Response to *Pieris* eggs in *Brassica nigra* – Chemical Analysis of Inducible Volatile Compounds

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Abstract

Plants can directly and indirectly respond to herbivorous insect eggs. The Black mustard *Brassica nigra* responds directly to butterfly eggs with the so called hypersensitivity response (HR). This plant can also arrest egg parasitoids through the emission of volatile compounds that are induced by egg deposition. This study aimed to investigate whether the egg parasitoid *Trichogramma brassicae* is attracted to *B. nigra* cues induced by eggs of one of its hosts, the Large Cabbage White butterfly *Pieris brassicae*, and whether the wasp's behavior can be correlated to a plant's volatile profile. Moreover, it was investigated if a qualitatively different volatile blend is produced by *B. nigra* plants that show hypersensitivity response compared to plants that do not respond directly. Simultaneous bioassays and volatile trapping were carried out in a dynamic airflow Y-tube olfactometer with wasps being released in groups. Organic volatile compounds were identified and quantified by gas chromatography-mass spectrometry. Results demonstrated that the minute wasps *T. brassicae* are attracted to volatile compounds induced by butterfly egg deposition. The volatile blend of *B. nigra* plants was composed largely of sesquiterpenes. β-Caryophyllene, 7-α and 7-β-siphilperfol-5-enes were the main compounds quantified in clean and infested plants. Mainly quantitative differences were observed in terms of volatile compounds produced by egg infested and non-infested *B. nigra* plants, but more analyses needs to be performed to confirm these results.

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Introduction

Plants need to defend themselves against various attackers. Through inducible defense mechanisms, plants can respond to insect attack directly or by employing natural enemies of the herbivores. In inducible defense mechanisms, compound biosynthesis is primed by arthropod damage (Schoonhoven et al. 2005). It is well known that plants damaged by herbivores produce secondary metabolites *de novo* or significantly increase volatile emission (Dicke and Hilker 2003). More subtle than damage by arthropod feeding is the plant response to insect egg deposition. Plants can activate direct and indirect defenses in response to herbivorous insect eggs (Hilker and Meiners 2006).

Direct responses to herbivorous insect eggs include killing the eggs and inducing changes in the plant tissue to isolate the future threat of the plant, the hatching larvae. It has been shown that rice plants produce an ovicidal substance, benzyl benzoate, against eggs deposited by the plant hopper *Sogatella furcifera* (Seino et al. 1996). The neoplasm formation that impedes pea weevil larvae from entry into the pod is mediated by long-chain α,ω-diols that are referred to as "bruchins" (Doss et al. 2000). The black mustard *Brassica nigra* can respond directly to butterfly eggs with a so-called hypersensitivity response (HR) that is expressed as the formation of necrotic tissue around the eggs, and is expected to kill the eggs. Shapiro and Devay (1987) have shown that a necrotic zone develops 24 h after egg deposition, and after 72 h the eggs dry out and often fall off. In individuals where the HR was observed all eggs were killed by this direct plant response (Shapiro and Devay 1987). My previous study showed that the observed hypersensitivity response of *B. nigra* to *P. brassicae* eggs is not an "all or none phenomenon" as described by Shapiro and Devay (1987) in their studies on a population found in California. In fact, a relative small percentage of the eggs are killed in the tested *B. nigra* plants (Lucas-Barbosa 2009). The mechanisms underlying this early type of plant response remain unknown, as well as which biotic and abiotic factors are relevant for the plant to effectively affect the eggs.

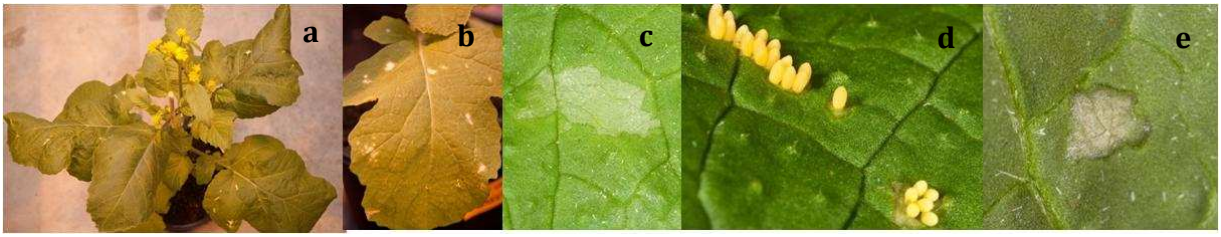

Figure 1. a) *Brassica nigra* plant viewed from the top with HR observed after 72 h; b) Leaf of *B. nigra* from the upper side with HR after 72 h; c) Close-up of HR after 24 h observed from the upper side of a leaf; d) HR after 72 h observed around an egg clutch of *P. brassicae*; e) Close-up of HR 72 h observed from the upper side of a leaf.

Plants can also recruit egg parasitoids through the emission of plant volatile compounds that are induced by egg deposition. Literature reports many cases of the use of plant cues by host searching egg parasitoids (Hilker and Meiners 2006). It has been shown that *Trichogramma* wasps respond to contact chemical cues induced by eggs of the Large Cabbage White butterfly, *Pieris brassicae*, deposited on Brussels sprouts plants (*Brassicae oleracea* gemmifera) (Fatouros et al. 2005, Fatouros et al. 2008). My previous study has demonstrated that *Trichogramma brassicae* wasps are arrested by oviposition-induced volatile compounds emitted by *B. nigra* plants infested with *P. brassicae* eggs using a static two-chamber olfactometer in which one wasp was released at a time. Indeed, chemical

cues from the first trophic level (plant) may play a role in host and host-habitat location (Wajnberg and Hassan 1994).

Attraction of egg parasitoids by oviposition-induced plant synomones has been shown for the pine, elm and bean systems (Meiners and Hilker 1997, Hilker and Meiners 2002, Colazza et al. 2004b). In the pine system, analyses of inducible compounds produced by plants infested with sawfly eggs revealed quantitative changes in terms of volatile emission. In particular, the sesquiterpene β-farnesene was emitted in significantly higher amounts (Mumm et al. 2003). Wegener et al. showed that β-caryophyllene and 4,8-dimethyl-1,3,7-nonatriene (DMNT) were the main compounds present in the volatile profile of elm leaves infested with eggs of the leaf beetle *Xanthogaleruca luteola* (Wegener et al. 2001). These compounds were also induced by feeding damage caused by the leaf beetle. Interestingly, the sesquiterpene β-caryophyllene was the only compound detected in significantly higher amounts in feeding damaged bean plants carrying egg masses when compared to plants with feeding damage only (Colazza et al. 2004a).

Production of inducible volatile compounds has been also reported for *Brassica* plants. Previous studies have shown changes in terms of inducible volatiles in *B. oleracea* due to feeding and oviposition (Conti et al. 2004). The monoterpene alcohol linalool was just detected in plants induced by egg deposition (Conti et al. 2008). Soler et al. (2007) found evidence of changes in the volatile profiles of *B. nigra* plants induced by root and leaf herbivores. Plants exposed to leaf herbivory produce higher amounts of β-farnesene and DMNT when compared to clean plants while plants exposed to root herbivory produced higher amounts of sulfide compounds (Soler et al. 2007). Allyl isothiocyanate and DMNT were the main compounds induced by *B. nigra* when plants were exposed to feeding damage by *P. brassicae* caterpillars. β-Caryophyllene was emitted by *Synapsis arvensis* and not by *B. nigra* plants infested with caterpillars (Gols et al. 2008).

Brassica crops are of worldwide economic importance. The black mustard *Brassica nigra* is widespread in Eurasia and naturalized in the USA. *B. nigra* provides an ideal system to study direct and indirect plant defense. Usually, the two plant response types are studied independently and mostly in cultivated plant species. The findings of my previous study showed that direct and indirect defense can be employed in concert in *B. nigra* (Lucas-Barbosa 2009). A synergistic effect of direct defense and indirect could be an important tool to control insect pests. This study aimed to further investigate behavioral and chemical aspects related to both defense types.

Research aim and questions

The objectives of this research were to investigate whether *T. brassicae* wasps are attracted to volatiles emitted by *P. brassicae* egg-infested *B. nigra* plants and whether the wasps' behavior can be correlated to the volatile compounds emitted by infested and non-infested plants using a novel Y-tube olfactometer set-up. In this set-up, wasp attraction to plant volatile blends was assessed while plant volatile blends were being trapped for further GC-MS analysis.

More specifically, I wanted to answer the following research questions:

1. Can *Trichogramma* wasps be released in groups while bioassays are carried out with a Y-tube olfactometer?

2. Are the wasps attracted to plant synomones and not just arrested?

3. Can the wasps discriminate between odors produced by an egg-infested and a non-infested plant when those odors sources are offered simultaneously?

4. Are the differences in terms of induced volatile compounds produced by clean plants and egg-infested (HR+ and HR-) plants qualitative or just quantitative?

Materials and methods

Plants and herbivores

Plant seeds of *Brassica nigra* L. (Brassicaceae) were obtained from the Centre for Genetic Resources (CGR, Wageningen, The Netherlands) from an earlier flowering accession CGN06619 (feral population collected in 1975 from the Peloponnesus, Greece). Seeds from 25 different individuals were mixed to represent this plant population. *B. nigra* plants were reared in a greenhouse compartment (22 ± 2°C, 70% r.h., L16:D8). Plants of 4-5 weeks old were used for the experiments.

Pieris brassicae (Pieridae) butterflies were reared on Brussels sprout plants in a climate room (22 ± 1°C, 50-70% r.h., L16:D8) and were kept feeding on saturated sugar solution. 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 *Pieris* adults, for at least 15 minutes to allow egg deposition. Plants carrying 3 to 5 egg clutches were used in the experiments. Later, the plants were kept in the greenhouse compartment (22 ± 2°C, 50-70% r.h., L16:D8) under a lamp of 400 W and in downwind position to the clean control plants.

Hypersensitivity Response

Twenty-four hours after egg deposition, plants were checked for hypersensitivity response. The strength of the hypersensitivity response was assessed visually. 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.

Severity	Visual description
+	Tissue on the upper side of the leaf above egg clutch is silver colored – pre-necrosis visible only from the upper side of the leaf.
++	Dead cells – necrosis visible from the upper side of the leaf on tissue above egg clutches
+++	Dead cells - visible from the upper side of the leaf + necrosis also visible around the eggs clutches

Parasitoids

Trichogramma brassicae (Trichogrammatidae) wasps were reared in *Ephestia kuehniella* eggs under laboratory conditions (23 ± 2°C, 50-70% r.h., L16:D8). The wasps were kept feeding on honey. An oviposition experience was given for a period of 17 h prior to the experiment with < 72 hrs old eggs of *P. brassicae*, deposited on *B. nigra* leaves. The wasps were always provided with a drop of honey prior to the experiment. Only female wasps were tested.

Host location behavior

Dynamic airflow Y-tube olfactometer with simultaneous volatile trapping

To test whether female *T. brassicae* wasps were attracted to volatiles from *B. nigra* induced by egg deposition by *P. brassicae*, bioassays were conducted in a dynamic air-flow Y-tube olfactometer with simultaneous volatile trapping. Pressurized air (Figure 2, no. 1) was filtered through activated charcoal (Figure 2, no. 3) and approximately 150 mg of Tenax-TA 25/30 mesh (Grace-Alltech) (Figure 2, no. 4) before being admitted into the system. Subsequently, air was humidified passing through a bottle containing 50 mL of tap water (Figure 2, no. 5). A total airflow of 400 mL min^{-1} was admitted into the system and read with a flow meter (Brooks Instrument B.V., Veenendaal, NL) (Figure 2, no. 2). The airflow was divided into two and each sub-flow was led through the odor source glass containers

(Figure 2, no. 7). Two glass containers each containing an odor source were closed with a glass lid, with a Teflon O-ring in between, and air-tight using a metal clamp. Air was admitted into the glass containers through an inlet on the lid. Subsequently, the two odor flows were led to each of the two arms of a glass Y-tube olfactometer (stem 9 cm, arms 8 cm, ID 1 cm) (Figure 2, no. 9) fitted with three female ground joints, through Teflon tubing and a two-way tube. The two-way tubes (Figure 2, no. 8) were fitted with a glass filter and screw joint at one of the extremities and a male ground joint on the other. They were also connected to round bottom flasks (Figure 2, no. 10) where the wasps were collected. The air flowed through the glass containers and was led to each of the arms the Y-tube through an outlet at the base of the containers. Connections between all glass parts were made with the use of Teflon tubing (Figure 2, no. 6). The airflow admitted through each arm of the olfactometer was 100 mL min^{-1}, which was read with a flow meter (Brooks Instrument B.V., Veenendaal, NL). In this way two well-separated laminar airflows were generated in the olfactometer. Headspace volatiles were collected on a glass tube filled with 90 mg of Tenax-TA 25/30 mesh (Grace- Alltech) (Figure 2, no. 11) for 5.5 h at a flow rate of 80 mL min^{-1} through an outlet on the lids of each of the glass containers. For that purpose a pump (PAS-500 SPECTREX, Redwood City, CA, US) (Figure 2, no. 12) equipped with 9 V rechargeable batteries was used to suck air at 100 mL min^{-1}. All glass parts of the Y-tube olfactometer system, including glass containers, were cleaned after every 3 trials or every time new plants were placed in the containers. The glass parts were cleaned with hot tap water, rinsed with ethanol 95% and dried in oven at 200 ^{0}C for at least 2 hours.

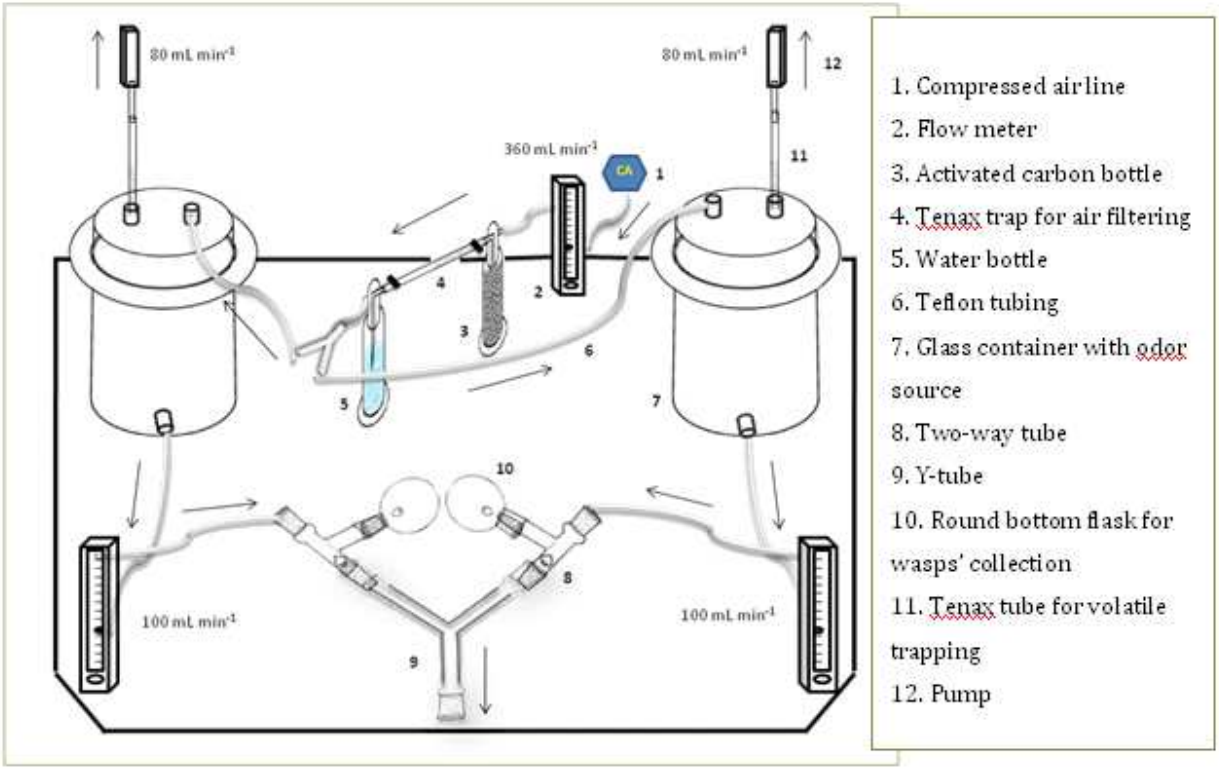

Figure 2. Schematic drawing of the dynamic airflow Y-tube olfactometer.

Bioassays and headspace collection

The experiments were carried out in the laboratory at 21 ± 2°C using a fiber optic light source (11 W, Philips) above the olfactometer and above the glass containers (2 of 18 W and 2 of 50 W, Philips) containing the plants as odor sources. Just before placing a plant in a glass container, the pot of the plant was removed and the roots and soil were packed

tightly in aluminium foil. The plants were placed in the air-tight closed glass containers. First, each of the glass containers with or without a plant, depending on the bioassay, was purged with air for 30 min through the containers. Volatile trapping started after this 30 min period and bioassays 1 hour after headspace volatile collection had started. Volatiles were trapped for 5.5 h. Thus, plants of different treatments were sampled simultaneously with the behavioral experiments.

A choice between two odor sources was offered to ten adult females of *T. brassicae* at the same time in the Y-Tube olfactometer in order to test their response to volatiles induced by the different plant treatments. The odor source consisted of clean air, clean plants and, plants infested with *P. brassicae* eggs, depending on the treatment. Infested plants are referred to as HR+ or HR- plants, whether direct response against the eggs was observed or not. On average, 150 wasps ere tested per treatment. The wasps were released in groups of 10. Three trials of 10 were carried out on 5 different days with 5 individual plants per treatment. After making a choice in the Y-tube, the wasps were directed through light placed above the Y-tube olfactometer, to two individual round bottom flasks' connected to each of the arms of the Y-tube airflow olfactometer through a two-way tube. After 30 minutes, the wasps collected in each of the flasks were counted. When a wasp did not make a choice within 30 minutes, it was recorded as a "non-response" (NR). Each wasp was used only once and then discarded. To exclude any bias effect, the odor source flowing through each arm of the Y-tube was exchanged after every trial. A balance (Mettler-Toledo B.V., Tiel, CZ) was used to weigh the aerial parts of the plants after the experiments.

Treatments

In treatment 1, the wasps were exposed to odors of clean plants placed in each of the containers. In this experiment, the effect of releasing the wasps in groups was tested. The wasps' distribution in the Y-tube olfactometer was expected not to differ from 50:50. The attraction of *Trichogramma* wasps to volatiles emitted by a clean plant or HR+/HR- plants infested with eggs was tested against clean air (treatments 2 to 4). In treatment 5 and 6, the behavior of *T. brassicae* to volatiles emitted by egg-infested (HR+ and HR-) plants was tested against clean plants. HR+ plants were also tested against HR- plants (treatment 7). In this experiment eggs were gently removed just before the bioassays. All treated plants were infested with eggs 24 h before the experiments.

Table 2. Overview of the dynamic air-flow Y-tube olfactometer bioassays.

No.	Treatment	Induction time (h)
1	**Clean plant vs. clean plant**	-
2	**Clean plant vs. clean air**	-
3	**HR+ plant with eggs vs. clean air**	24
4	**HR- plant with eggs vs. clean air**	24
5	**HR+ plant with eggs vs. clean control plant**	24
6	**HR- plant with eggs vs. clean control plant**	24
7	**HR+ plant vs. HR- plant (eggs removed)**	24

Headspace analysis

Headspace samples were analysed using a gas chromatograph with a thermodesorption unit (GC) (Agilent 6890 series, Santa Clara, USA) connected to a mass spectrometer (MS) (Agilent 5973 series, Santa Clara, USA). The collected volatiles were desorbed from the Tenax in a thermodesorption trap unit (Gerstel, Mülhein, Germany) by heating from 25 °C

to 250°C (5 min hold) at a rate of 60 °C min^{-1} in splitless mode. The released compounds were focused in a cold trap (ID 1.80 mm) filled with glass beads (d 0.75-1.00 mm) at a temperature of -50 °C. By heating of the cold trap to 250 °C at 12°C sec^{-1}, the volatiles were transferred to the analytical column (60 m x 0.25 mm ID, 0.25 µm film thickness, DB-5, J&W, Folsom, CA, USA). The oven temperature programme started at 50 °C (1 min hold) and rose at a rate of 20 °C min^{-1} to 100 °C, then it increased at a rate of 4 °C min^{-1} to 280 °C (1.5 min hold) and subsequently rose up to 300 °C at a rate of 10 °C min^{-1}. The column effluent was ionized by electron impact ionization at 70 eV. Mass scanning was carried out from 40 to 300 m/z with 5.36 scans sec^{-1}. The compounds were identified by comparison of the mass spectra with those of NIST, Wiley libraries and of the Wageningen Mass Spectral Database of Natural Products. The identity was confirmed by comparison of the retention index described in literature and the ones calculated during this study. Retention indices were calculated using traces of n-alkanes present in the samples as reference.

Quantification

Menthol (Merck), undecanal (Alfa Aesar), β-caryophyllene (Roth) and decanoic acid methyl ester (C10-FAME) (Merck) were used to quantify the identified compounds. A standard solution containing a known amount [0.02 mg/mL] of the four pure compounds dissolved in t-butyl methyl esther (MTBE) was analyzed by gas chromatography under the same oven conditions used to thermodesorb the plant volatiles. 1.0 µL was injected in triplicate *via* an autosampler in splitless mode (5 min of solvent delay). The amount of each of the identified compounds per 10 g of fresh plant material was calculated. Whenever a mass spectrum of a given compound could not be distinguished over the background signal it was considered as being below the detection limit.

Statistics

The choices of *T. brassicae* wasps between two odor sources in the Y-tube olfactometer were analyzed with two-sided binomial tests to investigate whether the wasps distribution differed from 50:50 (α=0.05; *, P≤0.05; **, P≤0.01; ***, P≤0.001; ns, not significant). The wasps that did not make a choice were excluded from statistical analysis. Data related to headspace analysis were not treated statistically because of the low number of replicates. Differences are given in percent.

Results

Hypersensitivity Response

The hypersensitive response (HR) in this study was checked at one time point: 24 h after plants had been infested with eggs. Aerial parts of the plants were weighed after the bioassays with the Y-tube olfactometer and then discarded. Fifty percent of the induced plants showed HR. Necrosis observed on 100% of the HR+ plants was visible from the upper side of the leaf (severity considered +, Table 1). In this study, I did not determine how HR affected subsequent development of *P. brassicae* eggs.

Host location behavior

Wasps were exposed to two similar odor source, namely a clean plant, to test whether they would affect each others' choice when released in groups. The wasps' distribution in the Y-tube olfactometer did not significantly differ from 50:50 (P=0.4661^{ns}, two-sided binomial test, Figure 3). Thus, the possibility of a grouping effect was excluded, and the wasps were released in groups in all subsequent experiments.

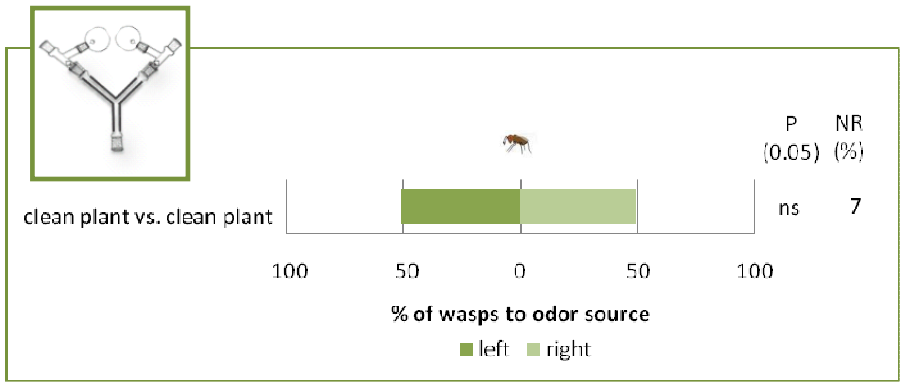

Figure 3. Percentage (%) of *T. brassicae* wasps attracted to odor source of two clean *B. nigra* plants is given. Two-sided binomial test (*, P≤0.05; **, P≤0.01; ***, P≤0.001; ns, not significant), 150 wasps tested. NR= "non response" or percentage of wasps that did not respond out of the 150 wasps tested.

T. brassicae wasps, released in groups of 10, were significantly attracted to volatiles emitted by intact HR+ and HR- plants carrying 24 h-old eggs when tested against clean air (^{HR+}P=0.002**, ^{HR-}P≤0.0001***, two-sided binomial test, Figure 4 and table 3). The wasps were not attracted to volatiles emitted by a non-infested clean plant (P=0.562^{ns}, two-sided binomial test, Figure 4).

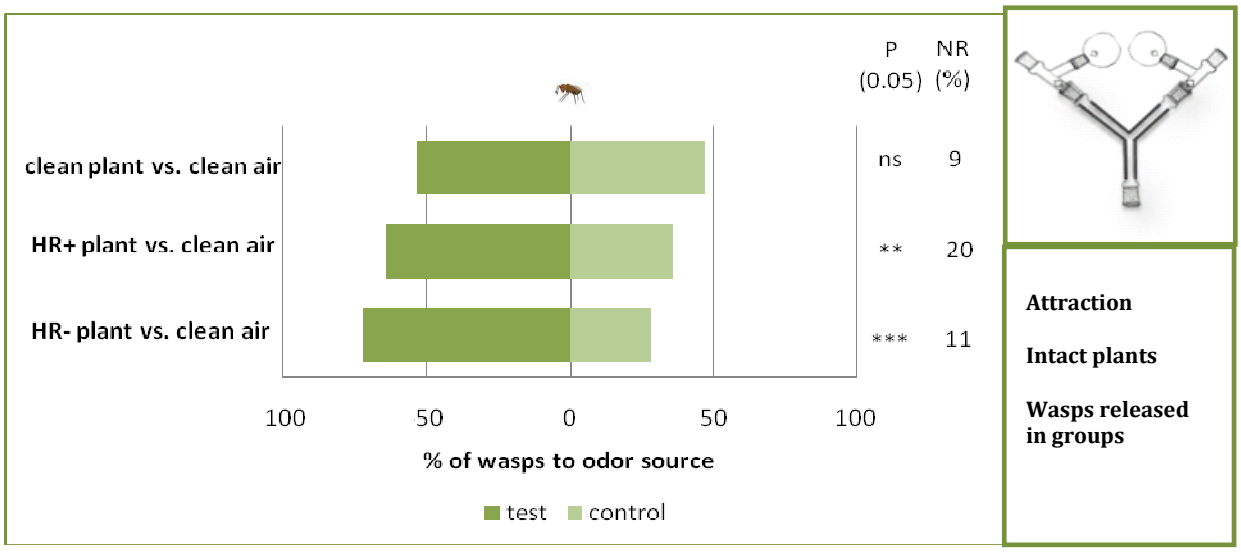

Figure 4. Percentage (%) of *T. brassicae* wasps attracted to odor source of plants submitted to different treatments and to clean air is given. Two-sided binomial test (*, P≤0.05; **, P≤0.01; ***, P≤0.001; ns, not significant), 150 wasps tested. NR= "non response" or percentage of wasps that did not respond out of the 150 wasps tested per treatment.

Wasps' choice between two different plant odor sources was also tested in the dynamic airflow Y-tube olfactometer. Results showed that *T. brassicae* was significantly more attracted to odors of an HR+ plant than to odors emitted by a non-infested plant (*P*=0.0462*, two-sided binomial test, Figure 5 and table 3). However, in this experiment 60 wasps were tested on two different days with only two different set of plants. Therefore, more replicates are needed.

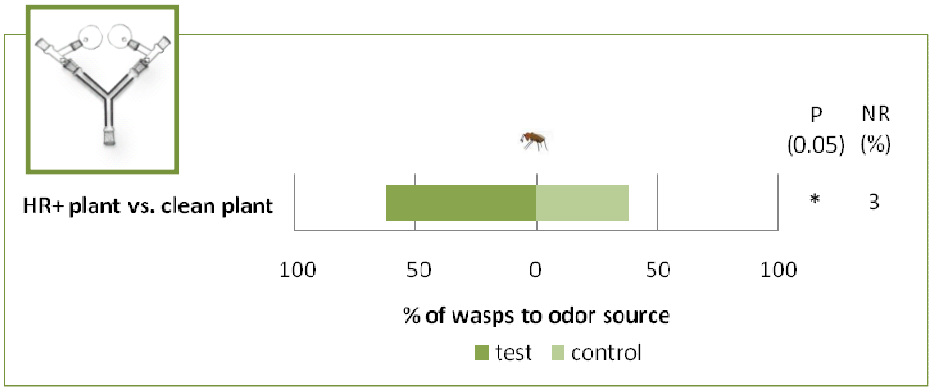

Figure 5. Percentage (%) of *T. brassicae* wasps attracted to odor source of HR+ plant and a clean plant is given. Two-sided binomial test (α=0.05; *, P≤0.05; **, P≤0.01; ***, P≤0.001; ns, not significant), 60 wasps tested. NR= "non response" or percentage of wasps that did not respond out of the 60 wasps tested.

The wasps did not discriminate between odors emitted by an HR+ plant and odors of an HR- plant (*P*=0.500*, two-sided binomial test, Figure 6 and table 3). More replicates are needed to confirm the result.

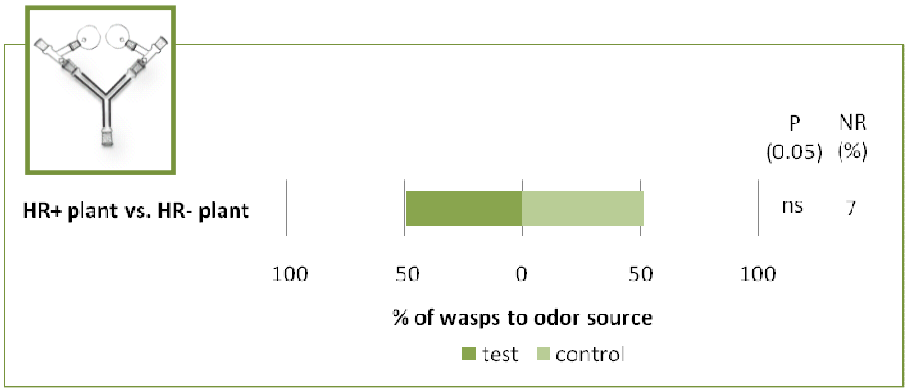

Figure 6. Percentage (%) of *T. brassicae* wasps attracted to odor sources of HR+ plant and HR- plant is given. Two-side binomial test (α=0.05; *, P≤0.05; **, P≤0.01; ***, P≤0.001; ns, not significant), 60 wasps tested. NR= "non response" or percentage of wasps that did not respond out of the 60 wasps tested.

Table 3. Preference (%) of *T. brassicae* wasps to different odor sources in the dynamic Y-tube olfactometer experiment. Two-side binomial test (*, P≤0.05; **, P≤0.01; ***, P≤0.001; ns, not significant), N= number of tested wasps.

treatment	Test (%)	Control (%)	P (≤0.05)	N
clean plant vs. clean air	53	47	0,5621^{ns}	150
HR- plant vs. clean air	72	28	0,0001***	146
HR+ plant vs. clean air	64	36	0,0016**	150
HR+ plant vs. clean plant	62	38	0.0462*	60
HR+ plant vs. HR- plant (with eggs removed)	52	48	0.5000^{ns}	60
HR- plant vs. clean plant (damaged by sciarid larvae)	49	51	0.6081^{ns}	60

HR- plants were also tested against clean plants. However, cotyledons of those plants were damaged by sciarid fly larvae. Sciarid fly larvae live from decay products of the soil but can

also feed on roots and soft leaves (Malais and Ravensberg 1992). Wasps' distribution in the Y-tube did not differ from 50:50 (P=0.608, two-sided binomial test, Table 3).

Headspace analysis

The main volatile compounds present in clean and egg infested plants have been identified and quantified. The chromatogram presented below shows a profile of the volatile blend produced by an HR+ plant (Figure 7). The numbered peaks were identified as plant compounds (Figure 7). Non-numbered peaks are considered not to be of plant origin. Most were plasticizers derived or present in the air, as the alkane traces used to calculate the retention indices. A few aldehydes (C8, C9 and C10 aldehydes) and dodecanoic methyl esther acid were also repeatedly found in the background samples. These compounds have been quantified in triplicate, as well from background samples, and considered not to be released by *B. nigra* plants, although they can be of plant origin. 2-Ethylhexanol can also be present in the air, but during this study was detected just once in background samples, therefore was considered to be emitted by the plants.

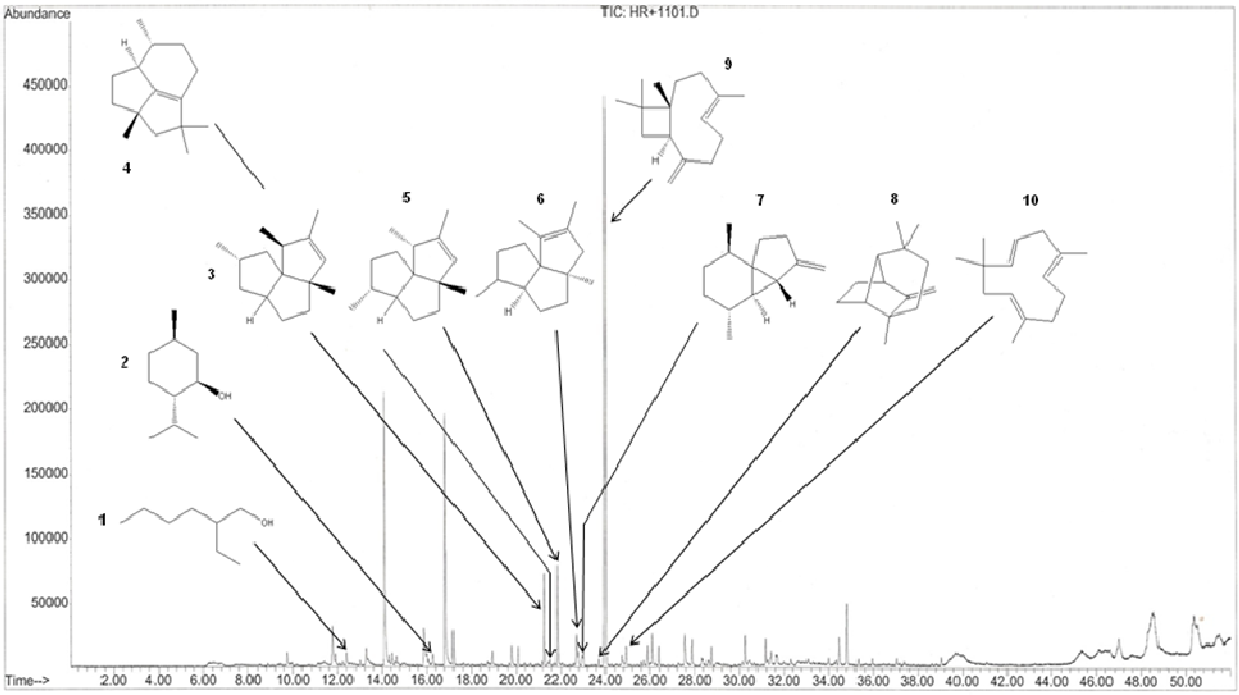

Figure 7. Total ion current (TIC) chromatogram of an HR+ plant and structures of the identified compounds. 1. 2-Ethylhexanol; 2. Menthol; 3. 7-α-Silphiperfol-5-ene; 4. Presilphiperfol-7-ene; 5. 7-β-Silphiperfol-5-ene; 6. 7-Silphiperfol-6-ene; 7. β-Cubebene; 8. Longifolene; 9. β-Caryophyllene; 10. α-Humulene.

Volatile compounds were identified by comparison with the mass spectra and their retention index. Whenever a mass spectrum of a given compound could not be distinguished over the background signal it was considered to be below the detection limit. Retention indices were calculated using traces of n-alkanes (C11 to C16) present in the samples. The retention time of decane (C10) was extrapolated as this alkane was not present in the samples. Calculated retention indices were compared with the ones reported in the literature. An overview of quantified and identified compounds as well as their calculated and reference retention indices are shown in table 4.

The volatile profiles of clean and HR+ plants are qualitatively and quantitatively different (Figure 8 and table 4). Headspace samples were analyzed in duplicate and results can be correlated with the outcome of the experiments with the Y-tube olfactometer, when HR+ plants were tested against clean plants. Most compounds identified in the volatile blend of *B. nigra* plants were sesquiterpenes. The sesquiterpenes β-caryophyllene and 7-α and 7-β-siphilperfol-5-ene were the main compounds present in both clean and HR+ plants headspace samples analyzed. β-Caryophyllene and 7-α-silphiperfol-5-ene were quantified in respectively 75% and 91% higher concentration in the headspace of HR+ plants than of clean plants. The alcohol 2-ethylhexanol and the sesquiterpene 7-silphiperfol-6-ene were present in higher amounts in clean plants than in HR+ plants. The average concentration of 7-silphiperfol-6-ene in clean plants was 59% higher compared to the profile of HR+ plants (Figure 8). 2-Ethylhexanol, β-cubebene and longifolene were detected only in one of the HR+ plant samples analyzed. β-Cubebene was absent in the headspace of clean plants.

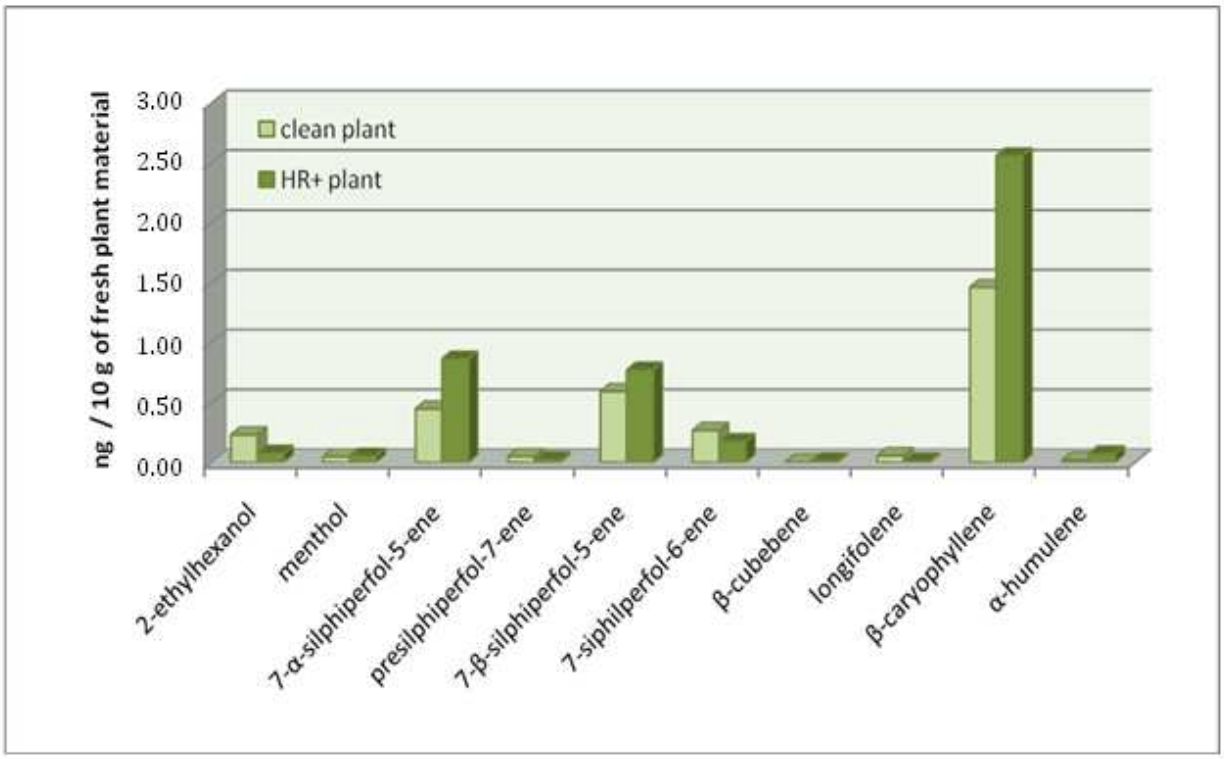

Figure 8. Identified and quantified volatile compounds from headspace samples of clean plants and HR+ plants. Mean in ng / 10 g fresh plant material is given. Analyses were carried out in duplicate (based on 2 plants per treatment).

Table 4. Identified and quantified compounds present in clean, HR+ and HR- plants. Retention time (RT), calculated and reference retention (Adams 1995) indices (RI) are given.

No.	RT (min)	Compound name	Compound class	RI (calculated)	RI (literature) (Adams 1995)	Clean plant [a]	HR+ plant [a]	HR-plant [b] (eggs removed)
1	12.6	**2-ethyl hexanol**	alifatic alcohol	1048	1029	0.23 ± 0.12	0.08 ± 0.11	---
2	16.3	**menthol**	monoterpene alcohol	1167	1172	0.05 ± 0.01	0.05 ± 0.02	---
3	21.3	**7-α-silphiperfol-5-ene**	sesquiterpene	1341	1329	0.45 ± 0.02	0.86 ± 0.33	0.35
4	21.5	**presilphiperfol-7-ene**	sesquiterpene	1346	1337	0.05 ± 0.04	0.02 ± 0.01	---
5	21.8	**7-β-siphilperfol-5-ene**	sesquiterpene	1360	1345	0.59 ± 0.03	0.77 ± 0.08	0.48
6	22.7	**7-silphiperfol-6-ene**	sesquiterpene	1389	1379	0.27 ± 0.18	0.17 ± 0.01	0.12
7	23.0	**β-cubebene**	sesquiterpene	1398	1388	---	0.01 ± 0.02	---
8	23.7	**longifolene**	sesquiterpene	1425	1407	0.06 ± 0.05	0.01 ± 0.01	---
9	24.0	**β-caryophyllene**	sesquiterpene	1434	1419	1.46 ± 1.37	2.56 ± 2.28	1.11
10	24.9	**α-humulene**	sesquiterpene	1466	1453	0.02 ± 0.03	0.07 ± 0.10	---

[a] (mean ng / 10 g fresh plant material ± standard deviation), samples analyzed in duplicate
[b] (ng / 10 g fresh plant material), single plan

A single analysis of a HR- plant was carried out. The eggs were removed just before the experiment and this plant was tested against a HR+ plant in the Y-tube olfactometer. β-Caryophyllene and the three silphiperfolenes previously identified in HR+ plants and clean plants are also presented in the volatile blend of the HR- plant. No other plant compounds were quantified; they are assumed to be absent or below the detection level (Table 4).

No clear quantitative difference between the sesquiterpenes present in the volatile blend of clean and HR- plants was observed in these analyzed samples. A few different volatile compounds were identified from headspace samples collected these from plants. All plants had been also exposed to feeding damage by sciarid fly larvae. Volatiles were trapped from egg-infested (HR- plants) and egg-free plants (clean plants damaged by sciarid fly larvae). The green leaf volatile, 3-hexen-1-yl acetate, and 4,8-dimethyl-1,3,7-nonatriene (DMNT) were present in the volatile blend of both, clean plants and infested plants (HR- plants) (Figure 9). β-Ocimene was present in only one of the HR- plant samples analyzed.

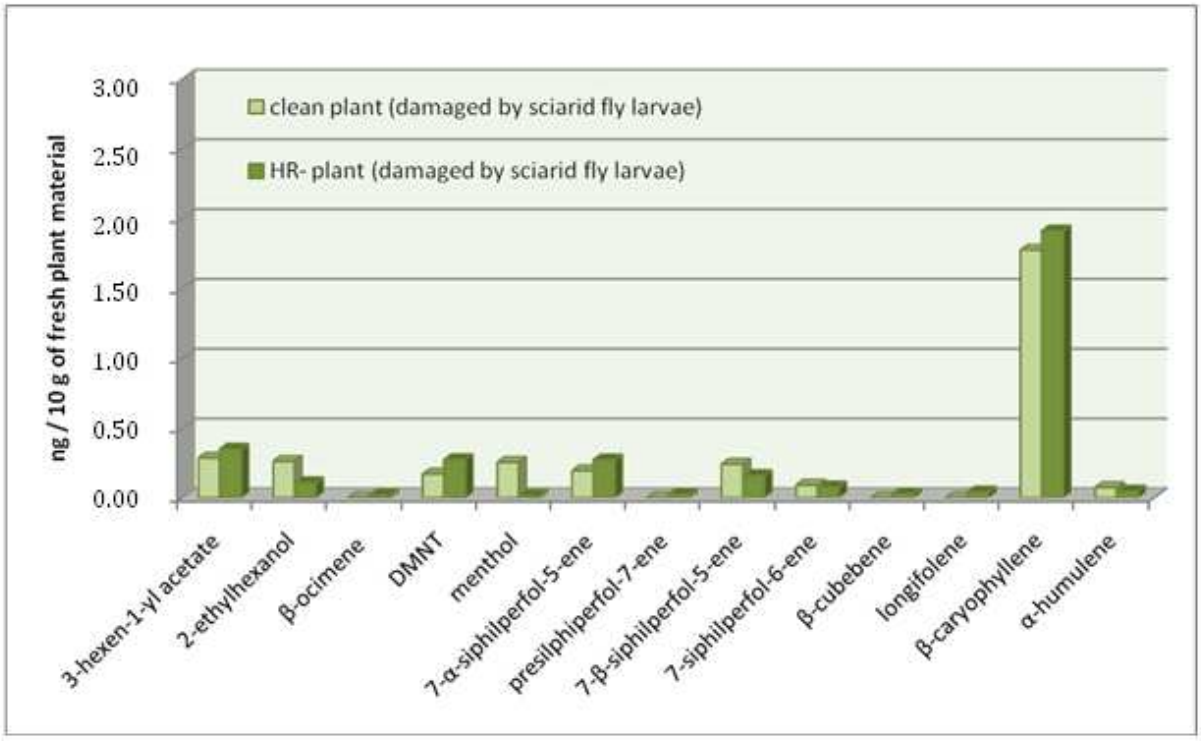

Figure 9. Identified and quantified volatile compounds from headspace samples of clean plants and HR- plants. Analyses of HR- plants were carried out in duplicate. Single analysis of the clean plant.

Discussion

In the experiments with the Y-tube olfactometer, *T. brassicae* wasps were released in groups. Although female wasps are not expected to follow other females, I wanted to exclude the possibility of any group effect to ensure that *Trichogramma* wasps could be tested in trials of 10 wasps at a time. When offering the choice between odors of two clean plants a bias effect as a consequence of difference in light or airflow conditions can also be excluded. Only one other study testing egg parasitoids in a Y-tube olfactometer of egg parasitoids reports the release of wasps in groups. However, grouping effect was not tested during the study (Yong et al. 2007). Equipment and ideal conditions used in this system were slightly different when compared to those described in literature (Table 5).

Table 5. General conditions reported in the literature for bioassays with eggs parasitoids tested in dynamic airflow Y-tube olfacometers.

Genus of studied egg parasitoid	Y-tube dimensions (stem, arms, internal diameter) (cm)	Parasitoids released individually or in group	Airflow through arms of the Y-tube (mL/min)	Attraction to kairomone, plant synomone or both	Authors
Anagrus	S 10, A 10, ID 1	individually	150	synomone	*(Lou et al. 2006)*
Telenorus	S 9, A 8, ID 1.5	individually	800	synomone	(Moraes et al. 2005)
Trichogramma	S 10, A 8, ID 3	individually	30	both	(Reddy et al. 2002).
Trichogramma	S 9, A 8, ID 1.5	group of 20	nq*	synomone	(Yong et al. 2007)
Trichogramma	S 9, A 8, ID 1.5	individually	30	kairomone	(Milonas et al. 2009)
Trichogramma	**S 9, A 8, ID 1**	**group of 10**	**100**	**synomone**	**this system**
Trissolcus	S 9, A 8, ID 1.5	individually	144	kairomone	(Colazza et al. 1999)
Trissolcus	S 9, A 8, ID 1.5	individually	30	synomone	(Colazza et al. 2004b)
Trissolcus	S 9, A 8, ID 1.5	individually	144	synomone	(Conti et al. 2004)

nq=not quantified.

The results of the bioassays in the dynamic airflow Y-tube olfactometer confirm that *T. brassicae* wasps are able to use volatile chemical cues produced by egg infested plants to find their host. The wasps were significantly attracted to volatiles emitted by HR+ and HR- plants when tested against clean air, and not attracted by volatiles emitted by a clean plant. These results are comparable to results obtained in my previous study using the static two-chamber olfactometer (Lucas-Barbosa 2009). Results with the static 2-chamber olfactometer showed that *T. brasssicae* wasps were arrested by HR+/HR- plant infested with *P. brassicae* eggs, and not by volatile compounds released by a non-infested plant. In this study, using the Y-tube olfactometer, I demonstrated that the wasps are also attracted to odor sources emitted by egg infested plants. Future studies are needed to investigate whether the observed wasps' responses to plant volatiles are innate, learned or triggered by sensitization.

Odor of an HR+ plant was offered against odor of a clean plant. Results showed that *T. brassicae* was significantly more attracted to odor of the infested plant (HR+ plant) than of a clean plant. Although more replicates are needed, these results indicate that the wasps can also choose between an infested and a non-infested plant in a two-choice situation. Headspace analysis indicates that this choice is based on quantitative changes in a few plant compounds. In this study, β-caryophyllene and 7-α-silphiperfol-5-ene were detected in respectively 75% and 91% higher concentration in the headspace of HR+ plants than of clean plants. Although more replicates are needed to confirm the results, these differences could explain wasps' preference during the bioassays. A number of studies showed that

terpenes can play a role in plant insect interactions (Mumm et al. 2008) and also more specifically on the recruitment of egg parasitoids (Hilker et al. 2002, Colazza et al. 2004a, Meiners et al. 2005). Simultaneous behavioral assays and volatile trapping can help us to investigate which are the chemical cues used by the insects.

T. brassicae wasps were significantly attracted to an HR- plant when tested against clean air, and not to odors of a clean plant. However, the wasps did not discriminate between odors of clean and HR- plants when tested against each other. In the latter situation, all plants had been also exposed to feeding damage by sciarid fly larvae. No clear quantitative difference between the sesquiterpenes produced by clean and HR- plants was observed in these analyzed samples. DMNT and 3-hexen-1-yl acetate were detected in clean and HR- plants. The quantitative or qualitative differences observed in the headspace samples could enlighten why the wasps do not discriminate between the two odor sources in the olfactometer. However, some variation in the volatile profile produced by clean or infested plants can be expected, and a small number of headspace samples have been analyzed during this study.

If quantitative differences in the volatile blend produced by clean and egg-infested plants are confirmed, wasps' attraction to the main compounds induced by egg infested plants could also be tested. The sesquiterpenes β-caryophyllene and 7-α-siphilperfol-5-ene would be the main candidates to be tested based in the headspaces analyses carried out during this study. However, not necessarily we could expect the wasps to respond to a pure compound or a mixture of few compounds. Increased amounts of a single sesquiterpene emitted by egg infested plants can attract egg parasitoids (Mumm et al. 2003, Colazza et al. 2004a). Although the egg parasitoids were attracted to infested pine twigs that produce increased amounts of β-farnesene, the wasps were not attracted to this sesquiterpene when tested as a single compound (Mumm et al. 2003, Mumm and Hilker 2005). However, the wasps did respond to β-farnesene when it was applied onto a clean plant. The authors argue that the wasps are attracted specifically to β-farnesene, but just when this compound is contrasted with the background odor of the host plant (Mumm and Hilker 2005). It may also be that a change in amount of β-farnesene simply represents another 'odor' sensed by the wasps. After detection of a complex odor mixture by the receptor cells in the sensory periphery, processing of the odor mixture in an insect's brain will eventually result in a response pattern encoding the identity of the blend (Perez-Orive et al. 2002). An enlarged concentration of a single compound or an addition of a new compound to a complex odor mixture leads to a different response pattern that may be recognized as a different odor in the insect brain. Wasps attraction to the main volatile compounds quantified in the volatile blend of egg-infested plant could be tested by applying these compounds onto a clean plant.

Whether *T. brassicae* wasps can choose between an infested and a non-infested plant in a two-choice situation needs to be further investigated. A few more experiments using simultaneous behavioral assays and volatile trapping should be performed. Odors of HR+ / HR- plants should be offered to the wasps against odors of clean plants. Whether it would be interesting to test odors of HR+ plants against odors of HR- plants, in the Y-tube olfactometer set up, should be reevaluated based on results of headspace analysis of HR+ and HR- plants.

Future studies should also investigate what is the elicitor of the direct and indirect plant responses to eggs in *B. nigra*. It has been demonstrated that oviposition-induced indirect plant responses can be elicited by compounds present in the oviduct secretion coating the eggs (Hilker et al. 2005, Fatouros et al. 2008). Extracts of pea weevil eggs and adults elicit

the direct plant response in *Pisum sativum*. The compounds that elicit the neoplasm formation were found predominantly in mature females (Doss et al. 1995, Doss et al. 2000). It would be interesting to investigate whether the indirect plant response to *Pieris* egg deposition is triggered by male-derived butterfly anti-aphrodisiacs as has been shown for indirect defense in *Brassica oleracea* (Fatouros et al. 2008). The plant response could be induced by spraying the compound(s) identified as the elicitor. An increasing number of studies are looking into how to apply defense mechanisms to biological control. Knowledge on the elicitor is one of the ways that could be explored to keep natural enemies of the herbivores in crop fields or to induce plant resistance.

Conclusion

This study demonstrated that the tiny parasitic wasp *T. brassicae* is attracted by volatile cues produced by *B. nigra* plants infested with eggs of the Large Cabbage White butterfly *P. brassicae* to find butterfly eggs. Simultaneous volatile trapping showed that β-Caryophyllene, 7-α and 7-β-siphilperfol-5-enes were the main compounds identified in headspace clean and egg infested plants. Mainly quantitative differences were observed in terms of volatile compounds produced by egg infested and clean plants during this study, but more analyses need to be performed to confirm these results. Qualitative and quantitative differences between the volatile blend produced by HR+ and HR- plants should also be further explored. Simultaneous behavioral assays and volatile trapping are not only important to explain which kind of compounds play a role in insects' attraction, but can be crucial to explain an insect's behavior in behavioral experiments.

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Appendix

In a CD enclosed to this report:

1. Mass Spectra *B. nigra*
   Mass spectra of the identified compounds

2. GC-MS Methods and Maintenance
   Methods used during this study to run samples, standards solutions and in the maintenance of the equipment.

3. Troubleshooting
   Description of main problems encountered and solutions related to headspace analysis

4. Y-tube Drawings

5. Report in pdf