, b). Attraction of such natural enemies of the herbivore by the plant might have a great advantage because they can be killed before the feeding damage (Hilker, 2006). However, it is still unclear whether these specific plant cues usage is adaptive for the egg parasitoids, since volatiles induced by feeding or wounding are more abundant than by oviposition, and the response by egg parasitoids needs of a fine-tuned sensory abilities (Fatouros *et al.* 2008b).

Trichogramma egg parasitoids can use plants cues induced by *Pieris* eggs (Fatouros *et al.* 2005b; 2008a; 2009). Egg deposition by the large and the small cabbage white butterfly *Pieris brassicae* and *P. rapae* on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera*), induces the change of plant surface chemicals that arrested *Trichogramma* wasps by contact in the eggs vicinity three days after egg deposition (Fatouros *et al.* 2005b; 2009; Pashalidou *et al.* 2010). Furthermore, the elicitor that triggers this oviposition-induced synomone are the anti-aphrodisiac compounds benzyl cyanide (BC) (for *P. brassicae*) or indole (for *P. rapae*), which are released together with the egg secretion during oviposition (Fatouros *et al.* 2008a; Fatouros *et al.* 2009).

In contrast to the Brussels sprouts system, *Trichogramma brassicae* wasps were shown to be attracted to volatiles of the Black mustard (*Brassica nigra*), a wild crucifer, 24 h after oviposition by *P. brassicae* (Lucas Barbosa, 2009, 2010). Additionally, *B. nigra* plants can respond directly to oviposition by Pierid butterflies through the formation of necrotic tissue around the eggs, i.e. hypersensitivity response (HR), a locally restricted

necrotic zone of cells at the oviposition site that eventually kills the eggs (Shapiro and DeVay, 1987). This direct defense to egg deposition is addressed by tissue changes that either kill the eggs or isolate hatching larvae in the HR formation place (Shapiro and DeVay, 1987, Balbyshev and Lorenzen 1997). Although the HR response as direct defense could be contrasting with the indirect defense, because it may cause the killing of all available eggs for the parasitoid, there is evidence of no conflict in both mechanism in *B. nigra*, since *T. brassicae* wasp can be arrested by oviposition-induced volatiles in plants which show HR. Only about 20 % of the eggs were killed by HR (Lucas Barbosa, 2009).

Previous research on oviposition-induced plant volatiles in *B. nigra* system was done with the large cabbage butterfly *P. brassicae*, it remains unknown how specific the response to insect eggs is: do eggs of two other abundant lepidopteran herbivores on *Brassica*, the small cabbage butterfly *Pieris rapae* and the cabbage moth *Mamestra brassicae*, induce direct and/or indirect defenses? The goal of the present study is to test whether eggs of different lepidopteran species induce volatiles in *B. nigra* that attract *T. brassicae* wasps. Additionally, I monitored natural parasitism rates by *Trichogramma* wasp and *Pieris* egg mortality in correlation with HR on wild *B. nigra* plants.

Research questions:

1. Do *B. nigra* plants infested with *Pieris rapae* or *Mamestra brassicae* eggs release volatiles that attract *Trichogramma* wasps?

2. Do *B. nigra* plants express HR induced by the two host species in the laboratory and/ or in nature? Are those plants attractive to the wasps?

3. What are the natural parasitism rates of *Trichogramma* in the two host species on *B. nigra*?

4. Are eggs on plants showing HR still parasitized by *Trichogramma* wasps? Is there a correlation between the HR occurrence, parasitism rate, and egg mortality?

Material and methods

Plants and insects

Plant seeds of *Brassica nigra* L. were used from the Center for Genetic Resources (CGR, Wageningen, The Netherlands), from an earlier flowering accession CGN06619 (feral population collected in 1975 from Peloponnesos, Greece). A seed mixture of 25 individuals represented the used plant population were sowed in pots. The plants were reared in a greenhouse compartment (22 ± 2°C, 70% r.h., L16:D8). Four and five week old plants have been used for the experiments. Entomophatogenic nematodes (*Steinernema sp.*) were applied to the soil to avoid sciarid fly larvae infestation.

The small cabbage white butterfly *Pieris rapae* L. (Lepidoptera: Pieridae) was reared on Brussels sprouts plants in a climate room (22 ± 1°C, 50-70% r. h., L16:D8) and kept feeding on saturated sugar solution. *Mamestra brassicae* L. (Lepidoperta: Noctuidae) caterpillars were reared on Brussels sprouts plants in large cages in a climate room (22 ± 1°C, 50-70% r.h., L16:D8). The adult moths were feeding on saturated sugar solution.

The egg parasitoid *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) was reared in eggs of the moth *Ephestia kuehniella* under laboratory conditions (23 ± 2°C, 50-70% r.h., L16:D8). The wasps were kept feeding on honey. For the bioassays, 2-5 day old mated naïve wasps were used that had no contact with the herbivore or plant before testing.

Plant treatments

For the experiments, on each day, a *B. nigra* plant was placed into a large cage, kept in a climate room with more than 100 adults of *P. rapae* butterflies, for app. 10 minutes to allow oviposition of 10 to 20 eggs. Later, the plants were kept in the greenhouse compartment (22 ± 2°C, 50-70% r.h., L16:D8) under a lamp of 400W and upwind position in relation to the clean control plants, for 24 hours.

In the case of *M. brassicae* induction, 7 to 10 adult mated female moths were collected and placed together with two *B. nigra* plants in a separate cage overnight, to get 1 or 2 egg clutches per plant until the next morning. Oviposition of the nocturnal *M. brassicae* is not as reliable as *P. rapae*, because they oviposit during the scotophase and the oviposition/ induction time could only be estimated between 22-30 h. After induction, the plants were treated similarly as the *Pieris* placing them in a cage separately from the clean plant that were going to be used as control.

Hypersensitivity response

After 24 h of infestation, plants were checked for hypersensitivity response (HR). The strength of the HR was assessed visually (how long) until eggs were hatching. The severity was noted using a semi-quantitative (+, ++, +++) scale as presented in table 1.

Table 1. Semi-quantitative scale and visual description of HR observed symptoms (Lucas Barbosa, 2010).

Severity	Visual description
0	No tissue damage underneath or around the eggs visible
+	Tissue above egg clutch is silver colored – pre-necrosis visible from the upper side of the leaf on tissue above egg clutches (Figure 1a)
++	Dead cells – necrosis visible from the upper side of the leaf on tissue above egg clutches (Figure 1b)
+++	Dead cells - visible from the upper side of the leaf + necrosis also visible around the eggs clutches (Figure 1c)

Figure 1: Hypersensitivity response levels of *B. nigra* to *P. rapae* eggs: a) Severity + b) severity ++ and c) severity +++ (Photo credits: Ilich Figueroa).

Y-tube olfactometer experiments

To test whether *T. brassicae* wasps are attracted to volatiles of *B. nigra* induced by egg deposition of *P. rapae* or *M. brassicae*, bioassays were conducted in a dynamic air-flow Y-tube olfactometer (See figure 2, for details see Lucas Barbosa 2010). The experiments were carried out in the laboratory at 21 ± 2°C using a fiber optic light source above the olfactometer. A choice of two different odor source, clean air or clean plant vs. treated plant was offered to a group of ten adult *T. brassicae* females in order to test their response to volatiles emitted by the different plant treatments.

Table 2: Overview of the four treatments

No.	Treatment	Induction time (h)
1	*P. rapae* egg-induced plant vs. clean air	24
2	*P. rapae* egg-induced plant vs. clean plant	24
3	*M. brassicae* egg-induced plant vs. clean air	30
4	*M. brassicae* egg-induced plant vs. clean plant	30

Intact plants of *B. nigra* with roots and soil wrapped in aluminum foil were placed in a glass container, in order to reduce interference of volatiles emitted also from the soil. The air flowed through the odor source glass containers and was led to each of the arms of the Y-tube through an outlet at the base of the containers. Connections between all glass parts were made by using Teflon tubing. The airflow admitted through each arm of the olfactometer was 100 mL min-1, which was read with a flow meter. In this way two well-separated laminar airflows were generated in the olfactometer (Figure 2).

In total 120 wasps were tested per treatment. Trials of 20 wasps were carried out on 6 different days with 6 different plants. Ten wasps were released at a time. After making a choice in the Y-Tube, the wasps were directed through phototropism to independent collector flasks connected to each of the arms of the Y-tube (Figure 2). After 30 minutes, the wasps collected in each of the flasks are counted and the odor source switched to exclude biased effects. If the wasp did not make a choice within 30 minutes (i.e. did not reach the end of one of the arms), it was recorded as a "no response" and not considered for statistic. Each wasp was used only once and then discarded.
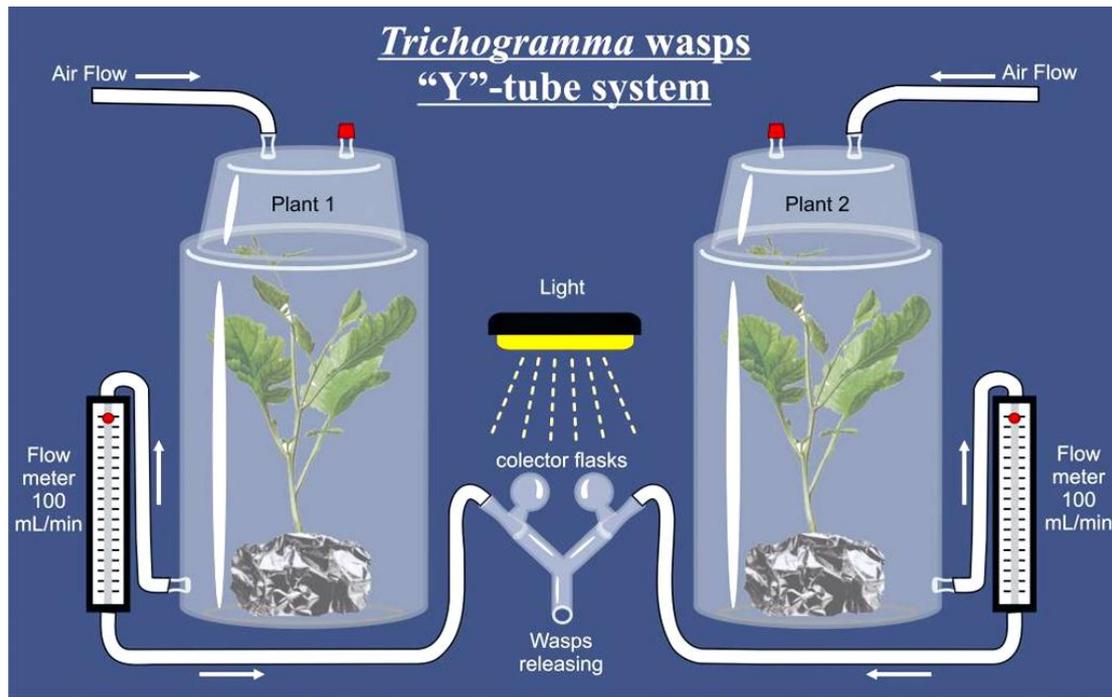

Figure 2: Schematic drawing of the "Y"-tube olfactometer system for tests with *Trichogramma* wasps (Draw credits: Ilich Figueroa).

Field egg and insect collection

A field survey was carried out where observations and data collection were performed. Different patches of *Brassica nigra* plants were marked and regularly visited to check the plants for butterfly eggs (Figure 3). All found single or clutches eggs were marked and checked for parasitism by *Trichogramma* wasps, presence of hypersensitivity response and/or egg death. Parasitized eggs were collected and reared in the laboratory until wasps emerge and further identification.
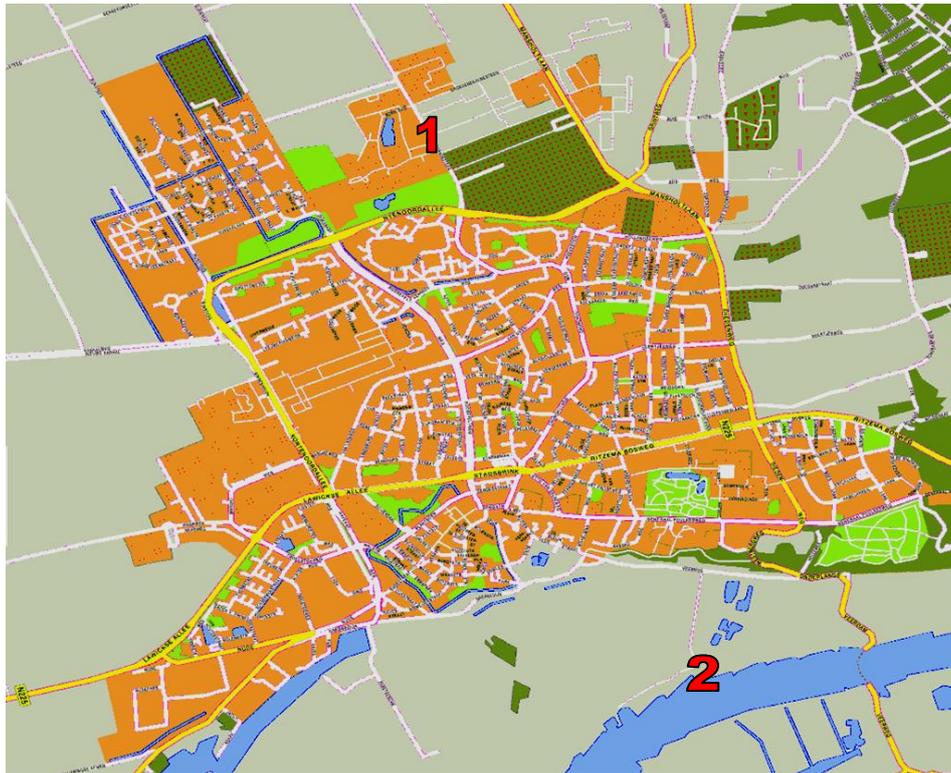
Figure 3: Map of egg-collection patch location (1) *B. nigra* **in patch of Bornsesteeg, north Wageningen and (2)** *B. nigra* **in the Rhine river bank south Wageningen.**

Statistics

Result of the Y-tube experiments were analyzed by the non-parametric two-sided binomial tests. Results from field experiment were analyzed with contingency tables 2x2 following the Chi-square test for independency and correlation of Spearman, in order to determine the level of dependency of the HR reaction with the parasitism rate and egg mortality. Pair-wise comparison between proportion of parasitism and mortality within the HR samples were done using the Chi-square test for homogeneity. All tests were carried out at α=0.05 in the statistical program SPSS version 11.0.1.

Results

Attraction to volatiles of *B. nigra* infested with *P. rapae* eggs.

T. brassicae wasps were significantly attracted to plant volatiles of *B. nigra* infested with *P. rapae* eggs 24 h after oviposition. In both tested combinations: egg-induced plant against clean air as well as egg-induced plant vs. clean plant; the wasps significantly chose odors of the infested plant (a: P<0.001; b: P=0.003, binomial test, Figure 4).
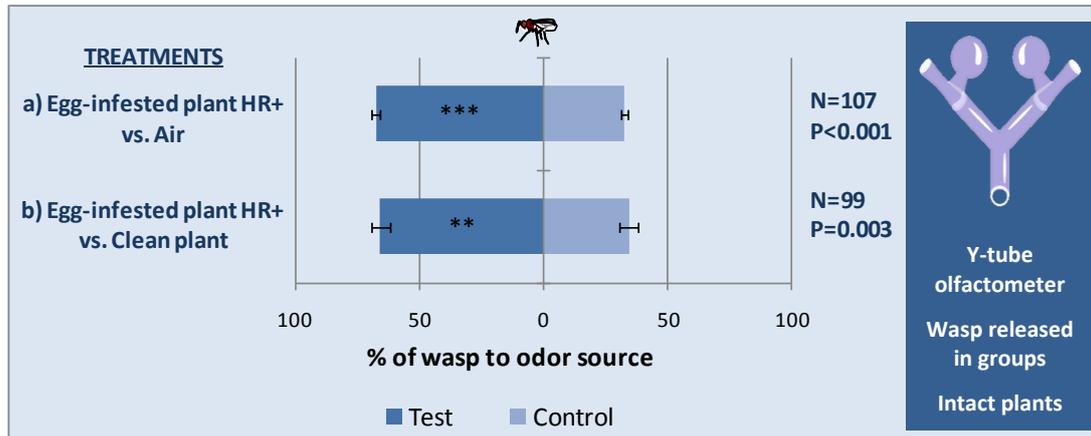

Figure 4: Arrestment of *T. brassicae* by *B. nigra* plant volatiles 24 h after oviposition with *P. rapae* eggs. Treatments: a) Egg-induced plant (HR+) vs clean air and b) egg-induced plant (HR+) vs clean plant. Percentage of wasp to odor source (test and control), standard error, number of responding wasps (N) and P-values are given. Significant differences in the two-sided binomial test: *** P<0.001 and ** P<0.01

Attraction to volatiles of *B. nigra* infested with *M. brassicae* eggs.

The wasps were significantly attracted by volatiles of plants infested with *M. brassicae* eggs ca. <30h. after oviposition when tested against clean air (P<0.001, binomial test, Figure 5). However, when volatiles of *M. brassicae* egg-infested plants were tested against volatiles of clean plants, the wasps did not discriminate between the two odor sources (P=0.395, binomial test, Figure 5).
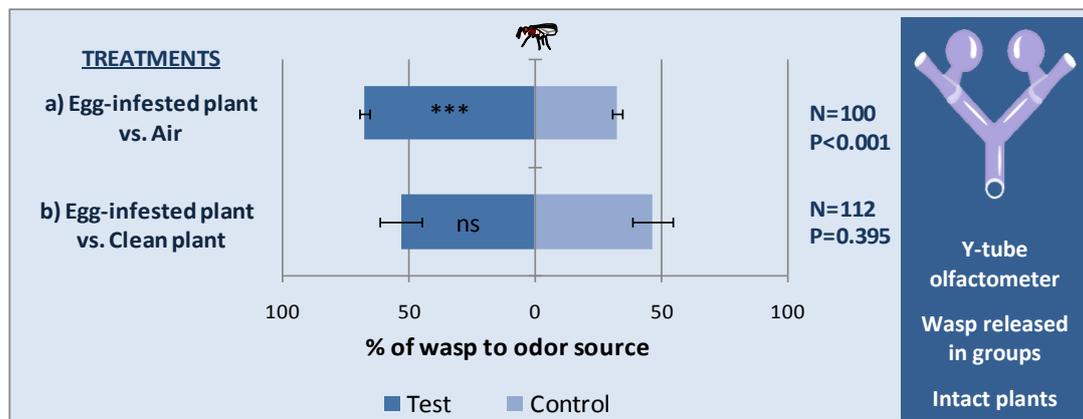

Figure 5: Arrestment of *T. brassicae* by *B. nigra* plant volatiles <30h after oviposition with *M. brassicae* eggs. Treatments: a) egg-induced plant vs air and b) egg-induced plant vs clean plant. Percentage of wasp to odor source (test and control), standard error, number of responding wasps (N) and P-values are given. Significant differences in the two-sided binomial test: *** P<0.001 and ns=not significant.

Hypersensitivity response against *P. rapae* and *M. brassicae* eggs in laboratory and field.

Under laboratory conditions, all the 12 *B. nigra* plants induced with *P. rapae* eggs that were used for the Y-tube experiments, showed different levels of HR expression severity 24 h after egg infestation: 6 plants showed the level 1 (+, subtle) and 4 plants showed the second level (++, middle). Only two plants showed a strong necrosis with the maximum severity level of HR (Figure 6).
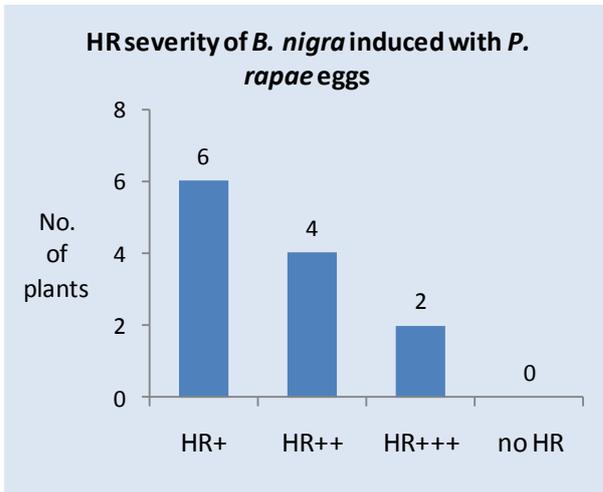
In the case of plants induced by *M. brassicae* eggs, none of the 12 plants showed HR against *M. brassicae* eggs, when followed until eggs hatched.

Figure 6. HR severity levels of *B. nigra* plants induced by *P. rapae* eggs, used for the Y-tube experiments.

The field collection of pierid eggs on *B. nigra* was done almost exclusively in the Rhine patch (see map on figure 3). At the time of collection (August and September), many plants were already seeding and a few new ones were growing around those, therefore there was less choice to oviposit for the butterflies and the plants showed high density of butterfly eggs. From the *B. nigra* plants monitored in the field I collected 455 *P. rapae* eggs. From all collected eggs, 275 were laid on plants that showed HR expression (60% of HR+, Figure 7). The HR expression in this particular population was strong, since some of the plants that, at the moment of collection did not show HR, developed it the following days until the plant dehydration. Neither *M. brassicae* eggs nor *Pieris brassicae* egg clutches were found on *B. nigra* plants monitored in the field.
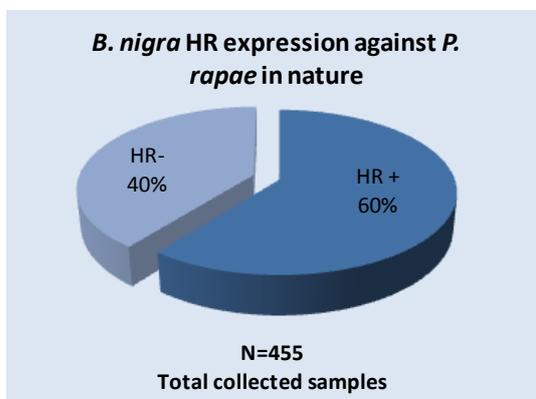

Figure 7. Percentage of HR expression against *P. rapae* eggs collected on *B. nigra* plants . Field site was located on the "Rhine patch", Wageningen.

Natural parasitism rates of *Trichogramma* wasps in *P. rapae* eggs.

From the 455 collected *P. rapae* eggs, 130 (29%) were parasitized by *Trichogramma* wasps, 169 (37%) were already dead during collection or died shortly after, due to reasons different than *Trichogramma* parasitism. From 156 (34%) eggs a caterpillar hatched (Figure 8).
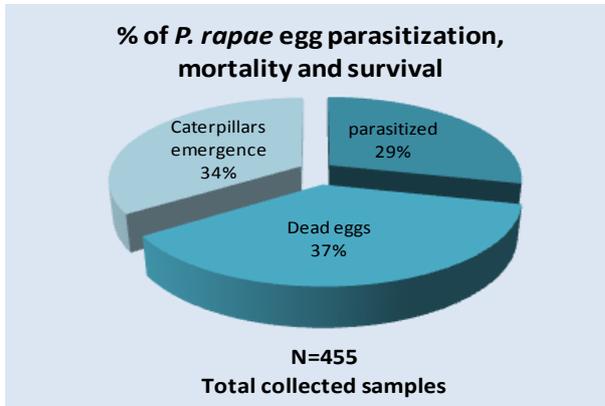

Figure 8. Percentage of egg parasitism, egg mortality, and egg survival of *P. rapae* eggs collected on wild *B. nigra*.

Correlation between *B. nigra* HR, *Trichogramma* parasitism and egg mortality.

Results from the field collection showed that 60% of the sampled *P. rapae* eggs triggered HR expression in different levels of severity on the plants (see figure 7). When analyzing the recorded HR expression with the *Trichogramma* parasitism, I showed a significant correlation between these two factors (χ^2=29.38, P<0.001. Contingency table 2x2 - Chi-square test for independency or Spearman correlation analysis Co=0.254, P<0.001). From all collected *P. rapae* eggs, 77 % died on HR+ plants, compared to 45 % on HR- plants (χ^2=48.32, P<0.001. Contingency table 2x2 - Chi-square test). Thirty seven percent (N=104) of the collected eggs triggering HR were parasitized compared to 15% (N=26) of the eggs on plants that did not express HR (figure 9).
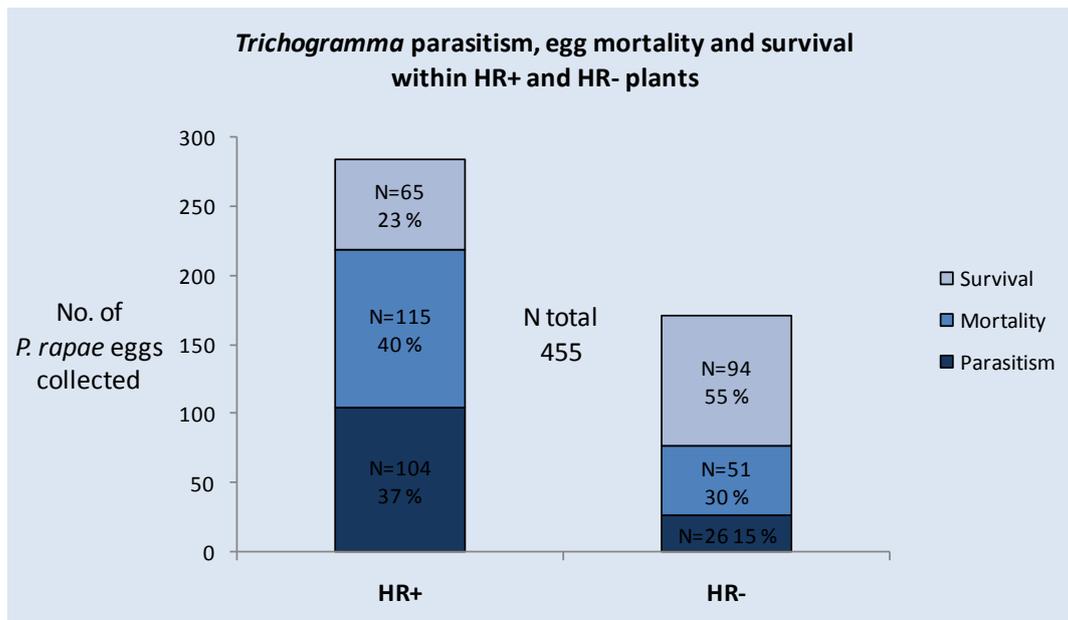

Figure 9: *Trichogramma* parasitism, mortality and survival from collected *P. rapae* eggs on wild *B. nigra* plants with positive and negative egg-induced HR expression. Percentage, number (N) of collected eggs of both HR+ and HR- plants and N total are given.

When correlating the HR expression and egg mortality, different from those eggs dying by parasitism, results showed that HR has a significant dependency of the variable mortality (χ^2=37.78, P<0.001 Contingency table 2x2 - Chi-square test for independency, Spearman correlation analysis Co=-0.34, P<0.001). Forty percent (N=115) of the eggs on HR+ plants died compared to 30% (N=51) on HR- plants (figure 9). Parasitism by *Trichogramma* wasps and egg mortality in plants with HR are shown in figure 10 a, b.

Figure 10: Hypersensitivity response of *B. nigra* against *P. rapae* eggs: a) *P. rapae* egg parasitized by *Trichogramma* wasp with strong necrotic zone around the egg b) shrank *P. rapae* egg with strong necrotic zone around the egg. (Photo credits: Ilich Figueroa).

Finally, the proportion of parasitized eggs and mortality eggs with respect to HR was tested. Results showed a highly significant difference between parasitized eggs on HR+ plants (80%) with those on HR- plants (χ^2=46.48, P<0.001 - Chi-square test for homogeneity, Figure 11a). When looking at egg mortality with respect to HR there was also a highly significant difference: 69% of the dead eggs are related with the positive HR expression (χ^2=24.67, P<0.001 - Chi-square test for homogeneity, Figure 11b).
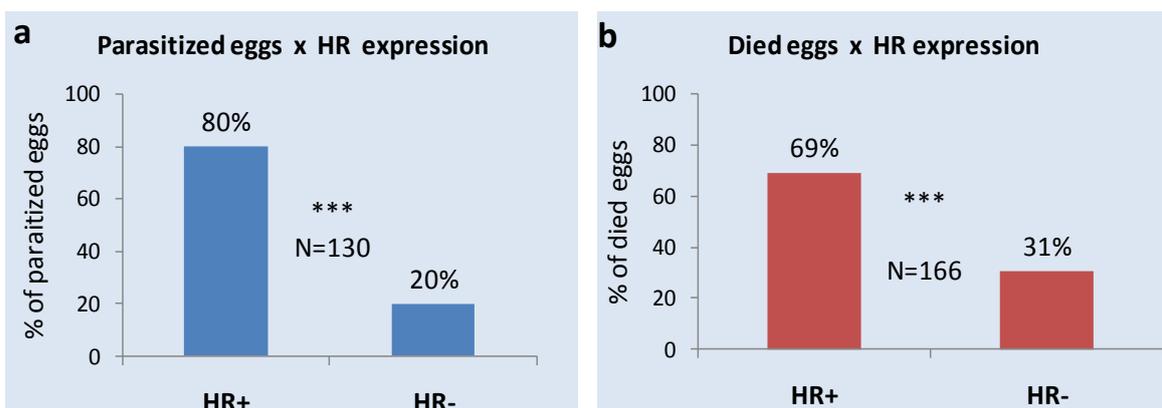

Figure 11: Proportion of parasitized *P. rapae* eggs on wild *B. nigra* plants with respect to their HR expression a) Percentage of parasitized *P. rapae* eggs laid on HR+ and HR- *B. nigra* plants b) Percentage of dead *P. rapae* eggs on HR+ and HR- *B. nigra* plants (egg mortality apart of *Trichogramma* parasitism). N=total number of examined *P. rapae* eggs. ***=P<0.001, pair-wise comparison with Chi-square test for homogeneity.

Discussion

Results of the bioassays performed in the dynamic air flow Y-tube olfactometer system, showed that volatile compounds produced by *B. nigra* plants infested with *P. rapae* eggs 24h after oviposition significantly attracted the egg parasitoid *Trichogramma brassicae*. The wasps were able to discriminate between egg-induced plant volatiles when tested against volatiles of uninfested plants, confirming that this wasp species is attracted directly toward the egg-induced plant emission. This strong attraction to *P. rapae* egg-infested plant volatiles is comparable with the results shown by Lucas Barbosa (2010), where bioassays were performed with *P. brassicae* egg-induced plants showing an attraction of *T. brassicae* 24 h after oviposition. That study, together with my results shows that the black mustard is a plant with a defense response to *Pieris* egg-infestation. It is able to develop both direct and indirect defense against *Pieris* egg depositions by expressing egg-killing HR and releasing egg parasitoid attracting cues at the same moment. In contrast, in the Brussels sprout system, *T. brassicae* uses close-range plant cues induced by the two butterfly species 72 h after oviposition (Fatouros *et al.* 2005b; 2008; 2009). There was neither an indication for the emission of egg-induced volatiles nor for HR. Because of the long-range attraction of volatile cues of HR+/HR- *B. nigra* plants, it is likely that the wasps can more easily find eggs on *B. nigra* than on *B. oleracea*. Eggs collected on Brussels sprouts plants showed significantly less *Trichogramma* parasitism than those collected on *B. nigra* (N. Fatouros, personal communication).

Whether *Mamestra brassicae* eggs induce volatiles in *B. nigra* that attract *T. brassicae* wasps still remains unclear. *Trichogramma brassicae* wasps were attracted by egg-infested plants when tested against clean air but could not discriminate between the odors of an *M. brassicae* egg-infested and uninfested plant. This might have several reasons; such as a) no emission/releasing of oviposition-induced volatiles, b) an attraction to host-kairomones (egg odour, scales, etc.) of *M. brassicae* apparent only when tested against clean air (Renou, *et al.* 1992), or c) a small difference in the blends between induced and no-induced plants. A chemical analysis of the plant volatiles could give further insight.

All plants tested in the bioassays, expressed HR in all levels of severity when egg infestation was done with *P. rapae*. However, there was no visible HR in plants infested with eggs of *M. brassicae*. Since both *Pieris* egg-induced HR+ and HR- plants attracted *Trichogramma* wasps (Lucas Barbosa, 2009) a lack of HR expression in response to *M. brassicae* oviposition could give evidence that HR plays an important role in the induction of volatile organic compounds in *B. nigra* attracting *Trichogramma* wasps. Eggs of the pierid butterfly seem to have an HR triggering effect that *M. brassicae* do not. The HR elicitor may be the same that was shown to mediate egg-induced plant defense in Brussels sprouts plants, namely the anti-aphrodisiac pheromones released in minute amounts with the egg secretion (Fatouros *et al.* 2008a; 2009). *Pieris brassicae* oviposition also causes a localized response in *Arabidopsis* with similarity to an HR, including the induction of defense and stress-related whereas the expression of single *P. rapae* oviposition was weaker (Little, *et al.* 2007). If the HR elicitor is the same that triggered the indirect defense in pierid butterflies, *M. brassicae* moths cannot induce the production of plant volatiles and this could explain why *T. brassicae* is not able to distinguish between volatiles of *Mamestra* eggs-infested and uninfested plants. Thus, the plant response against lepidopteran eggs seems to be specific for *Pieris* butterflies, whereas cabbage moth eggs are not defended. However, further studies with different egg ages of *M. brassicae* are needed to confirm this.

The fact that HR and parasitoid recruitment does not result in a conflict in *B. nigra* but rather leads to higher egg mortality by a synergistic effect is underlined by my results from the field collections. In total only 23% of the eggs survived on HR+ plants, compared to 55% on HR- plants. The correlation between HR+ plants infested with *P. rapae* eggs, and the parasitism and egg mortality rates are highly significant, showing that higher mortality and parasitism rate by *Trichogramma* wasp is closely related with high expression of HR in *B. nigra*. A very recent study performed with the larval parasitoid *Cotesia glomerata* showed a similar effect: HR+ *B. nigra* plants infested with 96 h old *P. brassicae* eggs significantly attracted the wasps in a two choice bioassay in the wind tunnel when tested against clean plants (Vafia, 2010). Lucas Barbosa (2009) stated that there is no conflict between HR (direct defense) and plant volatile emission (indirect defense) for the *Trichogramma* arrestment in *B. nigra* when infested with *P. brassicae* egg deposition and later, Vafia (2010) confirmed this statement in *B. nigra* showing that *C. glomerata* is also attracted by plants that expressed HR.

Nevertheless, little evidence in literature is showed about synergistic activity among direct and indirect plant defensive mechanism against herbivores. The spider mite *Tetranychus urticae* induced in tomato *Lycopersicon esculentum* both: direct defense by increasing in protease inhibitor activity one day after infestation and indirect defense by emission of plant volatiles 4 days after infestation, which attract predatory mites (Kant *et al.* 2004). The authors concluded that the tomato plant activates its indirect defenses (volatile production) to complement the direct defense response against spider mites. In the case of wild tobacco *Nicotiana attenuata*, Steppuhn and Baldwin (2007) described that this plant prevent the feeding of *Manduca* caterpillars by regulating the presence of antifeedant toxin nicotine (secondary metabolite), with work together with the negative digestibility impact of trypsin proteinase inhibitors; concluding that *N. attenuata* counters an insect adaptation with a defensive synergism of multiple compounds.

Some plants defense traits that act together gave evidence for multiple trait combinations evolving across species (plant and herbivores) as a characteristic of the so-called plant defense syndromes hypothesis (Agrawal and Fishbein. 2006), where association with specific ecological interactions can result in convergence of defensive traits (Agrawal, 2010). These few examples show some evidence that multiple plant resistance traits may provide a higher level of resistance than could be predicted from their independent action, and in these cases, the opposite of a trade-off is predicted (Agrawal, 2010). My results from laboratory bioassays together with field collection confirm and reinforce this synergy in defense mechanism, since the HR expression to pierid egg deposition (direct trait) works in concert with egg parasitoid recruitment (indirect trait) instead of a being trade-off in terms of energy costs and/ or offspring production. Nevertheless, more research is needed in order to elucidate why in some plant species like *B. nigra*, the synergy of direct and indirect defense works effectively against herbivore egg infestation.

Conclusions

Brassica nigra plants induced by egg oviposition of the small cabbage butterfly *P. rapae* release volatiles that attract the egg parasitoid *Trichogramma brassicae* 24h after oviposition. However, whether the attraction of *T. brassicae* to *Mamestra brassicae* infested plants is due to egg-induced plant volatiles still remains unclear.

B. nigra plants induced by *P. rapae* oviposition expressed several levels of positive HR under laboratory conditions, and still released plant volatiles attractive to *T. brassicae* wasps. None of the tested plants expressed HR against M. brassicae showing a specificity of the defense response against *Pieris* eggs.

In nature, *B. nigra* plants of the Rhine population (Southern Wageningen) expressed high levels of HR when infested by *P. rapae* eggs. This HR expression strongly correlated with *Trichogramma* parasitism and mortality on pierid eggs.

The synergy of direct defense (HR expression) and indirect defense (releasing of egg induced plant volatiles) in *B. nigra*, works effectively against pierid egg deposition but seems not work against cabbage moth egg deposition.

Future directions

To understand the behavior of the *Trichogramma* wasps, it is necessary to chemically identify the cues that take part in the parasitoid attraction/arrestment and the chemical changes involved in plants or plants population that expresses high HR. Furthermore, different egg ages of *M. brassicae* need to be tested to reveal whether older eggs do induce a plant synomone attracting *Trichogramma* wasps.

More research is needed in order to understand the mechanisms of the synergistic activity of direct and indirect plant defense against egg deposition in *B. nigra*, and whether these traits go beyond the herbivore oviposition i.e. the defense against feeding activity.

Acknowledgements

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