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2 **Attraction of egg-killing parasitoids toward induced plant volatiles in a**
3 **multi-herbivore context**

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1 **Abstract**

2 In response to insect herbivory, plants emit volatile organic compounds which may act as indirect
3 plant defenses by attracting natural enemies of the attacking herbivore. In nature, plants are often
4 attacked by multiple herbivores, but **the majority of the studies investigating** indirect plant defenses
5 **have focused on the recruitment of different parasitoid species in a single-herbivore context.** Here,
6 we investigated attraction of egg parasitoids of lepidopteran hosts (*Trichogramma brassicae* and *T.*
7 *evanescens*) toward plant volatiles induced by **different** insect herbivores in olfactometer bioassays.
8 We used a system consisting of a native crucifer, *Brassica nigra*, two naturally associated herbivores
9 (eggs and caterpillars of the butterfly *Pieris brassicae*; the aphid *Brevicoryne brassicae*), and an
10 alien invasive herbivore (eggs and caterpillars of the moth *Spodoptera exigua*). We found that
11 *Trichogramma* wasps are attracted by *P. brassicae*-egg induced volatiles but not by *S. exigua*-egg
12 induced volatiles indicating specificity of plant responses toward **lepidopteran** herbivores. Chemical
13 analysis shows significant differences between the volatile blends emitted by *P. brassicae*- and *S.*
14 *exigua*-egg induced plants **in agreement with behavioral observations.** We also investigated
15 attraction of *Trichogramma* wasps toward *P. brassicae*-egg induced volatiles in plants
16 simultaneously attacked by **larvae and nymphs of different** non-hosts. **Both *P. brassicae* and *S.***
17 ***exigua* chewing** caterpillars, but not **phloem-feeding** aphids, can disrupt *Trichogramma* species
18 attraction toward *P. brassicae*-egg induced volatiles. **Indirect plant defenses are discussed in the**
19 **context of multiple herbivory by evaluating the importance of origin, dietary specialization and**
20 **feeding guild of different attackers on recruitment of egg-killing parasitoids.**

21 **Keywords:** OIPVs, HIPVs, multitrophic interactions, parasitoid foraging behavior, **indirect plant**
22 **defences**

23

1 **Introduction**

2 As members of diverse ecological communities, plants and insects have coevolved for more than
3 400 million years (Sugio et al. 2014). Half of the described one million insect species are herbivores
4 and, among these, about 300.000 species lay eggs on plants (Schoonhoven et al. 2005). In response to
5 insect herbivory, plants emit complex mixtures of volatile organic compounds which may act as
6 indirect defenses by recruiting natural enemies of the attacking herbivore (Arimura et al. 2005;
7 Dicke 2009). These volatile compounds can be induced either by feeding (i.e. herbivore-induced
8 plant volatiles - HIPVs) or by egg-laying activity (i.e. oviposition-induced plant volatiles - OIPVs)
9 of insect herbivores (Kessler and Heil 2011; Hilker and Fatouros 2015).

10 Attraction of natural enemies of herbivores, such as predators and parasitoids, toward HIPVs and
11 OIPVs is a widespread ecological phenomenon recorded for at least 49 plant species belonging to 25
12 different families (Mumm and Dicke 2010) but the majority of these studies have been conducted
13 with plants that are attacked by a single herbivore species (Dicke et al. 2009). In nature, however,
14 plants are often attacked by multiple herbivore species, a scenario which may interfere with the
15 attraction of natural enemies as a result of modifications in the HIPV and/or OIPV blends (Dicke et
16 al. 2009). Multiple herbivory in a tritrophic perspective **has** received increasing interest recently, as
17 direct and indirect plant defenses may be **more often** shaped by a whole community of interacting
18 herbivores than by single pairwise interactions between species (Pilson 1996; Agrawal 2007; Dicke
19 and Baldwin 2010; Poelman and Dicke 2014). A growing body of literature suggests that, under
20 multiple herbivore attack, the emission of induced volatile blends can be altered in a specific manner
21 depending on insect feeding guild (biting-chewing or piercing-sucking), plant organ attacked (root-
22 damage or leaf-damage), herbivore density, order of colonization and time lag between arrivals of
23 the attackers (de Rijk et al. 2013 and references therein). Parasitoid recruitment as a consequence of
24 altered volatile emissions **is likely** disrupted when plants are simultaneously exposed to herbivore
25 species inducing different defense pathways (Zhang et al. 2009, 2013). This disruption effect can be

1 mediated by cross-talk between the main plant defense signaling pathways (Jasmonic Acid (JA)- and
2 Salicylic Acid (SA)-pathways) (Pieterse et al. 2009, 2012).

3 Here, we **investigated** the attraction of parasitoids toward plant volatiles emitted under conditions of
4 multiple herbivory. We used a native Eurasian species assemblage commonly found in the
5 Netherlands which consists of the black mustard, *Brassica nigra* L. (Brassicaceae) and two of its
6 naturally associated specialist herbivores, eggs and larvae of the large cabbage butterfly *Pieris*
7 *brassicae* L. (Lepidoptera: Pieridae) and nymphs of the cabbage aphid *Brevicoryne brassicae* L.
8 (Hemiptera: Aphididae). We also used eggs and larvae of the beet armyworm *Spodoptera exigua*
9 Hübner (Lepidoptera: Noctuidae) **which is an alien invasive herbivore**. The beet armyworm is
10 thought to have originated in Southeast Asia and it is a pest of several crops, including crucifers
11 (www.plantwise.org/KnowledgeBank/Datasheet.aspx?dsid=29808). It is a range-expanding species,
12 which in Europe was mainly confined to the Mediterranean basin up to the 1970s (CAB Distribution
13 Maps of Plant Pests 1972) but it has been moving up north and nowadays *S. exigua* is recorded
14 almost every summer in the Netherlands (Waring and Townsend 2006). Apart from **their different**
15 **origin and plant specificity**, these attackers were also selected based on the defense signaling
16 pathways they induce: while plant defenses against leaf-chewing caterpillars are induced via the JA-
17 signaling pathway, eggs and aphids are known to mainly induce the SA-signaling pathway
18 (Giordanengo et al. 2010; Reymond 2013).

19 **In this study we have investigated** the response to plant volatiles by two native egg-killing
20 parasitoids, *Trichogramma brassicae* Bezdenko and *T. evanescens* Westwood (Hymenoptera:
21 Trichogrammatidae). These two species can attack several lepidopteran hosts (Polaszek 2010) and
22 they can both also successfully reproduce in *S. exigua* eggs under laboratory conditions (A.
23 Cusumano, personal observations) leading to potential new host-parasitoid associations, **especially if**
24 ***Trichogramma* species respond to odors from plants induced with *S. exigua* eggs**. In fact, OIPVs are
25 known to play a key role in the attraction of egg parasitoids, including *Trichogramma* wasps, toward

1 plants infested with insect eggs (Hilker and Fatouros 2015). Depending on the nature of the attacking
2 herbivore, the emission of OIPVs **can occur** without plant wounding (Tamiru et al. 2011; Fatouros et
3 al. 2012) or it can be associated with plant damage caused by the female during oviposition (Meiners
4 and Hilker 2000; Hilker et al. 2002) or feeding (Colazza et al. 2004). In previous studies, it has been
5 shown that *B. nigra* emits volatiles in response to egg deposition by *Pieris* butterflies, which were
6 shown to attract both *Trichogramma* species, *T. evanescens* and *T. brassicae* (Fatouros et al. 2012,
7 2014), but these investigations were not carried out within a multiple herbivory context. In this study
8 we have addressed the following questions: 1) Are *Trichogramma* wasps attracted to *B. nigra*
9 volatiles emitted in response to *S. exigua* egg deposition? 2) Is *Trichogramma* attraction toward *P.*
10 *brassicae*-egg infested plants disrupted by concurrent feeding of *S. exigua* caterpillars? 3) Is
11 *Trichogramma* attraction toward *P. brassicae*-egg infested plants disrupted by concurrent feeding of
12 co-evolved herbivores? Volatiles emitted by the plants under various attack scenarios were studied
13 to link the chemical composition with parasitoid foraging behavior. Due to the different origin and
14 nature of herbivores, we expected them to have significant effects on the volatile profiles emitted by
15 the infested plants. This study will contribute to a better understanding of plant-mediated interactions
16 by evaluating the impact of **multiple** insect herbivores which induce different signaling pathways
17 **involved in indirect plant defenses**.

18

19 **Materials and methods**

20 **Plants and insects**

21 Seeds from black mustard plants (*B. nigra*) were collected from a local population growing along the
22 Rhine river in Wageningen (The Netherlands). Plants were grown in a greenhouse (22 ± 2 °C, 60–
23 70% RH, 16L:8D) and used in the experiments when they were four weeks old.

1 The native herbivores, *P. brassicae* and *B. brassicae*, were reared on Brussels sprout plants (*Brassica*
2 *oleracea* var. *gemmifera* cv. Cyrus) in a climate-controlled room or greenhouse at $22 \pm 2^\circ\text{C}$, 60–70%
3 RH 16L:8D. The alien herbivore *S. exigua* was obtained from a colony at the Laboratory of
4 Virology, Wageningen University, and reared on artificial diet as described by Vickerman and
5 Trumble (1999). The native parasitoids *T. brassicae* (strain Y175) and *T. evanescens* (strain ED16)
6 were reared on *Ephestia kuehniella* eggs (Koppert Biological Systems, Berkel en Rodenrijs, The
7 Netherlands) under standardized conditions in a climate chamber ($25 \pm 1^\circ\text{C}$, 50–70% RH, 16L:8D).
8 Only mated, 2–3 days old, wasps were used in the experiments. Wasps lacked previous contact with
9 any plant material or host residues and are referred to as inexperienced.

10

11 **Plant treatments**

12 Plants were incubated in a climate-controlled room ($25 \pm 1^\circ\text{C}$, 50–70% RH, 16L:8D) and were kept
13 in 35 x 35 x 60 cm mesh cages (Vermandel, Hulst, The Netherlands), one treatment per cage. In
14 addition to uninfested control plant (C), the following treatments were used for the behavioral test
15 and headspace analysis (fig. 1):

- 16 a) plant infested with an egg clutch of *P. brassicae* (EP);
- 17 b) plant infested with an egg clutch of *S. exigua* (ES);
- 18 c) plant infested with 20 L1 *P. brassicae* larvae (LP);
- 19 d) plant infested with 105 L1 *S. exigua* larvae (LS);
- 20 e) plant infested with 100 *B. brassicae* aphid nymphs (A);
- 21 f) plant infested with an egg clutch of *P. brassicae* and 20 L1 *P. brassicae* larvae (EP+LP);
- 22 g) plant infested with an egg clutch of *P. brassicae* and 105 L1 *S. exigua* larvae (EP+LS);
- 23 h) plant infested with an egg clutch of *P. brassicae* and 100 *B. brassicae* nymphs (EP+A);

24 The incubation period between the two lepidopteran species differs under our experimental
25 conditions :*P. brassicae* eggs start to hatch 120 h after oviposition whereas *S. exigua* eggs hatch 72h

1 after oviposition. *Trichogramma* wasps can parasitize *P. brassicae* eggs of different age performing
2 well in 72h old eggs (Fatouros et al. 2012) and wasps can develop in *S. exigua* eggs that are 24-36h
3 old (A. Cusumano, personal observations). Emission of OIPVs as a consequence of egg deposition
4 by *P. brassicae* in *B. nigra* starts 24h after oviposition and lasts until eggs are 96h old (Fatouros et
5 al. 2012, 2014). Therefore, we decided to induce plants with *P. brassicae* eggs 72h and *S. exigua*
6 eggs 24-36h before the bioassays. The induction of plant volatiles in response to aphids generally
7 takes longer than the induction of volatiles in response to caterpillar feeding (De Vos et al. 2005).
8 Therefore, plants treated with aphids were incubated for 72 h, whereas caterpillars were introduced
9 24 h before testing in the Y-tube olfactometer.

10 All biotic stresses were applied on fully developed leaves. Aphids and caterpillars were introduced
11 onto the first fully developed leaf whereas the focal stress, i.e. egg deposition, was obtained on the
12 younger adjacent leaf in dual-infestation treatments or a leaf of similar age in treatments with only
13 one biotic stress (fig. 1). For the treatments including *P. brassicae* eggs, oviposition was confined to
14 the selected leaf by covering the plant with a zippered mesh bag that allowed only one leaf to
15 protrude. Plants were then placed in a cage containing more than 100 *P. brassicae* adults, and were
16 removed once one egg clutch (about 30 eggs) had been laid. Any additional egg clutch was
17 immediately removed with a fine brush. Plants with *S. exigua* egg clutches (about 40-50 eggs) were
18 obtained by exposing them to 10 *S. exigua* females during the scotophase. These plants were
19 incubated for an additional day in a climate-controlled environment. Thus, *S. exigua* eggs were 24-
20 36h old when the plants were tested in the bioassays. Egg deposition was confined to the selected
21 leaf by using a bag to cover the plant apart from one leaf, as described for *P. brassicae* and plants
22 with more than one egg clutch were discarded.

23 Plant treatment induced by *P. brassicae* and *S. exigua* larvae differed in the number of caterpillars
24 used due to different sizes and feeding rates of their first instar larvae. In order to ensure that injured
25 plants received the same amount of damage and to exclude the potential effect of leaf area damaged

1 on parasitoid responses, we quantified the leaf area consumed in 24h by L1 caterpillars of both
2 species. To obtain a similar amount of damage, we used plants treated with either 20 L1 *P. brassicae*
3 caterpillars (leaf area consumed= $201.1 \pm 19.9 \text{ mm}^2$; n=10), or 105 L1 *S. exigua* caterpillars (leaf area
4 consumed= $207.9 \pm 27.5 \text{ mm}^2$; n=10) in the bioassays.

5 For treatments involving *B. brassicae* aphids, first and second instar nymphs were used to ensure
6 that no reproduction would occur during the induction period.

7 For each of the three treatments involving dual attack (fig. 1f-h), plants were induced in the same
8 manner as the treatments with one attacker. For treatments f-g, plants were first induced with *P.*
9 *brassicae* eggs and then 48h after oviposition, 20 first instar larvae of *P. brassicae* or 105 first instar
10 larvae of *S. exigua* were placed on the leaf immediately below and allowed to feed for a further 24h.
11 In the case of treatment h, plants were first induced with *P. brassicae* eggs and immediately after
12 oviposition, 100 *B. brassicae* aphids were placed on the leaf below and allowed to feed for 72h
13 before bioassays in order to obtain “quasi-simultaneous” dual attack.

14

15 **Y-tube olfactometer bioassays**

16 We tested the attraction of *T. brassicae* and *T. evanescens* to odors emitted by differently infested *B.*
17 *nigra* plants in dual choice conditions. Both single and dual infestation treatments were tested
18 against a control plant (=undamaged plant), with dually infested plants additionally being tested
19 against a *P. brassicae*-egg-infested plant. Bioassays were conducted in a dynamic airflow Y-tube
20 olfactometer as previously described by Fatouros et al. (2012). This olfactometer was adapted to
21 small wasps like *Trichogramma* spp. to be released in groups. Previously it has been established that
22 group release does not influence the behavior of these wasps (Fatouros et al. 2012). Ten adult
23 females of the first species were released and their preference for one of the two odor sources was
24 recorded. Thereafter, the position of the odor sources was exchanged and another group of 10 wasps
25 from the second wasp species was released to test its preference for the same two odor sources. After

1 30 min, the wasps present in the collection flasks placed at the end of the arms section of the
2 olfactometer were counted. When a wasp did not make a choice within 30 min, it was recorded as a
3 'no response'. Per odor source combination, 8 different plant pairs with one replicate per
4 experimental day were tested with 10 wasps of each species released per replicate
5 (80wasps/species/treatment). Each wasp was used only once. **The order of wasp species tested and**
6 **the position of the odor sources was randomly changed between replicates to avoid possible biases.**
7 Bioassays were performed in 4 blocks according to stage and herbivore identity of attackers
8 (herbivore eggs only, *P. brassicae* caterpillars, *S. exigua* caterpillars, *B. brassicae* aphids). Test
9 combinations were randomized within each block.

10

11 **Headspace collection of volatiles**

12 When testing the response of *Trichogramma* wasps to *B. nigra* volatiles using the Y-tube
13 olfactometer (see above), we simultaneously (**about 80% of the replicates**) or separately (**about 20%**
14 **of the replicates**) collected volatiles from the headspace of the same plant(s). **Regardless of whether**
15 **volatiles were collected simultaneously or separately, we regulated the air flow in the system in**
16 **order to maintain a constant flow rate of 100 mL min⁻¹ in each arm of the olfactometer and 200 mL**
17 **min⁻¹ through each collection cartridge.** Plant volatiles were collected in order to investigate whether
18 differences in volatile profiles could explain the observed behavior of parasitoids. For each
19 treatment, 10 replicates were sampled. In order to prevent any contribution from the collection set-up
20 to the plant volatile profile and to make necessary corrections, air from empty jars was sampled at
21 regular intervals. Volatiles were collected by sucking air with odors out of a glass jar at **the above-**
22 **mentioned** rate for 2h through a stainless steel cartridge filled with 200 mg Tenax TA (20/35 mesh;
23 CAMSCO, Houston, TX, USA) using an external pump (PAS-500 SPECTREX, US) directly
24 connected to the cartridge stainless steel tube with Tenax TA onto the outlet of the glass jars. The
25 Tenax-TA-filled cartridges with the trapped headspace samples were dry-purged for 15 min under a

1 nitrogen (N₂) flow at 50 mL min⁻¹ and stored at ambient temperature until chemical analysis were
2 performed. We followed the protocol for volatile analysis and compound identification as described
3 in detail in the Online Resource 1 of the Electronic Supplementary Material (ESM), using a Thermo
4 Trace GC Ultra in combination with Thermo Trace DSQ quadruple mass spectrometer (Thermo
5 Fisher Scientific, Waltham, USA) for separation and detection of plant volatiles.

6

7 **Statistical analysis**

8 To investigate whether parasitoid preference differed when various combinations of plant treatments
9 were offered, data were analyzed using logistic regression (i.e. a generalized linear model (GLM)
10 with a binomial distribution and a logit link function) with plant treatment and parasitoid species as
11 fixed factors. A quasi-binomial distribution was fitted in the model due to overdispersion. In the
12 comparisons with control plants (=uninfested plants), the number of wasps choosing the attacker-
13 infested plants out of the total number of responding wasps was used as response variable. In the
14 analysis of dual attack versus single attack (=plant infested with *P. brassicae* eggs), the number of
15 wasps choosing the dually infested plant out of the total number of responding wasps was used as
16 response variable. To determine under dual-choice conditions, whether there was a significant
17 preference for one of the offered plant treatments, we tested H₀: logit = 0. Data were analyzed with
18 the R statistical software (R Core Team 2013).

19 For the volatile emission patterns, measured peak areas divided by the above ground fresh mass of
20 the plant were analyzed through multivariate data analysis using projection to latent structures
21 discriminant analysis (PSL-DA). This projection method determines if samples belonging to the
22 different treatment groups can be separated on the basis of quantitative and qualitative differences in
23 their volatile blends. To do this, a Y-data matrix of dummy variables was included, assigning a
24 sample to its respective class. The PLS-DA extension in the SIMCA-P+ 12.0 software program
25 (Umetrics AB, Umeå, Sweden) then approximates the point 'swarm' in X (matrix with volatile

1 compounds) and Y in PLS components in such a way that the maximum covariation between the
2 components in X and Y is achieved. The results of the analysis are visualized in score plots, which
3 reveal the sample structure according to model components, and loading plots, which display the
4 contribution of the variables to these components as well as the relationships among the variables,
5 ranking based on the variable importance in the projection (VIP values) (Wold et al. 2001). Data
6 were log-transformed, mean-centered and scaled to unit variance before they were subjected to the
7 analysis. A Mann-Whitney-U-test was used to test differences in the corrected peak area per
8 compound between infested and uninfested control plants (Online Resource 2 in the ESM).

9 Differences in the total volatile blend between treatments were tested using one-way ANOVA
10 (SPSS, Chicago, IL, USA).

11

12 **Results**

13 **Specificity of OIPVs attracting *Trichogramma* wasps**

14 When testing the induction of plant volatiles by eggs of different herbivore species, there was a
15 significant effect of herbivore identity (GLM, $\chi^2=2.34$; $df=1$; $P<0.001$) but not of wasp identity
16 (GLM, $\chi^2=0.05$; $df=1$; $P=0.95$) on the distribution of the wasps' choices (Fig.2a). Both parasitoid
17 species, *T. brassicae* and *T. evanescens*, significantly preferred OIPVs induced by *P. brassicae* eggs
18 (GLM, T.b.: $t=2.75$; $n = 8$; $P=0.03$; T.e.: $t=5.45$; $n = 8$; $P<0.001$) but did not respond to volatiles of
19 plants infested with *S. exigua* eggs (GLM, T.b.: $t=0.47$; $n = 8$; $P=0.65$; T.e.: $t=-0.48$; $n = 8$; $P=0.64$)
20 when tested against clean control plants.

21

22 **Effect of OIPVs and/ or HIPVs on attraction of *Trichogramma* wasps**

23 When testing a combination of eggs and larvae of the native *P. brassicae*, we found that the
24 distribution of the wasps' choices was affected by the plant treatments (GLM, $\chi^2=6.32$; $df=1$;
25 $P<0.001$) as well as by wasp species (GLM, $\chi^2=0.68$; $df=1$; $P=0.04$) (Fig.2b). Only *T. brassicae* was

1 significantly attracted to volatiles emitted by plants induced with eggs plus L1 caterpillars of *P.*
2 *brassicae* over uninfested plants (GLM,T.b.: $t=2.87$; $n = 8$; $P=0.02$; T.e.: $t=1.14$; $n = 8$; $P=0.29$).
3 However, neither parasitoid species was attracted to odors induced by caterpillars alone when tested
4 against uninfested plants (GLM,T.b.: $t=-1.41$; $n = 8$; $P=0.20$; T.e.: $t=-0.66$; $n = 8$; $P=0.53$). In the
5 bioassays using *S. exigua* larvae, there were no significant differences between plant treatments
6 (GLM, $\chi^2=0.04$; $df=1$; $P=0.65$) or parasitoid species (GLM, $\chi^2=0.08$; $df=1$; $P=0.50$) (Fig.2c).
7 However, in paired choice conditions against uninfested plants, only *T. brassicae* was significantly
8 attracted to volatiles induced by *P. brassicae* eggs in combination with L1 caterpillars of *S. exigua*
9 (GLM,T.b.: $t=2.57$; $n = 8$; $P=0.04$; T.e.: $t=1.46$; $n = 8$; $P=0.19$). Neither parasitoid species was
10 attracted to volatiles induced by *S. exigua* caterpillars alone (GLM,T.b.: $t=1.59$; $n = 8$; $P=0.15$;
11 T.e.: $t=-0.44$; $n = 8$; $P=0.67$). When plants were induced by aphids, there were significant differences
12 between plant treatments (GLM, $\chi^2=5.30$; $df=1$; $P<0.001$) but not between parasitoid species (GLM,
13 $\chi^2=0.03$; $df=1$; $P=0.70$) (Fig.2d). Both parasitoid species were attracted to odors emitted by plants
14 infested with *P. brassicae* eggs plus aphids *versus* uninfested plants (GLM,T.b.: $t=2.53$; $n = 8$;
15 $P=0.04$; T.e.: $t=-2.32$; $n = 8$; $P=0.02$). Interestingly, volatiles emitted by plants infested with aphids
16 alone repelled *T. brassicae* wasps (GLM,T.b.: $t=-3.204$; $n = 8$; $P=0.02$; T.e.: $t=-1.95$; $n = 8$; $P=0.09$).
17 To test for more subtle effects of non-host herbivore identity on parasitoid attraction to OIPVs,
18 dually infested plants were also tested against plants induced with *P. brassicae* host eggs only
19 (Fig.3). There were neither significant differences between the different tested treatment
20 combinations (GLM, $\chi^2=0.03$; $df=2$; $P=0.85$), nor between parasitoid species (GLM, $\chi^2=0.30$; $df=1$;
21 $P=0.14$) on the distribution of wasp choices. Furthermore, preferences by *T. brassicae* and *T.*
22 *evanescens* were not significantly different from a 50:50 distribution in all paired tested conditions
23 (Fig.3).

24

25 **Headspace analysis of plant volatiles induced by different herbivore attackers**

1 A total of 32 different volatile compounds were detected in the headspace of the induced plants
2 across 6 treatments (ES, EP, LP, EP+LP, EP+LS, EP+A); 31 compounds were detected in 2
3 treatments (C, LS) and 30 compounds were detected in the treatment with aphids only (Online
4 Resource 2 in the ESM). Overall, all plants emitted the same compounds, but in different
5 proportions. Thus, the composition of the blend varied according to treatment. The total volatile
6 blend showed significant differences between treatments (ANOVA, $F = 2.236$; $df = 8$; $P = 0.03$)
7 (Online Resource 3 in the ESM).

8 A pairwise comparison by PLS-DA between plant samples infested with *P. brassicae* eggs and those
9 infested with *S. exigua* eggs resulted in a model with 3 significant Principal Components (PCs)
10 (Fig.4a). Thus the model largely separated volatiles emitted by *B. nigra* plants in response to egg
11 deposition by native and alien herbivores, in agreement with the behavioral observations. For this
12 model, 15 compounds had VIP values ≥ 1.0 which means that they strongly contributed to
13 explaining the differences between egg-infested treatments (Fig.4b). These compounds are: (*E*)-4,8-
14 dimethylnona-1,3,7-triene [(*E*)-DMNT], nerylisovalerate, α -caryophyllene, (*E,E*)-4,8,12-trimethyl-
15 1,3,7,11-tridecatetraene [(*E,E*)-TMTT], (*Z*)-3-hexen-1-ol,acetate, 7- α -H-silphiperfol-5-ene,
16 longifolene, presilphiperfol-7-ene, 7- β -H-silphiperfol-5-ene, α -funebrene, silphiperfol-6-ene,
17 farnesylacetaldehyde, allylithiocyanate and 2 unknown compounds.

18 An additional PLS-DA including all sampled plant treatments, resulted in a model with one
19 significant PC (Fig.5a). Despite the observed behavioral differences, this PC separated mainly the
20 volatile blends of plants with or without caterpillar infestation, regardless of the herbivore identity.
21 The only exceptions were the *P. brassicae* eggs + aphids and *S. exigua* egg-treatments, both of
22 which were not separated in the model from caterpillar-treated plants. Examination of the loading
23 plot shows that 9 compounds had VIP values ≥ 1.0 , thus contributing the most to explaining the
24 variation in the model (Fig.5b). These compounds are:(*E*)-4,8-dimethylnona-1,3,7-triene [(*E*)-
25 DMNT)], (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene [(*E,E*)-TMTT], allylithiocyanate, (*Z*)-3-

1 hexen-1-ol acetate, nerylisovalerate, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, Tricyclo[6.3.0.0(1,5)]undec-2-
2 en-4-one, 2,3,5,9-tetramethyl (TUTM) and silphiperfol-5,7(14)-diene.

3

4

5 **Discussion**

6 In the present study, we demonstrated that *B. nigra* volatiles induced by eggs of *P. brassicae* and by
7 eggs of *S. exigua* differ in terms of quantitative composition and parasitoid attraction. In fact, both *T.*
8 *brassicae* and *T. evanescens* are attracted by *P. brassicae*-egg induced plant volatiles but not by *S.*
9 *exigua*-egg induced plant volatiles indicating species specificity in the plants' response to egg
10 deposition. Considering the key role played by OIPVs in host location by egg parasitoids, we also
11 suggest that *Trichogramma* host shifts from *P. brassicae* to *S. exigua* are unlikely to take place in *B.*
12 *nigra*-associated insect communities. Plant specificity of responses observed in our study system
13 could be a direct consequence of (the lack of) plant-insect coevolution. Plants are expected to evolve
14 adaptive defense responses against coevolved herbivores but this hypothesis may not hold when an
15 alien herbivore invades native plant-insect food webs (Desurmont et al. 2014). However, other
16 factors could also play a role taking into account that plants seem to respond differently to egg
17 deposition according to the host specialization and/ or abundance of the herbivore (Hilker and
18 Fatouros 2015). In our study, parasitoid-attracting volatiles are induced only by the specialist
19 herbivore *P. brassicae* but not by the generalist species *S. exigua*. This hypothesis is also supported
20 by previous findings in *B. nigra* showing that *T. brassicae* did not respond to OIPVs induced after
21 24-36h by another generalist species, the native cabbage moth, *Mamestra brassicae* (Fatouros et al.
22 2012). Furthermore, in a different study system, it was found that oviposition by the abundant
23 specialist pine sawflies *Diprion pini* and *Neodiprion sertifer* on *Pinus sylvestris* induced the
24 emission of pine volatiles that attracted the egg parasitoid *Chrysonotomyia ruforum*, whereas eggs of
25 the less abundant pine sawfly *Gilpinia pallida* did not induce such a response (Mumm et al. 2005).

1 Further studies are required to elucidate the relative importance of factors such as herbivore origin
2 and coevolution as well as herbivore dietary specialization in triggering egg parasitoid attraction
3 toward OIPVs. While the mechanistic aspects of such specificity have not been demonstrated yet, it
4 is possible that the observed differences in induction between *P. brassicae*, *S. exigua* and *M.*
5 *brassicae* could be the result of species-specific elicitors associated with the eggs or their secretions
6 (Hilker and Fatouros, 2015). Alternatively, Fatouros et al. (2012) speculated that herbivore
7 specificity of plant–lepidopteran egg interactions could be related to egg attachment on the plant
8 surface: specialist herbivores such as *P. brassicae* are expected to attach their eggs more firmly on
9 brassicaceous plants compared with generalist herbivores such as *S. exigua* and *M. brassicae*. How
10 firmly eggs are attached to the leaf surface may affect different cells that are able to perceive
11 information about when an egg has been laid (Hilker and Fatouros, 2015). There is evidence that
12 egg-induced plant defenses differ between wild and cultivated plants (Fatouros et al. 2005, 2012,
13 Tamiru et al. 2011; 2015). In fact, *P. brassicae*-egg induced plant volatiles recruit *Trichogramma*
14 wasps when emitted by black mustard plants but not when emitted by Brussels sprouts plants,
15 suggesting that artificial plant breeding for traits related to yield might have caused a loss in
16 components of indirect plant defense. It is clear from our results that the composition of *B. nigra*
17 volatile blends differentially changed in a quantitative way in response to egg deposition by *P.*
18 *brassicae* and *S. exigua*, which suggests that *Trichogramma* parasitoids can detect changes in ratios
19 of compounds within the blends, adopting a so-called “ratio-specific odor recognition” foraging
20 strategy (de Boer et al. 2004; Bruce et al. 2005; Clavijo McCormick et al. 2012).

21

22 In this study we also investigated *Trichogramma* species attraction toward *P. brassicae*-egg induced
23 volatiles in plants concurrently attacked by different non-hosts. We found that *T. brassicae* displays
24 fine-tuned foraging responses as no disruption was found regardless of whether aphids, exotic or
25 native caterpillars were concurrently attacking the plant carrying *P. brassicae* eggs. On the contrary,

1 *T. evanescens* was still attracted to OIPVs only when aphids, but not caterpillars, were also attacking
2 the *P. brassicae*-egg infested plant. We did not find more subtle effects as neither parasitoid species
3 discriminated between volatiles emitted by dually infested plants and volatiles emitted by *P.*
4 *brassicae*-egg infested plants. This lack of discrimination suggests that key compounds associated
5 with egg deposition can still be emitted by dually infested plants, and perceived by the highly
6 sensitive antennal olfactory sensilla of *Trichogramma* wasps. These findings cannot be elucidated by
7 chemical changes in the volatile blend as the PLS-DA separated between OIPVs emitted by plants
8 induced by *P. brassicae* alone compared with OIPVs emitted by dually infested plants. **PLS-DA**
9 **analyses take into account all volatiles including those released as a consequence of feeding damage**
10 **by non-hosts, which induced dramatic quantitative changes in the whole profiles emitted by dually**
11 **infested plants.** However, the wasps likely use a specific subset of compounds of the total blend.
12 **Thus, a mismatch between behavioral responses and chemical analyses could be due to the fact that**
13 **the parasitoids focused on the subset of volatiles associated with *P. brassicae*-egg deposition which**
14 **constitute the active blend, whereas PLS-DA takes the total blend into account.**

15 In previous studies on dual herbivore attack, natural enemy attraction was disrupted, unaffected or
16 even enhanced by volatiles of dually-infested plants, indicating that the effect of multiple herbivory
17 on plant volatile emission is variable (Shiojiri et al. 2001; Rasmann and Turlings 2007; Soler et al.
18 2007; de Boer et al. 2008; Zhang et al. 2009, 2013; Erb et al. 2010; Bukovinszky et al. 2012;
19 Moujahed et al 2014; Ponzio et al. 2014). Despite the lack of general patterns, it is usually assumed
20 that plant **response** specificity to herbivory generates specificity in parasitoid responses toward
21 HIPVs (de Rijk et al. 2013). The results of our study extend this concept as we also found specificity
22 in plant responses and specific parasitoid attraction toward OIPVs. Interestingly, the induction of
23 volatiles by *S. exigua* or *P. brassicae* caterpillars had no effect on the attraction toward *P. brassicae*
24 egg-induced volatiles by *T. brassicae* but disrupted the attraction of *T. evanescens*, suggesting a
25 similar impact of **different** non-host lepidopteran species in a tritrophic perspective. In fact, we also

1 found that *Trichogramma* wasps are not attracted toward HIPVs emitted by *B. nigra* plants infested
2 with *P. brassicae* or *S. exigua* caterpillars. Such lack of response could be particularly adaptive
3 considering that moths and butterflies, including *P. brassicae*, usually do not lay eggs on already
4 infested plants in order to avoid intraspecific competition for food resources (Bruce et al. 2010;
5 Fatouros et al.2012). However, when moths with overlapping generations do not display such
6 avoidance behavior, HIPVs emitted by the non-host target stage could still be a reliable signal for
7 egg parasitoids **as larvae and eggs may be simultaneously present on the same plant** (Penaflor et al.
8 2011). On the contrary, the parasitoid responses toward HIPVs induced by non-host species are
9 generally assumed to be non-adaptive as such chemical cues are unreliable indicators of host
10 presence (Moujahed et al. 2014). Our results support this assumption considering that *T. evanescens*
11 does not respond to HIPVs from aphid-infested plants and *T. brassicae* is even repelled by these
12 induced volatiles.

13 Our results can be understood in the context of the plant defense pathways elicited by herbivores
14 belonging to different feeding guilds. Considering that eggs and phloem-feeding insects both
15 generally induce the same signal-transduction pathways, it was expected that aphids would not
16 interfere with egg parasitoid attraction toward OIPVs. On the other hand, feeding by leaf-chewers
17 activates a different signal transduction pathway than egg deposition, and interference of non-host
18 chewers on egg parasitoid foraging behavior has already been reported using a non-host chewing
19 herbivore (*Sitona lineatus*) whose adults attacks leaves but larvae feed on roots (Moujahed et al.
20 2014). Interestingly, *Vicia faba* plant damaged by either *S. lineatus* adults or larvae had a similar
21 disruptive effect on the attraction of the egg parasitoid *Trissolcus basalus* toward OIPVs induced by
22 the host *Nezara viridula*, suggesting that a common interference mechanism might be involved. In
23 our study we found an interference effect of both *S. exigua* and *P. brassicae* caterpillars on attraction
24 by egg parasitoids, but disruption occurred only for *T. evanescens*. The lack of interference of
25 chewers on the foraging behavior of *T. brassicae* indicates that OIPVs emitted by *B. nigra* in

1 response to *P. brassicae*-egg deposition provide a robust chemical signal for this egg parasitoid
2 which withstands disruption by non-host attackers, even when herbivores activate different plant
3 signal-transduction pathways. This phenomenon is not new, as larval parasitoids are known to
4 successfully locate caterpillar hosts using HIPVs emitted by plants concurrently infested with
5 phloem-feeding non-hosts (Erb et al. 2010;Ponzio et al. 2014).

6 The differences observed between *T. brassicae* and *T. evanescens* could be related to the lack of
7 oviposition experience of tested parasitoid females. To locate hosts in complex environments that
8 undergo spatial and temporal changes in infestation by both hosts and non-hosts with corresponding
9 changes in plant-derived odor cues, parasitoid females could rely on learning abilities. In fact, it has
10 been shown that plasticity in parasitoid responses after associative learning can be adaptive when
11 foraging in multiple herbivore scenarios especially for generalist species (Rasmann and Turlings
12 2007; Hoedjes et al. 2011; Moujahed et al. 2014). Previous studies have demonstrated that
13 associative learning is relatively more important for the foraging behavior of *T. evanescens* than for
14 *T. brassicae*. The former is known to parasitise a wide range of lepidopteran hosts (Polaszek 2010)
15 and is, therefore, considered to be more generalist. For example *T. brassicae* uses oviposition-
16 induced contact synomones, whereas *T. evanescens* shows less specificity in its response but can
17 learn to exploit such cues after an oviposition experience (Fatouros et al. 2007; Pashalidou et al.
18 2010). Similarly, associative learning after an oviposition experience may be required for *T.*
19 *evanescens* in order to exploit OIPVs emitted from plants concurrently attacked by *P. brassicae* or *S.*
20 *exigua* caterpillars when offered against odors of uninfested plants. Alternatively, learning can be
21 important for *T. brassicae* resulting in a preference for plants infested only with *P. brassicae* eggs
22 when tested against plants dually exposed to *P. brassicae* eggs and non-host caterpillar feeding.

23 The impact of alien non-host herbivores on tritrophic interactions has been investigated in another
24 model study indicating that the attraction toward HIPVs by the larval parasitoid *Cotesia glomerata*
25 was disrupted when host (*Pieris brassicae*) and non-host (*Spodoptera littoralis*) caterpillars were

1 simultaneously present on the same *Brassica rapa* plant (Chabaane et al. 2015). However, in this
2 study no additional non-host was tested so it is difficult to assess if exotic non-host herbivores
3 disrupt HIPV attraction in a different way than native non-hosts. Our results also indicate that alien
4 non-host herbivores have the potential to disrupt native tritrophic interactions **but the question**
5 **whether exotic herbivores interfere more strongly than native herbivores cannot be disentangled**
6 **because in our model study-system the herbivores differ in many traits other than plant-insect**
7 **coevolution.** Further studies comparing **phylogenetically similar species of** native and alien
8 herbivores are required to **investigate** if, regardless of the lack of co-evolution, chewers interfere
9 more strongly than phloem-feeders. In summary, our study contributed to a better understanding of
10 plant-mediated interactions under multiple herbivory by evaluating the impact of insect herbivores
11 **that differ in origin, specialization and** feeding guild on plant volatile emission and foraging
12 behavior **of different parasitoid species.**

13

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4

1 **Figure legend**

2 **Figure 1.** Visual summary of the main plant treatments including (a) plant infested with an egg
3 clutch of *P. brassicae* (EP); (b) plant infested with an egg clutch of *S. exigua* (ES); (c) plant infested
4 with *P. brassicae* larvae (LP); (d) plant infested with *S. exigua* larvae (LS); (e) plant infested with *B.*
5 *brassicae* aphid nymphs (A); (f) plant infested with an egg clutch of *P. brassicae* and *P. brassicae*
6 larvae (EP+LP); (g) plant infested with an egg clutch of *P. brassicae* and *S. exigua* larvae (EP+LS);
7 (h) plant infested with an egg clutch of *P. brassicae* and *B. brassicae* nymphs (EP+A).

8 **Figure 2.** Percentage (mean+SE) of female *Trichogramma* wasps choosing volatiles emitted by
9 infested plants *versus* uninfested control plants in a Y-tube olfactometer. *Brassica nigra* plants were
10 infested with: (a) eggs of *Pieris brassicae* (EP) or eggs of *Spodoptera exigua* (ES); (b) L1
11 caterpillars of *P. brassicae* (LP) or eggs and L1 of *P. brassicae* (EP+LP); (c) L1 of *S. exigua* (LS) or
12 eggs of *P. brassicae* and L1 of *S. exigua* (EP+LS); (d) aphid nymphs of *Brevicoryne brassicae* (A)
13 or eggs of *P. brassicae* and aphid nymphs of *B. brassicae* (EP+A). Bars represent the mean
14 percentage of choice displayed by *Trichogramma brassicae* (light grey) and by *T. evanescens* (dark
15 grey). Asterisks indicate a preference which is significantly different from a 50:50 distribution
16 within a choice test: *P <0.05; ** P <0.01; *** P < 0.001; “ns” is not significantly different (GLM).
17 Different letters indicate statistical differences (P<0.05) between differently infested plants (GLM).
18 Each treatment combination was replicated with eight plant pairs and 10 wasps of each species per
19 plant pair (n =80 wasps per treatment/species). N_{resp}=number of responding wasps.

20 **Figure 3.** Percentage (mean+SE) of female *Trichogramma* wasps choosing volatiles emitted by
21 infested plants *versus* *Pieris brassicae* egg-infested plants in a Y-tube olfactometer. *Brassica nigra*
22 plants were infested with eggs and L1 caterpillars of *P. brassicae* (EP+LP); eggs of *P. brassicae* and
23 L1 larvae of *Spodoptera exigua* (EP+LS); eggs of *P. brassicae* and aphid nymphs of *Brevicoryne*
24 *brassicae* (EP+A). Bars represent the mean percentage of choice displayed by *Trichogramma*

1 *brassicae* (light grey) and by *T. evanescens* (dark grey). “ns” indicates no significant differences
2 ($P < 0.05$) from a 50:50 distribution within a choice test (GLM) whereas the same letter indicates no
3 significant differences between treatments (GLM). Each treatment combination was replicated with
4 eight plant pairs and 10 wasps of each species per plant pair ($n = 80$ wasps per treatment/species).
5 N_{resp} = number of responding wasps.

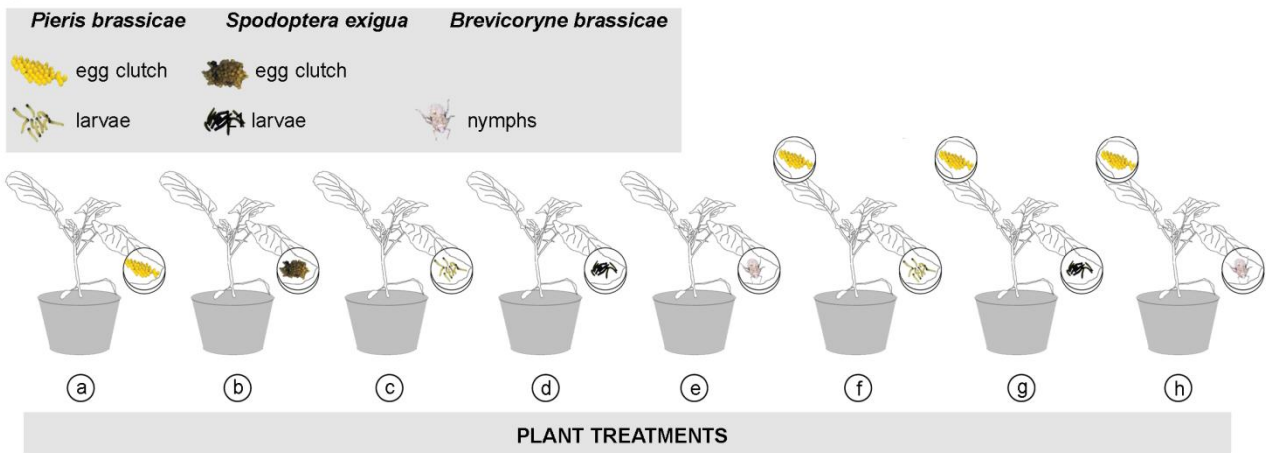
6 **Figure 4.** Projection to Latent Structures Discriminant Analysis (PLS-DA) of volatile compounds
7 emitted by *Brassica nigra* plants infested with eggs of *Pieris brassicae* (EP) or *Spodoptera exigua*
8 (ES). (A) Score plot visualizing the grouping pattern of the samples according to the first two
9 principal components (PCs) with the explained variance in brackets. The PLS-DA resulted in a
10 model with three significant components but only the first two PCs are shown for representational
11 purposes. The ellipse defines Hotelling’s T^2 confidence region (95%). (B) Loading plot of the first
12 two principal components shows the contribution of each of the compounds to the two PLS-DA
13 components. Markers of the two different treatments shown in the score plot are given. Each
14 treatment had 10 replicates. Numbers refer to compounds listed in the Online Resource 2 of the
15 ESM.

16 **Figure 5.** Projection to Latent Structures Discriminant Analysis (PLS-DA) of the volatile compounds
17 emitted by *Brassica nigra* plants. Plants were left uninfested (C) or induced with eggs of *Pieris*
18 *brassicae* (EP), eggs of *Spodoptera exigua* (ES), L1 larvae of *P. brassicae* (LP), L1 larvae of *S.*
19 *exigua* (LS); aphid nymphs of *Brevicoryne brassicae* (A); eggs and L1 larvae of *P. brassicae*
20 (EP+LP); eggs of *P. brassicae* and L1 larvae of *S. exigua* (EP+LS); eggs of *P. brassicae* and aphid
21 nymphs of *B. brassicae* (EP+A). (A) Score plot visualizing the grouping pattern of the samples
22 according to the first two PLS components. The PLS-DA resulted in a model with one significant
23 principal component only but the second axis is shown for representational purposes. The ellipse
24 defines Hotelling’s T^2 confidence region (95%). (B) Loading plot of the first two principal
25 components shows the contribution of each of the compounds to the two PLS-DA components.

- 1 Markers of the nine different treatments shown in the score plot are given. Each treatment had 10
- 2 replicates. Numbers refer to compounds listed in the Online Resource 2 of the ESM.

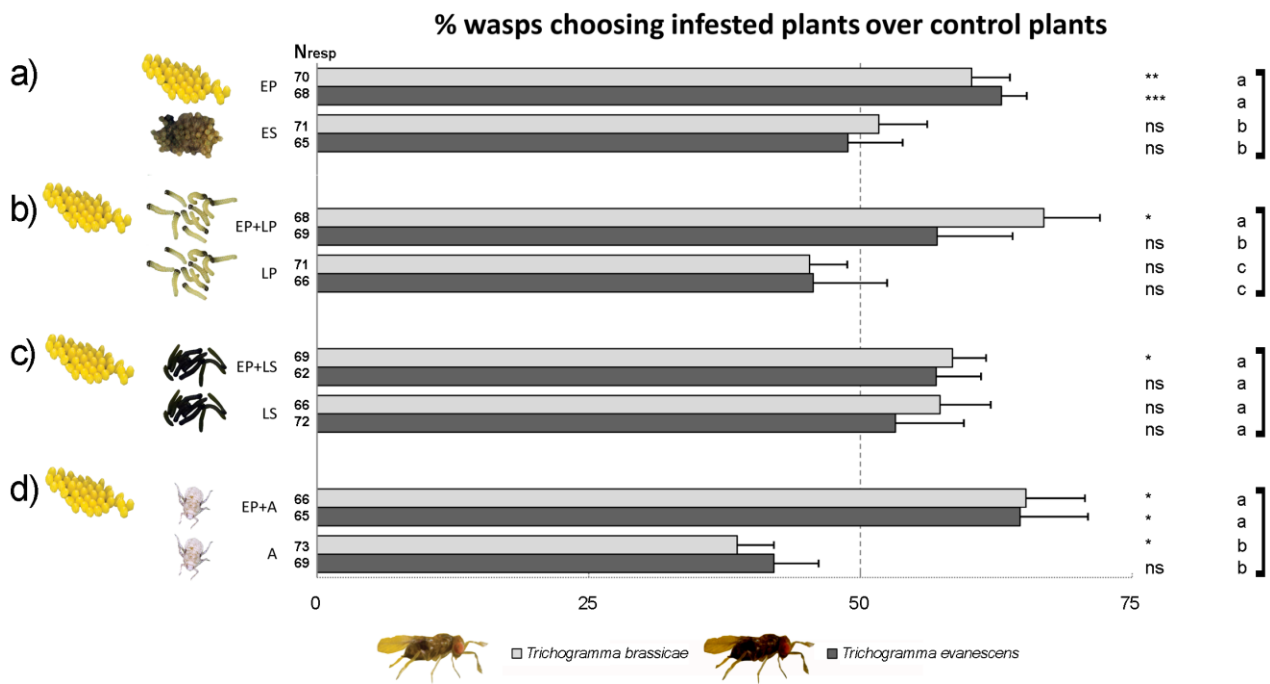
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1 **Figure 1**



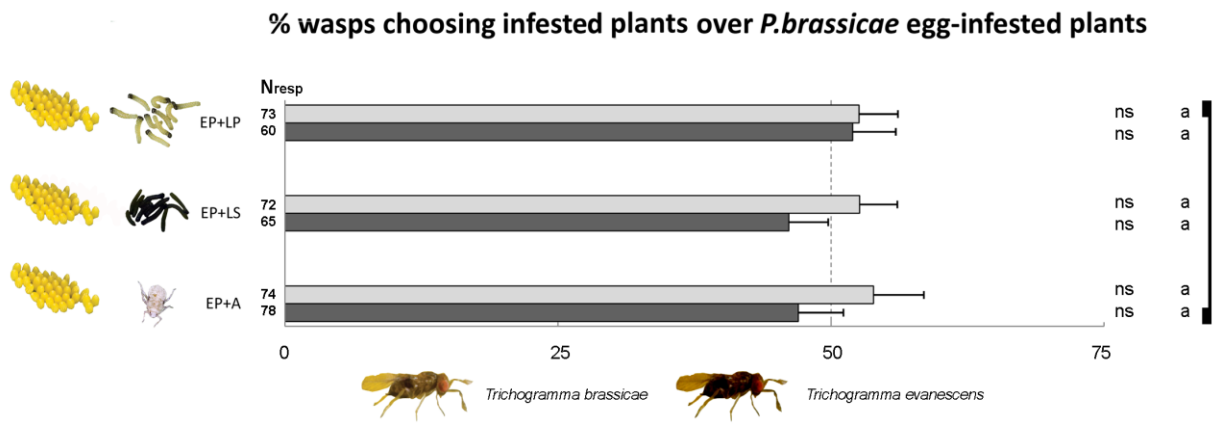
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1 **Figure 2**



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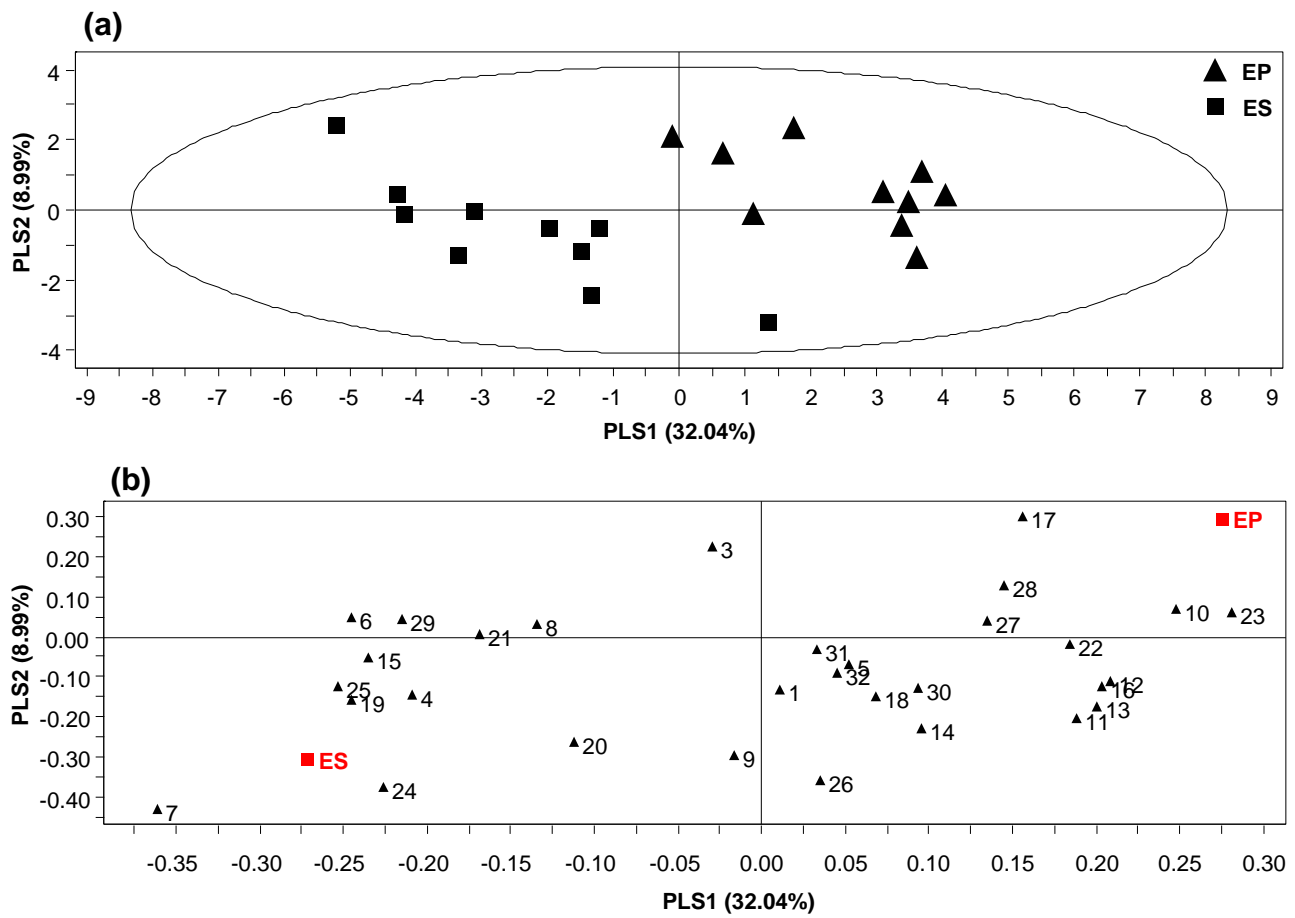
1 **Figure 3**



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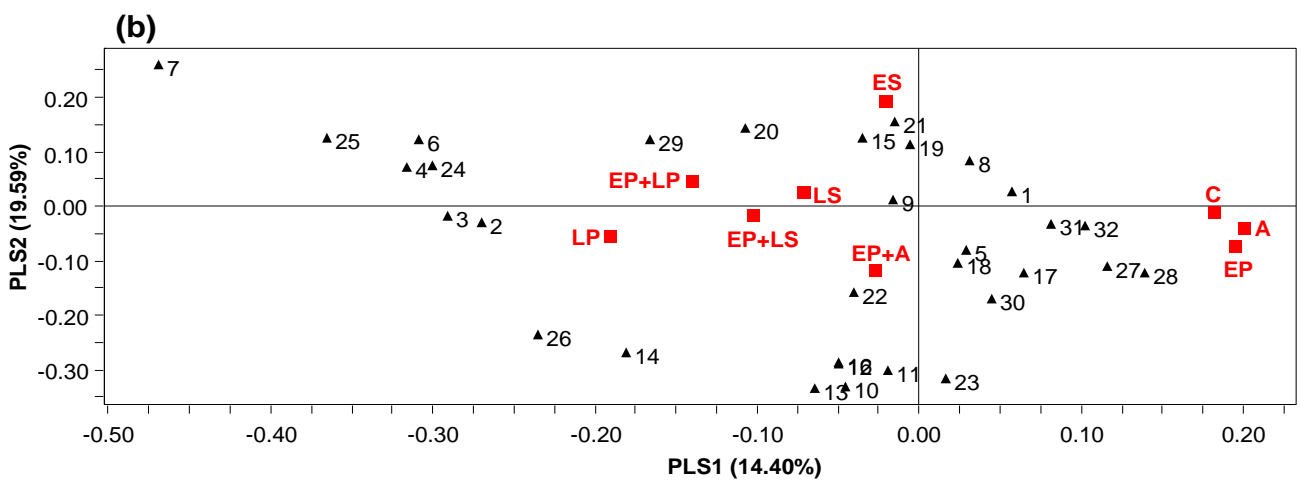
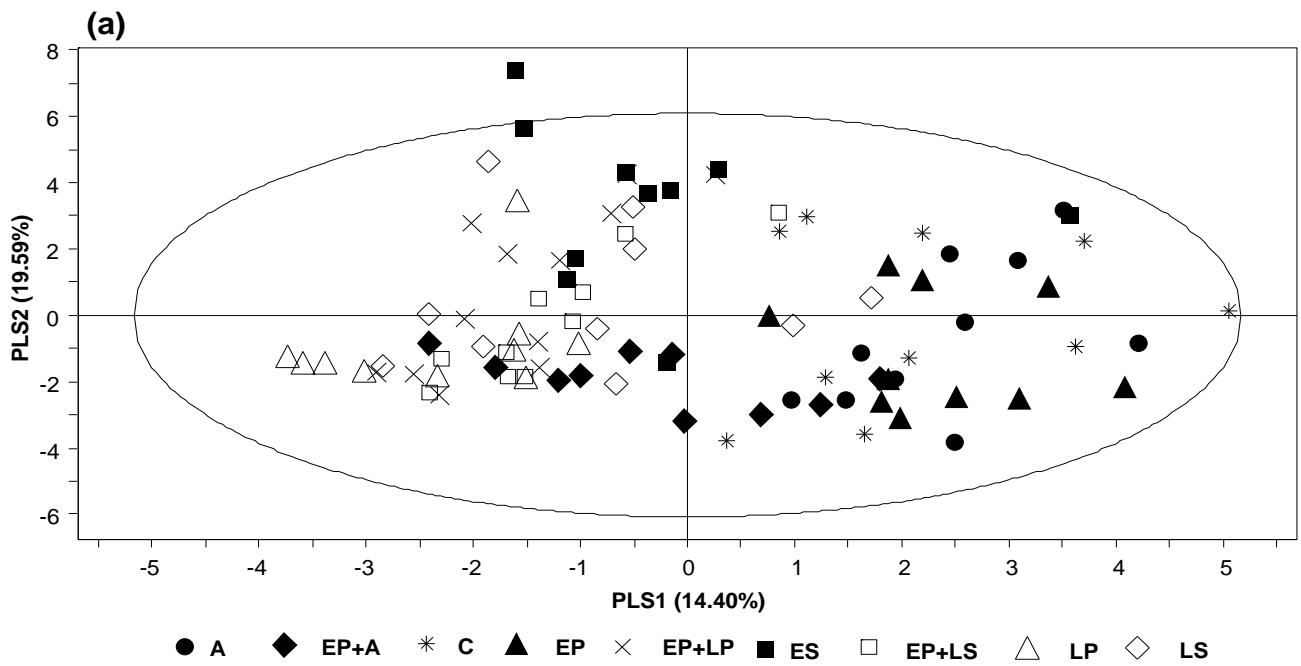
1 **Figure 4**



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1 **Figure 5**



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