

# Mechanisms and ecological functions of direct and indirect defenses induced by *Pieris* eggs in the Black mustard

*Brassica nigra*



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## Abstract

Plants have evolved a multitude of defense mechanisms to avoid or minimize damage caused by insect herbivores. By itself insect oviposition does not appear to be a threat for the plants' growth and development, but rather represent a future threat as they give rise to the feeding larvae. *Brassica* plants defend themselves against cabbage white butterfly (*Pieris*) eggs through induced direct and/or indirect defenses. *Brassica nigra* responds to *Pieris brassicae* eggs directly through a so-called hypersensitivity reaction (HR) that kills the eggs and indirectly by attracting *Trichogramma* egg parasitoids through induced plant volatiles. In *B. oleracea* var. *gemmifera*, eggs of *P. brassicae* induce leaf surface changes that arrest the egg parasitoid *Trichogramma brassicae*. The male-derived anti-aphrodisiac benzyl cyanide (BC), which is passed to the female butterfly during mating, was identified in the accessory reproductive gland (ARG) secretion of mated female butterflies and the responsible elicitor for the induced leaf surface changes. The objectives of the present study were to (1) identify the elicitor of the *P. brassicae* oviposition-induced direct (HR) and indirect (*Trichogramma* attraction) responses in *B. nigra* plants and (2) to investigate the correlation between HR occurrence, egg mortality and parasitism rate in a natural *B. nigra* population. Extracts of ARGs of both mated and virgin *P. brassicae* butterflies were shown to induce HR in *B. nigra*. Furthermore, I showed that BC is present in very small amounts in the ARG of mated females and when applied on *B. nigra* induces HR. In Y-tube olfactometer bioassays, the egg parasitoid *Trichogramma brassicae* was not attracted to volatiles of plants treated with ARG extracts of mated or virgin females, neither 24h nor 72h after treatment. In a field survey, I report that there is a strong correlation between HR occurrence, *Pieris* egg mortality and *Trichogramma* parasitism rate in a natural *B. nigra* patch. More eggs were found dead and/or parasitized on HR+ plants than on plants not expressing this direct defense trait. The results of this study suggest that the elicitor of HR is present in the ARG secretion of female *P. brassicae* butterflies and that other compounds than BC alone induce HR. The elicitor of volatiles induced by *P. brassicae* oviposition in *B. nigra* attracting the egg parasitoid remains unknown. The combination of a direct (HR) and indirect defense trait of some *B. nigra* seems to be a significant mortality factor for *Pieris* butterfly eggs and is likely to increase the plants' fitness.

**Key words:** Hypersensitivity response, elicitor, *Trichogramma* wasps, , accessory reproductive glands, benzyl cyanide.

## Introduction

Plants have evolved a multitude of defense mechanisms to avoid or minimize damage caused by various attackers, e.g. herbivores and pathogens. These defense mechanisms can be direct or indirect and either be constitutively expressed or induced upon attack (Schoonhoven et al., 2005, Walling 2000, Kessler and Baldwin 2002). Direct defense mechanisms are any plant characteristics such as toxins, secondary metabolites, trichomes, thorn etc. that have a negative effect on the performance and behavior of the herbivores (Kessler and Baldwin 2002, Chen 2008). Indirect defense mechanisms are plant characteristics that enhance the effectiveness of natural enemies of the herbivores, e.g. extra floral nectar as alternatively food source, domatia as shelter or the production of volatile organic compounds (VOCs), which provide information on the location of hosts or prey (Kessler and Heil 2011, Dicke 2009, Turlings and Wackers 2004, Kessler and Baldwin 2002). The majority of studies on induced plant defenses against insect herbivores mainly focus on the effect of plants defense towards feeding insects (Dicke 1999, 2009, Dicke et al., 2003, Turlings and Wackers 2004). Relatively little is known about the effect of induced plant defenses against insect egg deposition (reviewed by Hilker and Meiners 2002, 2006, 2009, 2011). While most plants show a defensive response to insect feeding directly after being damaged, the insect causes little or no damage during oviposition (Meiners and Hilker 1997, Hilker and Meiners 2002, Colazza et al., 2004, Fatouros et al., 2005, Little et al., 2007). Interestingly, plants are capable to detect egg deposition and activate direct and indirect defenses against insect eggs before larvae hatch and start feeding (Hilker and Meiners 2006).

Induced direct defenses against oviposition may affect eggs laid on the plant surface, the hatched larvae or the egg-laying gravid female (Hilker and Meiners 2002). The responses can be in various ways such as neoplasm formation, ovidical substance production and hypersensitivity responses (HR) (Doss et al., 2000, Shapiro and De Vay 1987, Seino et al., 1996). The formation of neoplasm is a tumor-like growth of undifferentiated cells developing below the eggs (Doss et al., 2000). This type of direct defense has been observed in some lines of pea (*Pisum sativum L.*) whereby the pea pod forms neoplasm in response to bruchid weevil egg deposition. This causes the bruchid eggs to raise above the plant surface, hence accelerate desiccation and eventually eggs drop off from the leaf surface. Also it increases the risk of the hatched larvae to carnivorous (Doss et al., 2000). Some rice varieties produce an ovidical substance containing benzyl benzoate in response to egg deposition of the white backed plant hopper (*Sogatella furcifera*) (Seino et al., 1996). This chemical weapon causes high mortalities in eggs of *S. furcifera* in rice fields (Seino et al., 1996). In some plants oviposition induces the production of deterrents compounds (i.e. host marking pheromones) that deter conspecific females to lay eggs on the same host (Nufio and Papaj 2001). Induction of oviposition deterrents is a well known example is cabbage plants whereby egg deposition by large cabbage white butterflies induce the production of deterrents that hinder the conspecific female to oviposit on the same host (Blaakmeer et al., 1994). Ovipositional deterrents activity occurs systematically throughout the plant and by doing this, the plant controls the number of egg clutches laid on the surface, hence minimizes feeding damage from hatched larvae (Blaakmeer et al., 1994).

A further direct defense response induced by egg deposition is HR. This type of response commonly controlled by interactions between pathogen a virulence gene and plant resistance gene (gene for gene), and usually involves rapid cell death at the site of infection (Heath 2000). Moreover, some plant species also show HR against insect herbivory at the site of oviposition or feeding (Fig 1) (Shapiro and De Vay 1987, Balbyshev and Lorenzen 1997, Garza et al., 2001, Fernandes et al., 2003). defense response. By producing a necrotic zone at the base of the eggs, HR either kills the eggs through desiccation (Fig 1.) and/ or detachment from the plant surface and eggs fall on the soil where they are

subject to predation (Balbyshev and Lorenzen 1997, Garza et al., 2001 Shapiro and De Vay 1987). Hypersensitive response has been reported in black mustard plants (*Brassica nigra*), where it kills the eggs of *Pieris* butterflies (Shapiro and De Vay 1987). More evidence of egg-killing HR is reported in beans against *Apion godmani* (Coleoptera) eggs (Garza et al., 2001) and in potato (*Solanum spp*), where eggs of the notorious pest, the Colorado potato beetle, are detached from the plant surface and are predated by ground beetles (Balbyshev and Lorenzen 1997). In *Arabidopsis thaliana*, HR occurs in absence of cell death, hence no vivid necrotic zone is formed on the leaf surface. However, *A. thaliana* plants express HR related genes (*PR 1-5*) after Pierid butterfly egg depositions (Little et al. 2007).



**Fig 1.** Hypersensitivity response induced by *P. rapae* eggs on *B. nigra*. Shriveled and dead egg due to desiccation (left picture) and parasitized egg (right picture) both with strong necrosis zone at the base and collected in the field. (Photo credits: Mpoki Shimwela).

Additionally to the direct defenses, plants also employ indirect defenses against insect eggs. Insect egg deposition has been shown to induce the emission of plant volatiles or modifications of the plant surface chemistry resulting in attraction or arrestment of egg parasitoids (reviewed by Hilker and Meiners 2002, 2006, 2009, 2011). The induction of volatile organic compounds (VOCs) by egg deposition can occur locally at the site of oviposition and systematically in uninfested plant parts (Meiners and Hilker 1997, 2000, Hilker et al., 2002, Colazza et al., 2004). These tritrophic interactions have been observed in various systems: in elm trees, oviposition by the elm leaf beetle induces the emission of a plant synomone that attracts the eulophid wasp *Oomyzus gallerucae* (Meiners and Hilker 1997) whereas in pine trees, sawfly eggs induce the pine needles to produce odours that attract another eulophid wasp, *Chrysonotomyia rufum* (Hilker et al., 2002). Moreover, *C. rufum* needs a background odour of the non-attractive pine volatile (sesquiterpenes i.e. (E)- $\beta$ -caryophyllene and  $\delta$ -cadinene) in order to detect (E)- $\beta$ -farnescence induced by eggs deposition in pine (Mumm and Hilker 2005). Furthermore, in both systems the emission of the synomone is induced only after damage caused by the herbivore female during oviposition. In two annual bean plants, it was shown that eggs of the pentatomid bug (*Nezara viridula*) induce VOCs that attract the scelinoid wasp *Trissolcus basalis* (Colazza et al., 2001). In another crop, Brussels sprouts, eggs of cabbage white butterflies (*P. brassicae* and *P. rapae*) induce leaf surface modifications that arrest *Trichogramma* egg parasitoids 72h after egg deposition (Fatouros et al. 2005, 2009, Pashalidou et al., 2010). In contrast, it has been



shown that egg deposition of *P. brassicae* and *P. rapae* induces VOCs in black mustard plants, starting to be attractive to *T. brassicae* wasps 24h after egg deposition (Lucas-Barbosa 2009, Figueroa 2010).

According to Meiners and Hilker (2006) egg-associated secretions released during oviposition play a crucial role in eliciting direct and indirect plant defenses against insect egg deposition. The plant perceives these elicitors through damage caused by gravid females during oviposition or feeding (Meiners and Hilker 2006). To date only few elicitors inducing direct and indirect responses to insect egg deposition have been identified based on chemoecological and physiological studies (Hilker and Meiners 2010). Bruchins (3-hydroxypropanoate) are the only known elicitor for a direct defense response against egg depositions (Meiners and Hilker 2006, Hilker and Meiners 2011). These esters isolated from bruchid beetle females applied on pea pods mimic the formation of neoplasm (Doss et al. 2000). Shapiro and De Vay (1987) suspect that egg cementing material of *Pieris* butterflies contains the main elicitor of HR in *B. nigra*. So far only one study has investigated and confirmed this hypothesis (Put 1996). Nevertheless, the main eliciting compound(s) of HR remain unknown. Proteins present in the oviduct secretion of both elm leaf beetle and pine sawfly are responsible for the VOC emission attracting the egg parasitoids (Meiners and Hilker 2000, Hilker et al. 2005). Male *Pieris* butterflies are known to transfer anti-aphrodisiac compounds to females during mating, which render the female unattractive to other males (Andersson et al., 2003). The same anti-aphrodisiac compounds, benzyl cyanide (in *P. brassicae*) and indole (in *P. rapae*), are released in minute amounts with the egg secretion during egg deposition and were shown to be responsible to elicit the leaf surface changes in Brussels sprouts that arrest *Trichogramma ssp.* (Fatouros et al., 2008, 2009, Palishadou et al., 2010).

### Research objectives and questions

The objectives of this study are to (1) identify the elicitor of the *P. brassicae* oviposition-induced direct (HR) and indirect (*Trichogramma* attraction) responses in *B. nigra* plants and (2) investigate the correlation between HR occurrence, egg mortality and parasitism rate in a natural *B. nigra* population. More specifically I address the following research questions:

- (a) Does the ARG secretion of *P. brassicae* females induce HR and/or *Trichogramma* attraction in *Brassica nigra*?
- (b) Does mating change the eliciting function of the ARG secretion?
- (c) What is the amount of BC in the ARG secretion of mated female butterflies of the Swiss *P. brassicae* population?
- (d) Is benzyl cyanide (BC) involved in eliciting HR and/or parasitoid attraction? If so, what is the amount of BC needed to induce HR/ parasitoid attraction?
- (e) Are *Pieris* eggs on HR+ plants more parasitized than eggs on HR- plants in a natural population?
- (d) Do eggs on HR+ plants show higher egg mortality than on HR- plants of the same *Brassica nigra* population?

## Materials and Methods

### Plants and Insects

The black mustard (*Brassica nigra* L.) plant accession CGN 06619 (Feral population collected in 1975 from the Peloponnesus, Greece) was used for the experiments. The plants were grown in a greenhouse compartment ( $22 \pm 2$  °C, 70% rh, L16:D8). Plants of 4 to 5 weeks old were used for the experiments.

*Pieris brassicae* were reared on Brussels sprouts plants in a climate room ( $21 \pm 1$  °C, 50-70% rh, L16:D8). Compared to studies of Fatouros et al., (2008) the used *P. brassicae* population was obtained from a colony from the Swiss Federal Institute of Technology in Zurich, Switzerland. The male and female butterflies were separated during the pupal stage; virgin females and males were kept separately until dissection or mating. One day after eclosion, the butterflies were allowed to mate for the first time. The observed mated couples were separated in order to obtain mated females for dissection. Accessory reproductive glands were dissected from mated and virgin females, 3 days after eclosion according to Fatouros et al., (2008, 2009).

*Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae), strain Y175, was reared in irradiated eggs of the moth *Ephestia kuehniella* Zeller (Lepidoptera; Pyralidae), received from Koppert B.V., The Netherlands, at  $25 \pm 1$  °C, 50-70% RH, L16: D8 in a climate chamber. Only mated, 2-5 days old, naïve female wasps were used for the experiments.

### Dissection and preparation of ARGs

To obtain samples for plant treatments, the ARGs were dissected from mated or virgin *P. brassicae* females (3 -4 days old). Soon after dissection three glands were transferred to 200 µL Phosphate buffered saline (PBS; PH 7.2) and homogenized. Thereafter, the homogenate was centrifuged at maximum speed for 5 minutes, and the supernatant was used for application onto the plants. For the quantification of benzyl cyanide (BC) in the glands of mated females, an ARG was dissected and transferred to a vial containing 50 µL dichloromethane (DCM) plus 0.5 µL phenyl cyanide as internal standard. 14 ARGs were analyzed. The ARGs of virgin females were not analyzed because a previous study already proved that no BC was present (Fatouros et al., 2008).

### Plant Treatments

For the bioassays and HR expression tests, 100 µL of ARG supernatant were applied with a fine brush onto the edge of *B. nigra* leaves, on the abaxial side of the leaves (a 100 µL ARG homogenate equivalents 1.5 glands per plant). In a similarly way, control plant were treated with PBS only (compare Fatouros et al. 2008). Thereafter, treated plants were kept for either 24h or 72 h in a climate chamber ( $22 \pm 2$  °C, 70% HR, and L16:D8 h). Since HR is the strongest at 72h, it was recorded at this time point. To examine whether pure BC induces HR 72h after application a 100 µl of 0.5, 1.5 and 2 ng BC/µl diluted in Ethanol (70%) was applied as described above. Control plants were treated with 100 µl ethanol only. Ethanol was chosen in this study because other solvents i.e. dichloromethane (DCM) and methanol, which were used in previous studies to treat the very waxy and thick Brussels sprouts leaves (Fatouros et al., 2008, 2009), caused severe damage to the less waxy *B. nigra* leaves even at very low concentrations.

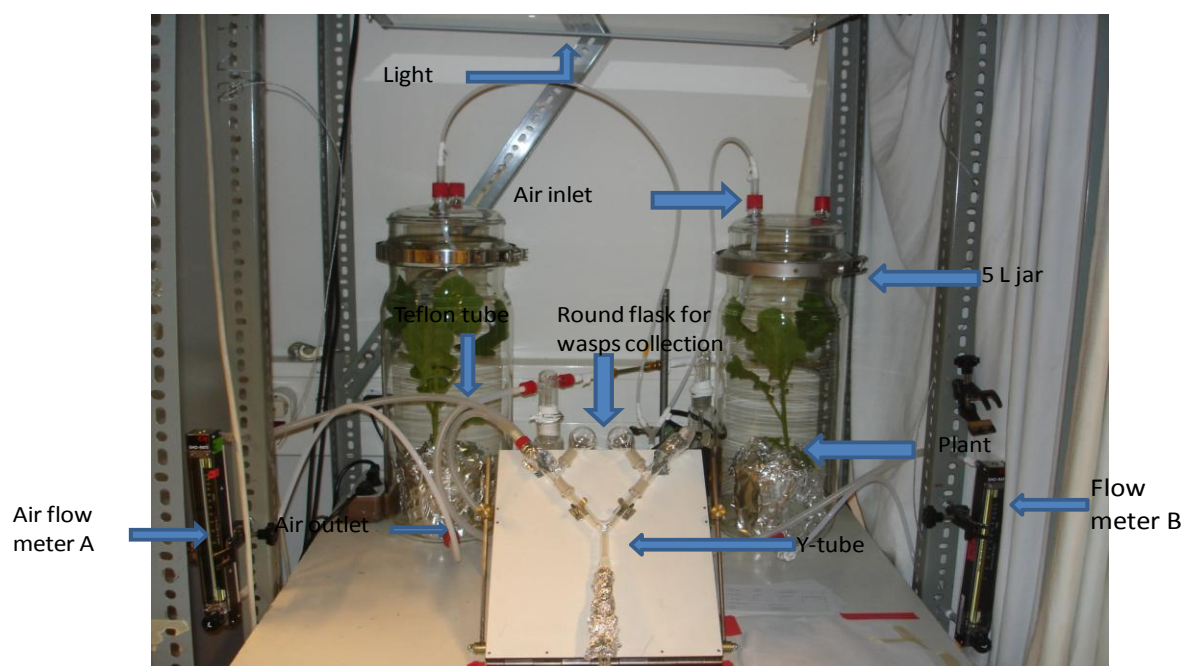
### Bioassays

Dual-choice tests were carried out using a dynamic air-flow Y-tube olfactometer, described in detail by Lucas Barbosa (2010). It consisted of two glass arms shaped as a Y tube (1 cm inner diameter, 9cm stem and 8 cm arms at 100° angle) and two 5L glass containers holding the odour sources (Fig. 2). Air was pumped through activated charcoal, humidified and split into two air stream that were fed to the

glass containers and subsequently to each arm of the olfactometer at a flow rate of 100 mL/min (Flow meter , Brooks Instruments B.V. Veenendaal, NL) (Fig. 2). All connections between glass and flow meters were made using Teflon tubing. All tests were conducted at constant temperature  $24 \pm 1^\circ\text{C}$ . Above the Y tube and container was a light using 4 fluorescent tubes (24W, Viva lite) (Fig. 2). Before being placed into the glass containers the pots were removed from the plants and soil and roots were covered with aluminium foil (see fig. 2.). This enables avoidance of unwanted volatiles emitted from soil and roots.

A choice between two odour sources was offered to a group of ten adult female *T. brassicae* (2-5 days old) for 30 minutes. Thereafter the odour sources and Y-tube were switched in order to exclude biased effects; hence in total 20 wasps were used per treatment per day on five different days. The response was recorded when the wasps reached the end of one of the two arms. All wasps that did not reach the end of the arms (no choice within 30min) were regarded as non responders and were excluded from statistical analysis. The following choice combination were evaluated:

Combinations No.	Treatment	Induction time
1	AGR (Virgin) vs PBS (control)	24h and 72 h
2	AGR (Mated) vs PBS (control)	24h and 72h
3	AGR (Mated) vs AGR (Virgin)	72h



**Fig. 2.** Dynamic airflow Y-tube olfactometer set - up for bioassays with *Trichogramma* wasps (Photo credits: Mpoki Shimwela).

### Quantification of BC in ARG

An 7890A gas chromatography (GC) coupled to 5975C mass spectrometry (MS) (Agilent Technologies, Palo Alto, CA, USA) was used for separation and detection of BC in glands of mated female *P. brassicae*. Two micro liter per sample extract were injected in splitless mode, with a splitless time of 2 min at an injector temperature of  $250^\circ\text{C}$  using an 7683B series auto sampler (Agilent). The separation was carried-out using a ZB-5MS analytical column with dimensions – 30m L x 0.25 mm I.D. x 0.25 m F.T. (Phenomenex, Torrance, CA, USA). The column was operated at an



initial temperature of 45 °C for 2 min and raised at 10 °C min<sup>-1</sup> to 280 °C and held for a less than a second under a column flow of 1 ml min<sup>-1</sup>. The MS was operated in a scan mode with a mass range of 45 – 450 amu at 3.54 scans s<sup>-1</sup> and ionization was performed in EI mode at 70 eV. MS transfer line, ion source, and quadrupole were set at 280 and 230 and 150 °C, respectively. Identification of BC and internal standard phenyl cyanide was performed by comparing the mass spectra with those in the NIST 2005 MS libraries and retention times of pure standard (98% purity, Sigma – Aldrich). The amount of BC in the glands was quantified based on comparison with the internal standard phenyl cyanide . Preliminary tests revealed very low BC amounts and hence different BC concentrations were quantified by calibration series (1ppb, 10ppb, 100ppb, 1ppm and 10ppm).

### Field survey

A survey was conducted to record *Trichogramma ssp.* parasitism, HR and survival of *Pieris* eggs on individual *B. nigra* plants in a natural population. The survey was conducted in a *B. nigra* patch along the river Rhine bank in Wageningen from early May to mid-July 2011. Eggs were collected and checked for the presence of HR on the leaf and/or parasitism by *Trichogramma* wasps. After collection, eggs were kept in a climate chamber at (25±1 °C, 50-70% RH, L 16: D8 ) until caterpillars or wasps emerged. All hatched, dead and parasitized eggs were recorded. Hatched caterpillars were fed with black mustard/cabbage leaves until 4<sup>th</sup> instar for species identification. The emerged wasps were preserved in 100% ethanol and kept in freezer at -25°C for future identification using molecular tools.

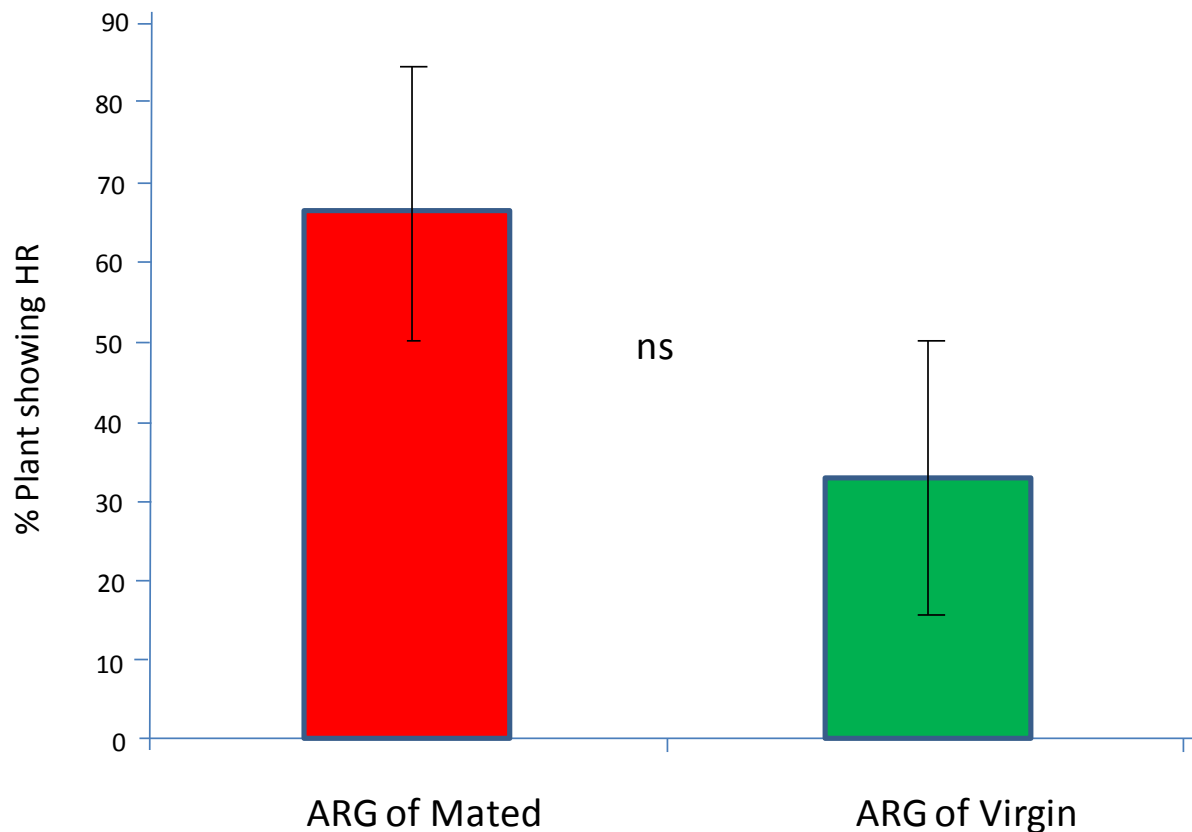
### Statistics

A non-parametric two-sided binomial test (0.50) was used to determine the choice of *Trichogramma brassicae* between two odour sources. Development of HR between plants treated with ARG secretion from mated or virgin females was analyzed using a chi-square test for independency. Pairwise comparison between parasitism rate and mortality rate due to HR was analyzed a 2\*2 contingency table followed a by chi-square test for homogeneity .

## Results

### Effect of ARG secretion on HR

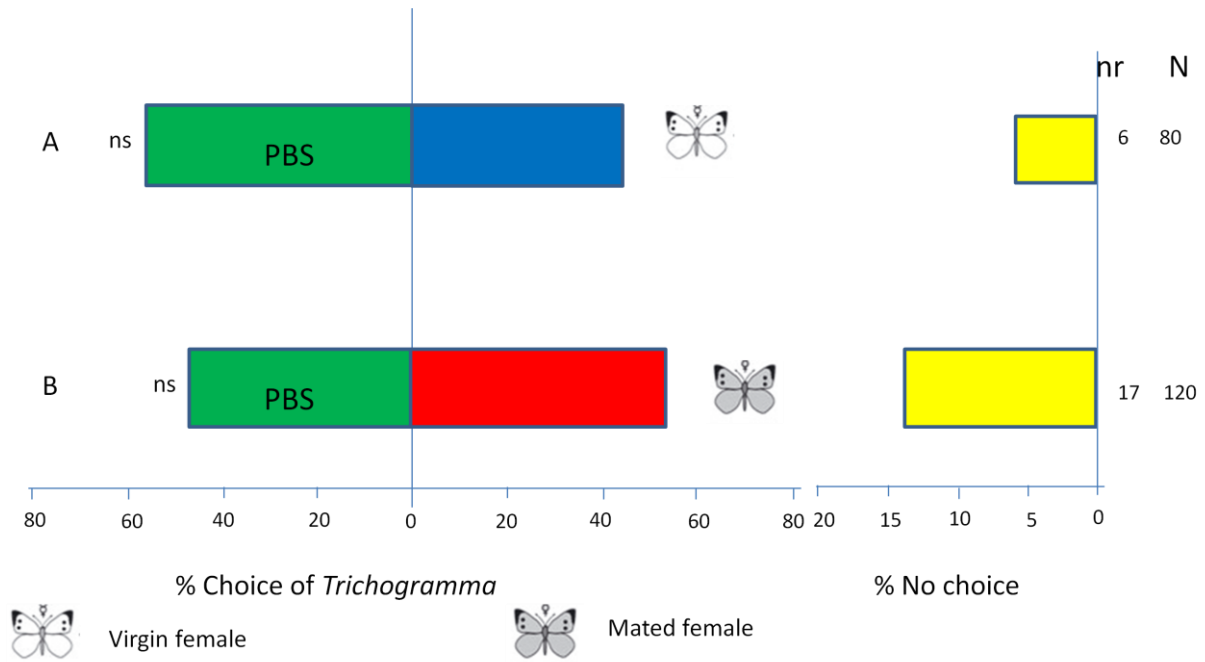
To test whether ARG secretion induces HR and whether mating plays a role in it, *Brassica nigra* plants were treated with ARG secretion of mated and virgin females. The number of plants that developed HR did not significantly differ between plants treated with ARG secretion of mated or virgin females ( $P = 0.1407$ , Chi square test with 2\*2 contingency, Fig 3.). However, there was a trend that ARG secretion of mated females induced HR in a higher number of plants than ARG secretion of virgin females. In all ARG-treated plants expressing HR, a necrotic zone started to develop within 48h after application. No plant expressed HR after treatment with PBS.



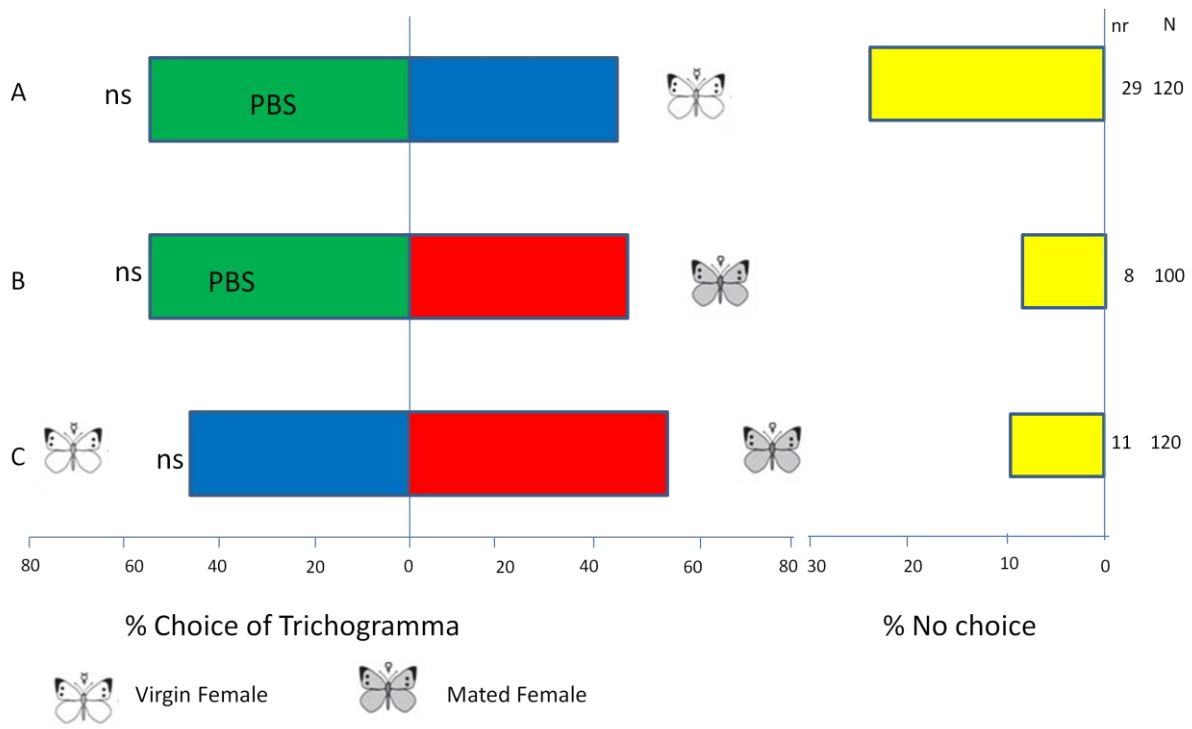
**Fig 3.** Percentage of plants showing HR 72 h after application of ARG extract of mated (red column) or virgin (green column) female *P. brassicae* butterflies. (Each bar represents percentage  $\pm$  SE of each treatment,  $N = 23$ , ns not significant,  $\text{Chi}^2$ -test.)

### Effect of BC on indirect defense

To evaluate the effects of ARG secretion on the induction of plant volatiles attracting *Trichogramma brassicae* wasps, a series of tests were performed in the y-tube olfactometer. Naïve female *T. brassicae* did not discriminate volatiles of plants treated with ARG secretion of mated females or virgin females 24h after treatment when tested against volatiles of PBS-treated plants ( $P = 0.694$  and  $P = 0.356$  binomial test, Fig 4a,b). Likewise no discrimination was observed 72 h after treatment ( $P = 0.402$  and  $P = 0.466$ , binomial test, Fig 5a,b). Furthermore, *T. brassicae* did not show a significant response when offered a choice between volatiles of plants treated with ARG secretion of mated females against volatiles of plants treated with ARG secretion of virgin females ( $P = 0.338$ , binomial test, Fig 5c).



**Fig 4.** Response (% choice ) of *T. brassicae* to volatiles emitted by *B. nigra* plants 24 h after treatment with ARG extracts of mated (red bar) or virgin (blue bar) female *P. brassicae* or PBS (green bars). Bars with yellow color indicate the percentage of non-responding wasps. N=, total number of tested wasps, nr = number of non responding wasps, ns not significant, Binomial test.



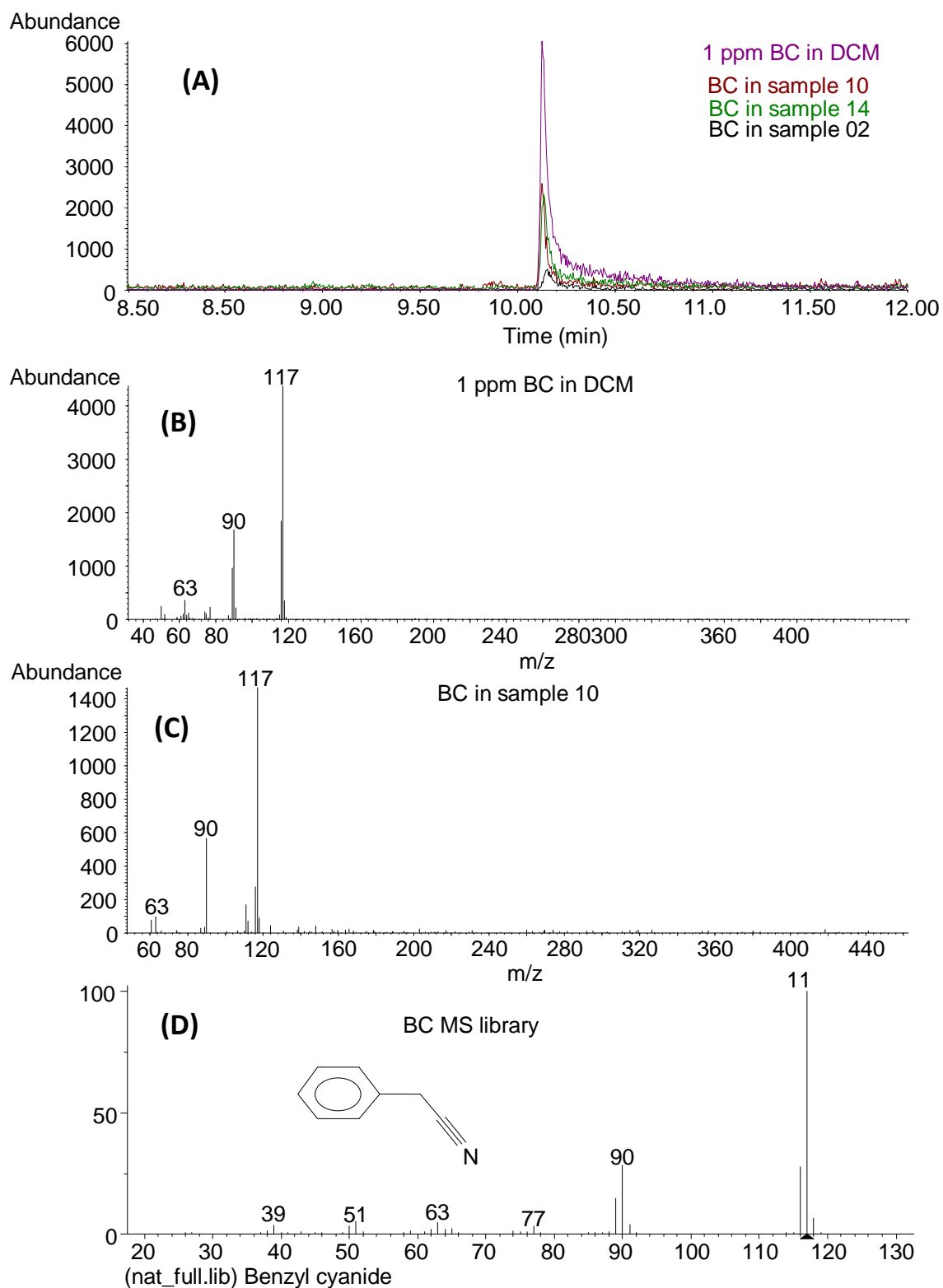
**Fig 5.** Response (% choice) of *T. brassicae* to volatiles emitted by *B. nigra* plants 72 h after treatment with ARG extracts of mated (red bars) or virgin female (blue bars) *P. brassicae* or PBS (greens bars) indicate percentage of wasps responding to different choices. Bars with yellow color indicate percentage of non-responding wasps. (N=, total number of tested wasps, nr = non responding wasps, ns not significant, Binomial test ).

#### Amount of BC in ARGs of mated females

To evaluate whether BC induces HR in *B. nigra*, and whether it is present in the ARG of mated females of the new population of *P. brassicae*, the glands were chemically analyzed and the amount of BC measured by GC-MS (Fig 6.). Benzyl cyanide was detected in 7 of the 14 analyzed ARGs. Only 1ppm and 10pmm detected by machine, other concentration 1ppb, 10ppb, and 100ppb, were not detected. Based on this concentration of BC estimated to range from 0.1 ng < 1 ng BC/μl dichloromethane ARG.

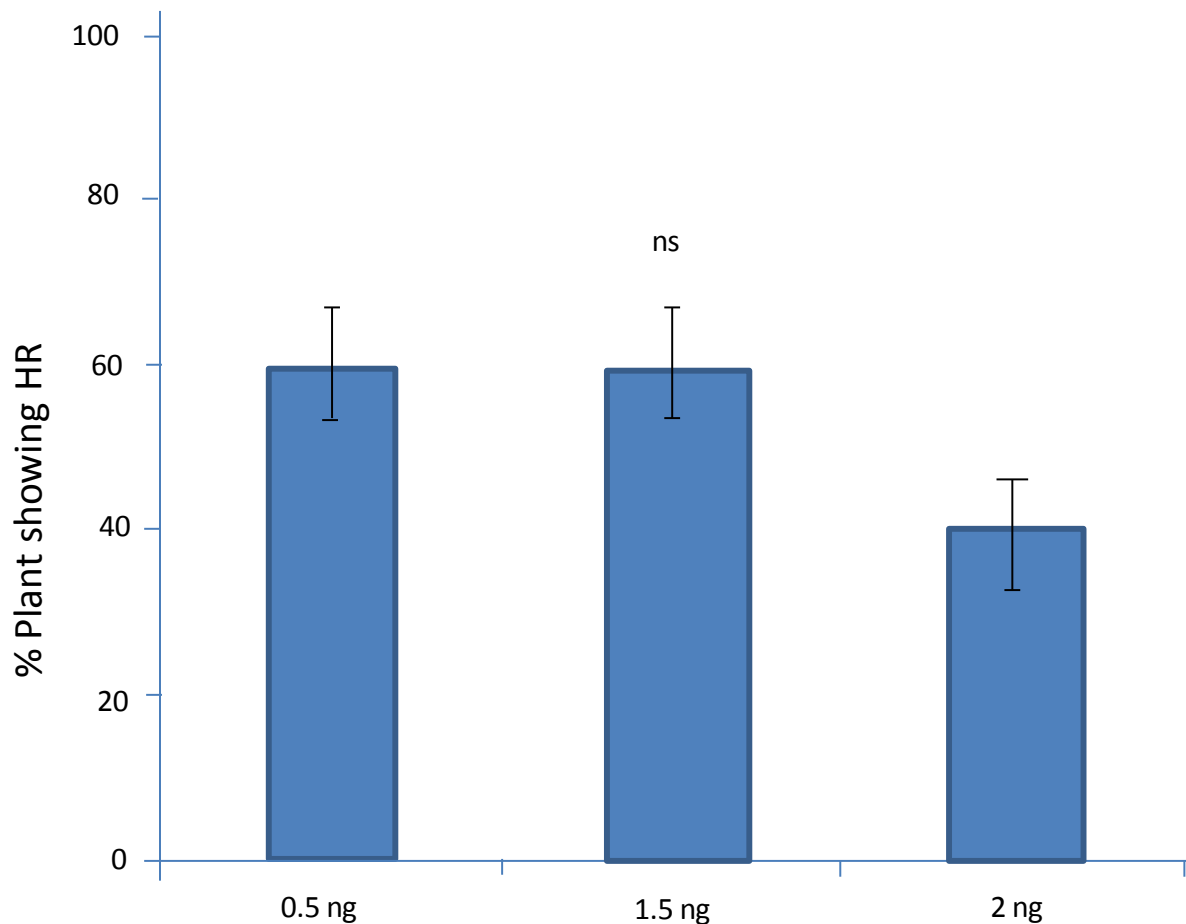
#### Effect of BC on HR expression

Different concentrations of synthetic BC (0.5 ng, 1.5 ng and 2ng) were prepared and applied onto the leaves. Percentage of BC-treated plants expressing HR was 60% at 0.5 ng, 60% at 1.5 ng and 40% at 2 ng BC 48-72 h after treatment (Fig. 7). This percentage was not statistically significant. Moreover, no HR was observed in any of the control plants.



**Fig 6.** (A) Overlaid chromatograms displaying BC in 3 out of the 14 analyzed ARG samples and 1 ppm BC standard both in dichloromethane; (B) Mass spectra (MS) of synthetic BC from 1 mg L<sup>-1</sup> concentration; (C) MS of BC from DCM extract of ARGs, and (D) MS of BC from the Wageningen MS database of natural products.





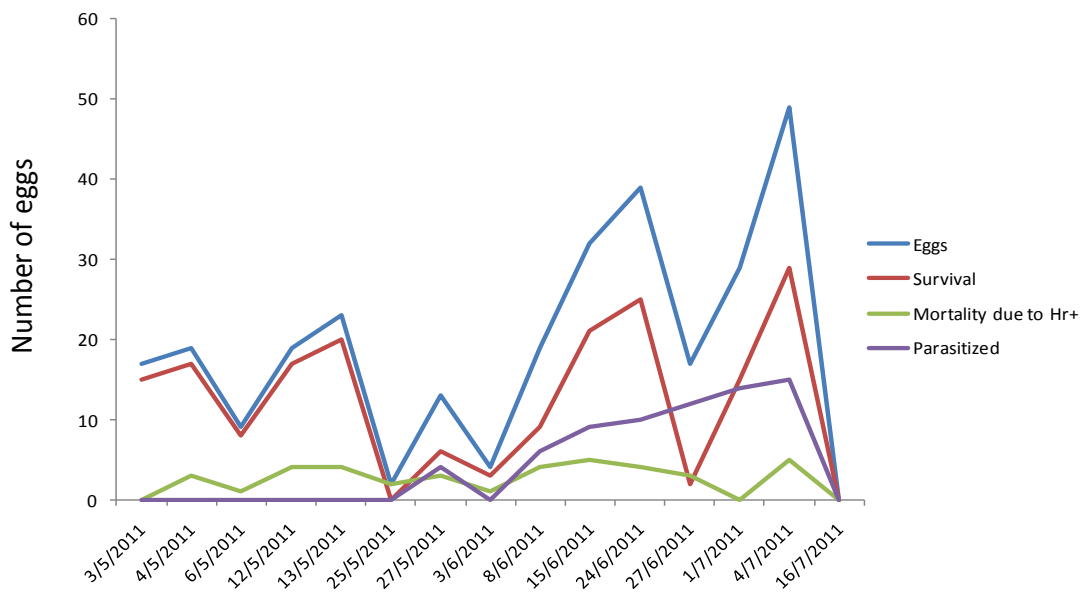
**Fig 7.** Percentage of plants showing HR 72h after application of BC in different concentrations (0.5, 1.5 and 2 ng). Each bar represents percentage  $\pm$  SE of each concentration, Total number of tested plants = 15, ns not significant, Binomial test).

#### **Egg mortalities in a natural population of *B. nigra***

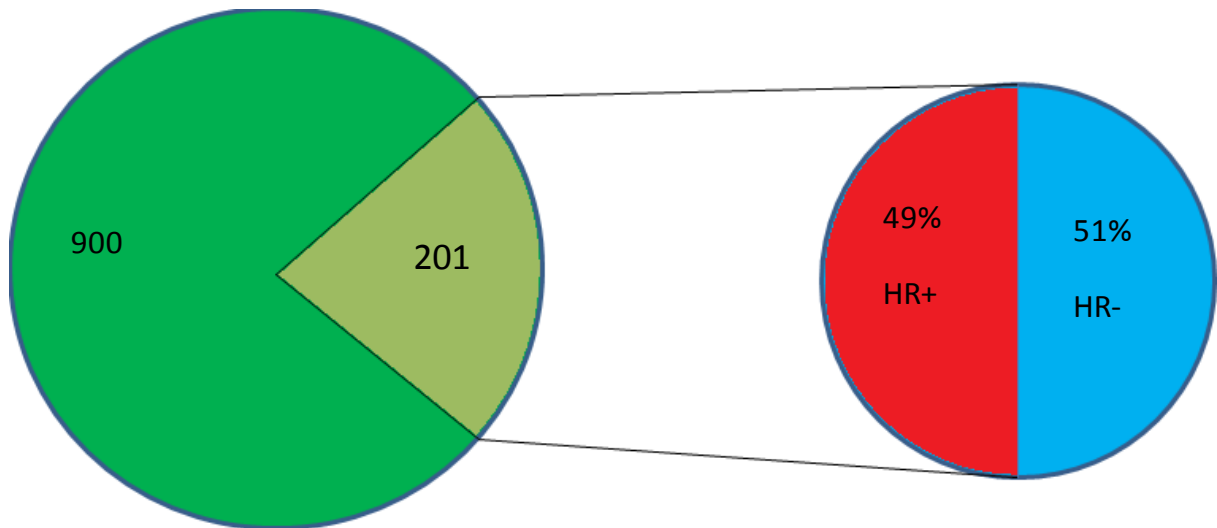
A path divided the surveyed area into two patches and the total area monitored was approximately 50 m<sup>2</sup>. The average number of plants in the area was estimated to be of approximately 900 individuals. At the start of the survey in early May, most plants were very young (few weeks old) and only 5-20 cm high (Fig. 8), while at the end of the field survey in mid July plants were of 1- 1.5 m height. Generally, the peak of the number of eggs collected was in the whole month of June (Fig. 9). A total of 291 *Pieris* eggs was collected from 201 plants, out of which 98 (49%) plants showed HR and 103 (51%) plants did not express HR against the monitored *Pieris* eggs (Fig. 10).



**Fig. 8.** Height of the monitored *B. nigra* plants on which most of the eggs were collected.



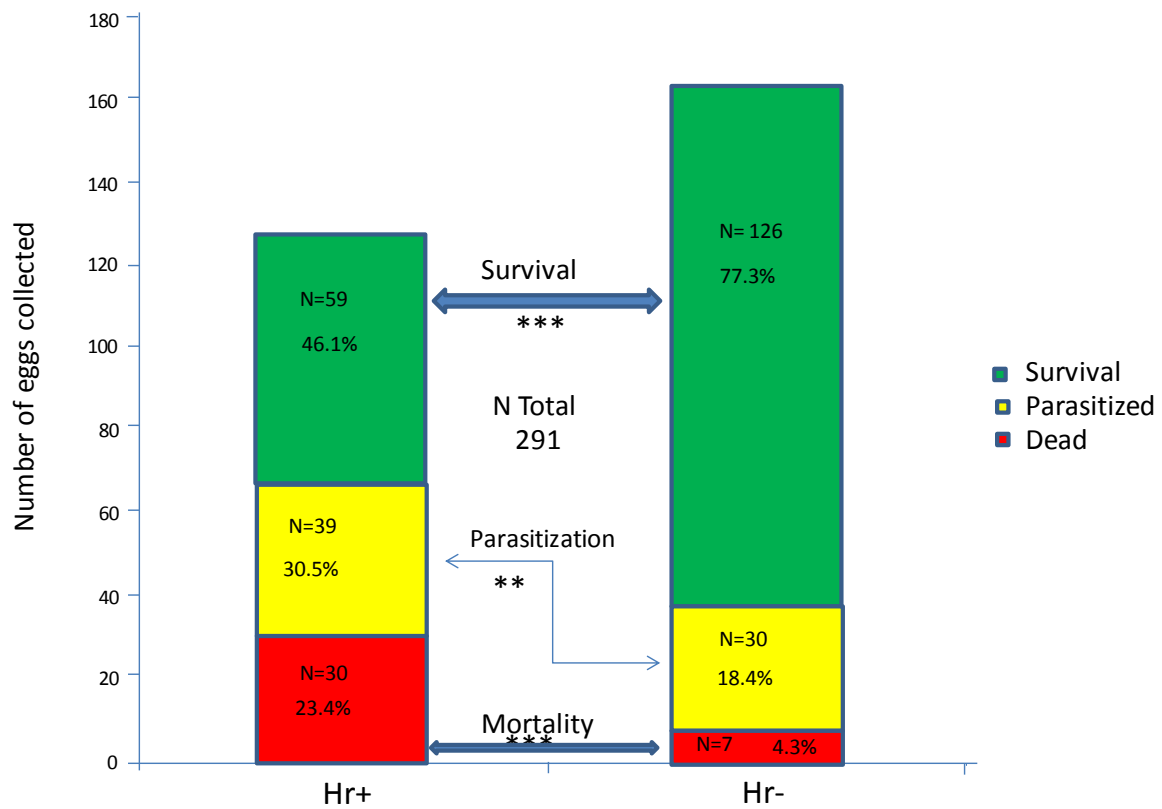
**Fig 9.** Number of *Pieris* eggs collected from 3/5/2011 to 16/7/11, survival rate, mortality due HR and parasitism rate.



**Fig 10.** Average number of plants in the patches (N=900), and total number of plants were *Pieris* eggs were collected from (N=201). Percentage of plants with no hypersensitive response (HR-) and percentage of plants with hypersensitive response (HR+) against egg deposition are shown in the right pie.

All collected *Pieris* eggs were checked for parasitism by *Trichogramma* wasps and whether they were surrounded by a necrotic zone (HR). The first eggs parasitized by *Trichogramma* wasps were collected on May 25<sup>th</sup> (Fig. 9). Significantly more parasitized eggs were found on HR+ plants than on HR- plants (P=0.0237, Chi-square test 2\*2 contingency table Fig. 11).

The mortality of *Pieris* eggs is significantly higher on HR+ plants (P < 0.001, 2x2 contingency table, Chi<sup>2</sup> -test, Fig. 11). Of all 291 collected eggs from the field only 37 eggs died (12.7 %) 2 to 3 days after sampling, of which 81% died due to hypersensitive response, while only 19% of the collected eggs died due to other reasons, i.e molds or dried out due to lack of moisture in the petridish. In total, 46.1 % of the collected eggs hatched on HR+ plants, significantly less than the 77.3 % eggs hatching on HR- plants (P<0.001, 2x2 contingency table, Chi<sup>2</sup> test, Fig. 11). From all hatched eggs 97% were identified as *Pieris rapae* and only 3% were *Pieris napi*. No egg clutch of *P. brassicae* was collected in the monitored *B. nigra* population.



**Fig. 11.** Number of collected eggs on *B. nigra* plants (HR+/ HR-). Total number of collected eggs, survival rate (green), parasitism rate (yellow) and mortality (red) are shown for eggs collected on HR+ and HR- plants. \*\*\* P<0.001, \*\*P<0.01, 2x2 contingency table followed by Chi<sup>2</sup> test.

## Discussion

Earlier studies have shown that the eggs and accessory glands of mated female *Pieris* butterflies induce a local hypersensitivity response (HR) on leaves of *Brassica nigra*, starting 24 h after deposition or treatment (Shapiro and De Vay 1987, Put 1996). In Brussels sprouts, Fatouros et al., (2008, 2009) showed that an extract of accessory reproductive glands (ARG) of mated *Pieris* females induced a local plant response that arrested *Trichogramma* egg parasitoids 72 h after application. Surprisingly, results of the present study show that the extract of accessory reproductive glands (ARG) of both mated and virgin females induces HR in *B. nigra* leaves. This finding is conversely to what I have expected, namely that only the ARGs of mated females induce HR when applied on the leaves of *B. nigra*. This is the first report, which shows that ARG of virgin female *P. brassicae* butterflies induces a direct response. This finding raises the question on what other compounds present in the glands of both mated and virgin female butterflies could elicit HR. In this study, I found variation among *B. nigra* plants in their direct response to eggs: when ARG extract was applied from the same group of butterflies, some plants showed very strong HR while others did not show any sign of HR even after 72 h. This finding is in agreement with my field results in which 49% of sampled plants showed HR. Moreover, Lucas-Barbosa (2009) and Shapiro and de Vay (1987) also observed differences in HR expression among individual plants of the same *B. nigra* population in the Netherlands and in California, U.S.

Recently, the anti-aphrodisiac benzyl cyanide (BC) was detected in the ARG of mated female *P. brassicae* and shown to elicit indirect defense in Brussels sprouts plants (Fatouros et al., 2008). Similarly, the anti-aphrodisiac compound of *P. rapae* indole was detected in the ARG from mated females and identified as responsible to elicit phytochemical leaf changes induced by *P. rapae* eggs that arrest *Trichogramma* wasps in Brussels sprouts plants (Fatouros et al., 2009, Pashalidou et al. 2010). According to Fatouros et al., (2008) about  $1.4 \pm 1.5$  ng BC/ $\mu$ l DCM/ ARG was detected from mated females of a different *P. brassicae* population from The Netherlands, whose exact time of mating was unknown. In the present study, concentrations of analyzed glands range from  $0.1 \text{ ng} < 1 \text{ ng}$  BC/ $\mu$ l DCM/ARG in half of the analyzed ARGs of mated *P. brassicae* females of the Swiss population. This is about a 1/4 of BC what has been shown to be present in the glands of the Dutch *P. brassicae* population and could be the reason, why the applied gland extracts did only partly induce HR in *B. nigra*. To date, only the Bruchins in the secretion of bruchid beetles are chemically identified as an elicitor of direct defense induced by oviposition (Doss et al. 2000). The present study is the first showing that BC induces HR when applied onto *B. nigra* leaves. However, the fact that BC was isolated only from glands of mated female *P. brassicae* (Fatouros et al., 2008), and that in the present study ARG of both mated and virgin females induced HR, shows that BC seems not the only compound involved in eliciting HR in *B. nigra*. It is likely that other compounds present in ARG of mated and virgin female butterflies are also involved in inducing HR. For example, Hilker et al., (2005) identified a proteinaceous substance present in the oviduct secretion of the sawfly *Diprion pini* eliciting the emission of plant volatiles that attract the egg parasitoid to pine trees.

Studies on Brussels sprouts plants showed that *P. brassicae* eggs and the ARG secretion of mated females induce leaf surface changes that arrest *Trichogramma* wasps 72h after application (Fatouros et al., 2005, 2008, 2009, Pashalidou et al., 2010). In contrast to the cropplant, the wild crucifer *B. nigra* responses to *P. brassicae* egg deposition with a change in the volatile emission attracting *T. brassicae* wasps 24h after oviposition (Lucas-Barbosa 2010). Although the indirect defense response in the two *Brassica* species is different, I expected that the ARG extract of mated females is involved in the



elicitation of both oviposition-induced plant synomones. Surprisingly, results of the present study showed that ARG secretion of mated and virgin females did not induce plant volatiles attractive to *Trichogramma* wasps 24 h - 72 h after treatment. So far, an eliciting function of the herbivores' oviduct secretion has been shown in all previous studies on oviposition-induced plant volatiles (Hilker et al., 2002, 2005, Fatouros et al., 2008, 2009, Meiners and Hilker 2000). Again, I suspect that low concentration of BC detected in the ARGs extract of mated females of the Swiss population compared to the studies by Fatouros et al. (2008) done with a different *P. brassicae* strain might be a reason for the lack of elicitation of attractive volatile to *Trichogramma* wasps.

Occurrence of HR induced by *Pieris* eggs has been reported in several studies of Dutch *B. nigra* populations (Put 1996, Lucas-Barbosa 2009, Figueroa 2010). However to date, no data are available on the proportion of individual *B. nigra* plants responding to *Pieris* eggs with HR. In the present study, 49% of all plants on which *Pieris* eggs were collected expressed HR. This shows that the plants of this natural *B. nigra* population are highly adapted of using HR as direct defense trait towards *Pieris* eggs. Lucas-Barbosa (2009) showed in greenhouse studies that HR is age-dependent, in which most of the young individual responded with HR to *P. brassicae* eggs. Similar trends have been observed in this study: mainly young plants and/or young leaves responded directly to *Pieris* eggs by HR (data were not shown).

For the first time, Lucas-Barbosa (2009, 2010) reported a synergism between HR (direct defense) and attraction of *Trichogramma* wasps by oviposition-induced plant volatiles (indirect defense). Furthermore, Figueroa (2010) showed that, natural parasitism rates of *Pieris* eggs by *Trichogramma* wasps were highly correlated with HR occurrence: 40% of the parasitized eggs were collected on HR+ plants compared to 30% on HR- plants. In total, 77% of the collected eggs died on HR+ plants compared to 45% on HR- plants. These findings are in parallel with my results, which show that *Trichogramma* wasps parasitized significantly more *Pieris* eggs on plants that show HR. In the present study parasitization started late May and increased afterward, about 30.5% eggs collected from leaves showing HR+ were parasitized, while only 18% parasitized eggs were collected from leaves lacking HR.

Apart from increased parasitism rates, HR also accelerated *Pieris* egg mortality rates under natural conditions (Figueroa 2010, this study). Various studies reported that HR is an important local defense response to control insect populations under natural conditions (Garza et al., 2001, Balbyshev and Lorenzen 1997, Bentur and Kalode 1996, Shapiro and De Vay 1987, Fernandes et al., 2003). According to Shapiro and De Vay (1987), HR kills eggs of *Pieris* butterflies through desiccation. In beans, eggs of *Apion godmani* are killed and encapsulated by a necrotic tissue (Garza et al., 2001). Similarly in the present study, most of the collected *Pieris* eggs with a surrounding necrosis died due to desiccation. About 23.4% eggs collected from leaves showing HR+ were killed and only 4.3% killed by other factors like mold in HR- leaves. Differences in the season of eggs collected in the present study (May-July) and by Figueroa 2010 (August – September), might be a reason for the different mortality rate. In contrast to HR in *B. nigra*, Balbyshev and Lorenzen (1997) observed that the necrosis itself had no direct effect on the mortality of Colorado Potato beetle eggs, however, it causes the eggs to detached from the leaves and fall down on the soil where they subjected to predators i.e ground beetle. In this way the plant with HR trait minimized the damaged caused by the herbivores. Hence this is a positive results for potential utilization of host-plant resistance and biological control in integrated pest management.

### Conclusions and future directions

This study reveals that the ARG extracts of both mated and virgin females *Pieris* butterflies induce HR 72 h after application on *B. nigra*. However, I observed a low frequency of HR on plants treated with ARG extract of virgin female *Pieris* butterflies, and the majority of plants treated with the secretion of mated female butterflies expressed HR. Moreover, the majority of BC-treated plants expressed HR. Hence, I conclude that BC plays a role in eliciting HR but other compounds that are not male-derived might be, involved as well which needs further evaluation. Moreover, I showed that volatiles of plants treated with ARG of mated or virgin females did not attract *Trichogramma* wasps. Therefore, I recommend: (1) to chemically analyze the volatile profile of plants treated with ARG extracts of mated and virgin female *P. brassicae* and compared it to that induced by *P. brassicae* egg depositions, which are attractive to *Trichogramma* wasps (Lucas-Barbosa 2010) and (2) to increase the number of glands by applying 3 glands per plants, instead of 1.5 gland per plants used in present study. In the field about half of the plants where *Pieris* eggs were collected showed HR, which showed an increased *Pieris* egg mortality as well as parasitism rate. Hence, those *B. nigra* plants that use a combination of a direct (HR) and indirect defense trait are expected to have a higher fitness than those individuals that use only a single defense trait.

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