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Impact of the invasive painted bug *Bagrada hilaris* on physiological traits of its host *Brassica oleracea* var botrytis

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Abstract *Bagrada hilaris* is a herbivorous insect native of Asia and Africa which has invaded southern Europe and North America where it causes major damage to cole crops. Laboratory experiments were conducted to assess how the infestation of this invasive species damages the host *Brassica oleracea* var botrytis, and to evaluate the interaction between plant emission of volatile organic compounds (VOC) and *B. hilaris* adults. Plant responses to insect feeding were evaluated through changes in photosynthesis, stomatal conductance, VOC emission, and visual damage on leaves. The impact of *B. hilaris* was compared with that of *Nezara viridula*, a polyphagous species distributed worldwide. Plant VOC role in host plant detection was tested with electroantennography bioassays on *B. hilaris* antenna. Photosynthesis and stomatal conductance were consistently reduced in plants infested with 40 *B. hilaris* adults for 24 h. The feeding activity of a single *B. hilaris* caused larger discolored spots on host leaves in comparison with *N. viridula*. VOC emitted by *B. oleracea* changed significantly in response to *B. hilaris* and *N. viridula* infestation. In particular, production of limonene was strongly reduced by the infestation of the two pentatomids, while an increase in the emission of acetic acid and 2-ethyl-1-hexanol was observed. EAG dose–response tests using the main plant VOC showed *B. hilaris* antennal responses to benzaldehyde, octanal, nonanal, and acetic acid, which indicates a role of these compounds in host location.

Keywords *Bagrada hilaris* · *Nezara viridula* · Photosynthesis · Stomatal conductance · Visual damage · Volatile organic compounds (VOC) · Electroantennography (EAG)

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Introduction

Invasions by phytophagous insects are an important consequence of global change, as alien insects threaten indigenous species, and contribute to biodiversity loss, ecosystem degradation, and impairment of ecosystem services worldwide (Kenis et al. 2009). Alien insects can affect native biodiversity through direct interactions, e.g., herbivore feeding on a native plant (Jenkins 2003), but also indirectly, e.g., competing for food or space with native species (Roques et al. 2009). However, the differences in terms of insect–plant interactions between invasive and local phytophagous pests have rarely been investigated (Paris et al. 2010).

It is well known that plants can respond to herbivore feeding by changing the emission of volatile organic compounds (VOC) at the quantitative or qualitative level, also producing "herbivore induced plant volatiles" (HIPV) (Dicke and Van Loon 2000). Changes in the VOC profile may be accompanied and/or associated with a change in the physiology of the host plant (Paré and Tumlinson

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1997; Holopaainen and; Gershenzon 2010). The feeding mechanism of phytophagous species (chewing or piercing-sucking) also plays an important role in influencing host plant physiology (Welter 1989). In the case of true bugs (Hemiptera: Heteroptera), they penetrate plant tissues with stylet mouthparts and display different feeding behaviors classified as stylet sheath feeding, lacerate-and-flush feeding, macerate-and-flush feeding, and osmotic pump feeding (Hori 2000). Generally, these phytophagous insects cause less mechanical damage to leaves compared to chewing herbivores, and the overall impact on plant physiology is also reduced (Hare and Elle 2002). Nevertheless true bugs are also responsible for serious crop losses and are a major threat to agriculture worldwide because of their typical aggregation behavior on host plants and their potential to transmit pathogens (Schaefer and Panizzi 2000). In recent decades, the impact of true bug infestation on plants VOC emission was investigated in some plant-pentatomid systems (Williams et al. 2005; Velikova et al. 2010; Moujahed et al. 2014).

The painted bug, *Bagrada hilaris* Burmeister, is an invasive pentatomid species feeding mainly on brassicaceous hosts, and particularly dangerous to crop production in newly invaded areas (Huang et al. 2014). This pest, native to Asia and Africa, was first reported in Europe in 1978 in Pantelleria Island (Italy), and in Malta, where it feeds on *Capparis spinosa* L., caper bush (CAB 1981; Colazza et al. 2004). In Europe, *B. hilaris* has been so far confined to these two islands. In 2008, the painted bug was first reported in California, probably introduced by commercial trade, and rapidly expanded its range to the brassicaceous crops of coastal California and southwestern Arizona (Palumbo and Natwick 2010). Successively, it was reported in Nevada, New Mexico, and Utah (Bundy et al. 2012). More recently, *B. hilaris* has been observed in México (Sánchez-Peña 2014) and on the Big Island of Hawaii (Palumbo et al. 2016). Since its introduction in North America, *B. hilaris* had exerted a strong negative impact on agriculture; it has been estimated that about 90% of the broccoli acreage planted in USA has been infested by the painted bug, with yield losses often exceeding 10% of production (Huang et al. 2014). The bug damage appears as circular or star-shaped chlorotic spots on plant tissues that become necrotic (Palumbo et al. 2016).

Seedlings and young plants of several *Brassica* species are highly susceptible to feeding damage on cotyledons, newly emerged leaves, and apical meristems (Sánchez-Peña 2014), with significant reductions in leaf area, chlorophyll content, and dry weight (Huang et al. 2014).

The aggressiveness of this species poses questions about the real impact of the invasive *B. hilaris* feeding activity on physiology of *Brassica* host plants. The aim of this study was to investigate the effects of artificial *B. hilaris* infestation on *Brassica oleracea* var botrytis by measuring visual damage, photosynthesis, stomatal conductance, and plant VOC emissions. These aspects have been rarely investigated among pentatomids, e.g., on the harlequin bug *Murgantia histrionica* Hahn (Conti et al. 2008; Velikova et al. 2010). The effects of the invasive painted bug infestation were compared with those of *Nezara viridula L.*, a pentatomid of African origin with a wide range of host plants, but long established in Europe and America. Finally, the potential role of plant volatile compounds in host location by *B. hilaris* was investigated by EAG dose–response tests.

Materials and methods

Plants and insects

Cauliflower seeds (*B. oleracea* L. var botrytis, cv Alverda) were planted individually in plastic pots (8×8 cm), filled with soil (COMPO BIO, Compo Italia srl, Italy). Cauliflowers were grown in a greenhouse at 20–25 °C, 60–80% RH with a 16/8 (light/dark) photoperiod. Plants were irrigated daily and fertilized with a 1 g l^{-1} solution of Flory 9 Hydro (N-P-K 15-7-22) (Planta Regenstauf). Forty days after planting, seedlings were selected for dimensional uniformity. Selected plants were moved to a (4×6×2 m) walk-in growth chamber programmed with 16-h photoperiod, photosynthetic photon flux density (PPFD) of 600 µmol photons m^{-2} s^{-1} at the top of the foliage, CO_2 concentration of 420 µmol mol^{-1}, 24 ± 2 °C temperature, and 65% relative humidity. Plants were acclimated to the climatic conditions of the chamber for 2 days before insect infestation. Plants with 6–7 expanded leaves were used for the experiments.

Insect species were reared in an environmentally controlled room (25 ± 1 °C, 70 ± 10% RH, photoperiod 16L:8D), inside wooden cages (25×25×40 cm) with two 5-cm diameter mesh-covered holes for ventilation. The colony of *B. hilaris* was established and restocked regularly with insects collected from caper fields (*C. spinosa*) located in the island of Pantelleria (Italy). The insect colony was provided with cauliflower, cole-seed, and cabbage plants, depending on seasonal availability. As *B. hilaris* lays eggs in the soil, dishes (6-cm Ø) with a mixture of sand, silt, and clay (33% for each soil component) were placed inside the cages to allow oviposition. Dishes were changed weekly, and those with glued eggs were kept in separated cages until emergence of nymphs.

Similarly, a colony of *N. viridula* was established from locally collected individuals. Insects were fed with sunflower seeds and seasonal fresh vegetables. For both insect species, separate rearing cages were used for nymphs and adults. Adults used for the experiments were randomly selected among individuals 5–10 days old.

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Plant damage

The impact of *B. hilaris* and *N. viridula* feeding activity on host plants was investigated in terms of change of ecophysiological parameters and visual damage. Impact on photosynthesis and stomatal conductance was evaluated by infesting a single *B. oleracea* plant placed in a Plexiglass cage (40 × 40 × 40 cm) with the ceiling made of net for ventilation. The following treatments were made: (a) plants infested with 15 adults of *B. hilaris* ($N = 6$); (b) plants infested with 40 adults of *B. hilaris* ($N = 6$); (c) plants infested with 15 adults of *N. viridula* ($N = 6$); (d) healthy plants ($N = 8$). Sex ratio of individuals used for this experiment was 1:1. Plants were infested for 24 h in the growth chamber in an environmentally controlled room maintained at 25 ± 1 °C, 70 ± 10% RH, photoperiod of 16:8 (light:dark). Immediately before measuring ecophysiological parameters, the insects were removed from the infested plant using an artist's paintbrush. Gas exchanges were measured using the LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE, USA). A portion (6 cm^2) of the leaf area was clamped in the gas-exchange cuvette and exposed to a 0.44 l min^{-1} flow of synthetic air, contaminant-free, and made by mixing N_2 (80%), O_2 (20%) and 400 ppm CO_2. Air humidity was set at 45–55%, PPFD at 800 µmol m^{-2} s^{-1}, and air temperature inside the cuvette at 25 °C. Photosynthesis (A), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were calculated using the LI-6400 software.

In order to compare visual damage, i.e., the area of foliar discolored spots caused by feeding of *B. hilaris* and *N. viridula* individuals, experiments were carried out in Petri dishes. A single *B. oleracea* leaf (mean surface 15.15 ± 1.00 cm^2) was cut at the base of the petiole, which was inserted in a 2-ml vial filled with water-soaked cotton to maintain turgidity, and placed in a plastic Petri dish (10-cm Ø) with 5-cm Ø mesh-covered holes on the lid for ventilation. Only females were used for this bioassay, as previous studies on *B. hilaris* revealed that females feed for significantly longer periods, and cause almost fivefold more damage than males (Huang et al. 2014). A single randomly selected female of *B. hilaris* or *N. viridula* was gently placed inside the Petri dish using a paintbrush. Females were food deprived for 24 h before the experiment. The bioassays were conducted in a climatic room at conditions of 25 ± 1 °C, 70 ± 10% RH, 16:8 photoperiod. After 24 h, insects were removed and the size (in mm^2) of each single discolored spot induced from *B. hilaris* or *N. viridula* individuals was estimated by measuring the areas under a stereomicroscope with the help of a transparent graph paper sheet (Tecnopen – DQ, Buffetti, Roma, Italy). Twelve replicates were conducted per treatment.

VOC emission

Plants used to estimate the ecophysiological parameters were also used for volatile collection immediately after measuring photosynthesis and stomatal conductance. To measure VOC emissions, the outlet of the gas-exchange cuvette was disconnected from the LI-6400 system, and the flow was diverted into a cartridge packed with 30 mg of Tenax TA and 30 mg of Carboxen 1000 (SRA Intruments, Cernusco sul Naviglio, Milano, Italy). A volume of 5 l of air was pumped through the trap at a rate of 0.3 l min^{-1}. The cartridge was stored in a refrigerator until analysis by GC–MS, using an Agilent 7820 GC equipped with a 5975 C MSD with EI ionization (Agilent Technologies, Wilmington, DE). A Gerstel MPS2 XL autosampler equipped with automated Thermal Desorption Unit (TDU) and liquid CO_2-cooled programmable temperature vaporizer (GERSEL CIS 4) was used for ensuring consistent VOC extraction and injection conditions. The following chromatographic settings were used: injector in splitless mode set at 260 °C; J&W Innowax column (30 m, 0.25 mm i.d., 0.5 µm df); oven temperature program starting with an initial temperature of 40 °C (1 min), then ramping at 5 °C min^{-1} until 200 °C, at 10 °C min^{-1} until 220 °C, and at 30 °C min^{-1} until 260 °C, and hold time at the highest temperature of 3 min. Helium was used as carrier gas at 1 ml min^{-1}. The MS detector was operated in scan mode in the m/z range 29–330, at three scans s^{-1}. Synthetic standards provided from Sigma–Aldrich (Milan, Italy) were used for chemical identification.

Electroantennography

Electroantennogram (EAG) dose–response recordings were conducted with the main VOC identified in the previous experiment: (*R*)-limonene, (*S*)-limonene, octanal, nonanal, decanal, benzaldehyde, acetic acid. All used VOC where ≥95% purity, obtained from Sigma-Aldrich (Milan, Italy). VOC were serially diluted 1:10 with hexane (Sigma-Aldrich, HPLC-grade, 99%) up to the concentration of 1 µg l^{-1}. For EAG experiments, a standard 1 µl aliquot of each test solution was pipetted onto a piece of filter paper (Whatman, grade 1), exposed to the air for 30 s to allow the solvent to evaporate, and then inserted into a glass Pasteur pipette. Puff stimuli were blown across insects' antennae using a flow controller (model CS-05; Syntech, Hilversum, The Netherlands) to generate a 1.5-s stimulus at 1-min interval, with a flow rate of 1.5 l min^{-1}. The signals generated by the antennae were passed through a high-impedance amplifier (model IDAC-4, Syntech) and recorded with specialized software (Syntech). At the beginning and the end of the stimulation of the antennae with each concentration of the compounds, 1 µl pure hexane was puffed

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as reference. Tested compounds were used for each active ingredient, using the amounts of 1, 10, 100, and 1000 µg. The sequence of the stimuli of all the compounds was provided starting from the lowest dose. The same antenna was used to test all the concentrations of a single compound. The response elicited from hexane (mean of the two puffs) was subtracted from the responses obtained by the test stimuli. Each dose of the chemicals was tested on one randomly selected antenna per insect ($N=14$; sex ratio 1:1). *Bagrada hilaris* adults were anesthetized by refrigerating them at about −4 °C for approximately 40 s. They were cut between head and prothorax. The anterior part (constituted by antennae and head) was used for the recordings. EAG connections were made by inserting the cut end of the head into a glass capillary (1.5 mm diameter) grounding electrode in contact with a silver wire, filled with 0.1 M KCl solution. The recording electrode was a similar glass capillary brought into contact with the distal cut end of the antenna. The capillary tubes were drawn to a fine point using a microelectrode puller (Narishige PC-10, Japan) to get an inner diameter wide enough to enable insertion of the preparation.

Statistical analysis

Mean values of photosynthesis and stomatal conductance were analyzed by one-way ANOVA and statistically separated by Tukey test. The mean surface of foliar discolored spots caused by *B. hilaris* and *N. viridula* feeding was compared by a *t* test for independent samples. VOC data were subjected to root square transformation and then analyzed by one-way ANOVA. The EAG responses of the adults to the different doses of each chemical were analyzed by repeated measures ANOVA with dose and chemical (VOC) as independent variables. Within each chemical, mean EAG responses were separated using least significant difference tests. Since both sexes of *B. hilaris* showed similar EAG responses ($F_{1,84}=1.32$; $P=0.25$; ANOVA), data from males and females were pooled together. All the statistical analyses were performed using Statistica 7.0 for Window (Statsoft 2001, Vigonza, PD, Italy).

Results

Plant damage

The infestation with 15 adult individuals of *B. hilaris* and *N. viridula* reduced photosynthesis by about 25 and 15%, respectively. However, these reductions were not statistically significant when compared to rates of healthy plants (Fig. 1a). The infestation with 40 individuals of *B. hilaris* determined a statistically significant reduction of photosynthesis by about 43% compared to healthy plants ($F_{3,\ 22}=3.13$, $P<0.05$; ANOVA). The infestation with 15 adult individuals of *B. hilaris* and *N. viridula* caused a small reduction of stomatal conductance, whereas the infestation with 40 individuals of *B. hilaris* caused a 43% reduction of the stomatal conductance compared to healthy plants (Fig. 1b). However, even the latter was not statistically significant due to the large variability between measurements ($F_{3,\ 22}=1.02$, $P=0.39$; ANOVA). The mean (±SE) values of intercellular CO_2 concentration (Ci) [ppm (CO_2)] were, respectively, 200 ± 15 in healthy plants, 195 ± 27 in plants infested with 15 individuals of *B. hilaris*, 230 ± 14 in plants infested with 15 individuals of *N. viridula*, and 207 ± 16 in

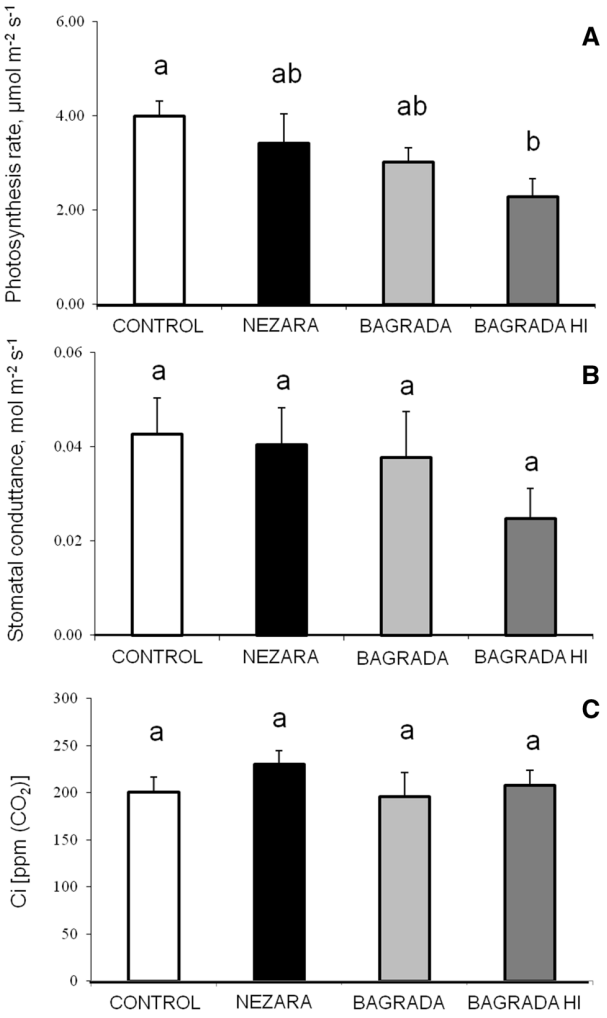

Fig. 1 Photosynthesis (**a**), stomatal conductance (**b**), and intercellular CO_2 concentration (**c**) of *Brassica oleracea* var botrytis leaves after a 24-h long exposure to *B. hilaris* (15 adults) (BAGRADA), *B. hilaris* (40 adults) (BAGRADA HI), *N. viridula* (15 adults) (NEZARA), or healthy plants (CONTROL). Mean values ± SE are given. *Different letters* indicate significant ($P<0.05$) differences as evaluated by ANOVA and Tukey test

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plants infested with 40 individuals of *B. hilaris*, with no significant differences among treatments (Fig. 1c).

Visual damage was significantly greater when caused by individual females of *B. hilaris* than by *N. viridula*. The area of discolored spots determined after a 24-h feeding on a single leaf was, respectively, 9.58 ± 2.67 mm^2 for *B. hilaris* and 2.62 ± 1.14 mm^2 for *N. viridula* (mean ± SE; $t = 2.16$; $df = 20$; $P = 0.046$).

VOC emission

Volatile blends of infested and healthy plants are shown in Fig. 2. Twelve VOC were identified by GC–MS analysis. There was no qualitative variation in the volatile emissions from healthy plants and bug-infested plants. Major volatile compounds were generally limonene, nonanal, acetic acid, and decanal. The infestation of the host plant with *B. hilaris* or *N. viridula* individuals affected significantly the quantity of volatile emissions of decanal, 2-ethyl-1-hexanol, acetic acid, nonanal, and limonene. Bug-infested plants emitted a significantly lower amount of limonene compared to healthy plants ($F_{3,21} = 5.19$; $P < 0.02$; ANOVA). Plants infested with 40 individuals of *B. hilaris* also emitted significantly higher amounts of acetic acid than healthy plants ($F_{3,21} = 2.20$; $P = 0.03$; ANOVA). Significantly higher amounts of 2-ethyl-1-hexanol were detected in plant infested with 40 individuals compared to plants infested with 15 individuals of *B. hilaris* ($F_{3,21} = 1.45$; $P < 0.05$; ANOVA), while no differences were observed in comparison to healthy controls and plants attacked by *N. viridula*. Plants infested with 15 individuals of *B. hilaris* emitted significantly less decanal than plants infested with 15 individuals of *N. viridula* ($F_{3,21} = 1.57$; $P < 0.05$; ANOVA), while no differences were observed with healthy plants. The amounts of all the other VOC did not differ statistically among the treatments.

Electroantennography

Antennae of *B. hilaris* adults showed positive dose-dependent responses to four out of the seven VOC tested: benzaldehyde, octanal, nonanal, and acetic acid (Fig. 3). Significant differences in EAG responses were observed among chemicals ($F_{6,84} = 5.05$; $P < 0.001$; ANOVA) and among doses ($F_{3,252} = 19.85$; $P < 0.001$; ANOVA). Also the interaction of the two factors provided a statistically significant effect ($F_{18,252} = 5.43$; $P < 0.001$). Significant EAG responses were already recorded at the dose of 10 µg for octanal ($F_{3,36} = 7.72$; $P < 0.001$; ANOVA) and benzaldehyde ($F_{3,36} = 10.48$; $P < 0.001$; ANOVA), while significant responses starting at the dose of 100 µg were determined for nonanal ($F_{3,36} = 2.97$; $P < 0.01$; ANOVA) and acetic acid ($F_{3,36} = 8.36$; $P < 0.05$; ANOVA).

Discussion

The feeding activity of *B. hilaris* not only determines damage that is visible immediately, but also influences ecophysiology and VOC emission of *B. oleracea* var. botrytis leaves. Feeding damage by pentatomids is determined by salivary secretions, considered phytoaggressive as enzymatically dissolve plant tissues and/or cause subsurface corking damage on plant tissues (Wiman et al. 2014 and reference therein). Our visual damage experiment indicated that single *B. hilaris* individuals, despite their smaller size compared to *N. viridula*, caused a greater amount of discolored spots after feeding. Whether this is due the injection of toxins that degrade chlorophylls or to the different feeding habit is not known. *Bagrada hilaris* feeds by a lacerate-and-flush method, in a way that the repetitive insertion of stylets between leaf epidermal layers causes mechanical damage to cellular tissue (Reed et al. 2013). On the other hand, *N. viridula* adults feed on leaf veins with a stylet sheath feeding mode destroying only a few cells and causing minimal mechanical damage (Miles 1972; Hori 2000). According to the generalist/specialist hypothesis, host plant defenses affect mainly the polyphagous species rather than the specialist ones (Van der Meijden 1996; Agrawal 2000; Ali and Agrawal 2012). In the case of brassicaceous plants, glucosinolates are reported to reduce the performance of generalist herbivores, while acting as feeding/oviposition stimulants for specialists (Fahey et al. 2001; Halkier and Gershenzon 2006; Hopkins et al. 2009; Müller et al. 2015). Consequently, the different host specificity of the two species could explain our results; in fact while *N. viridula* is polyphagous and feeds on members of many plant families, *B. hilaris* is more associated with brassicaceous plants.

Our results also show that the number of individuals infesting the plant was proportional to the damage inflicted. Only infestations by 40 individuals/plant of *B. hilaris* determined a remarkable reduction of leaf photosynthesis. In the case of pierce-sucking insects, including pentatomid species, the damage is often strictly related to the number of individuals that feed on the plant, as reported for *M. histrionic*a (Ludwig and Kok 2001), and for cauliflower and other different *Brassica* species infested by *B. hilaris* adults (Huang et al. 2014). Field observations indicate that *B. hilaris* often forms large aggregations of hundreds of both adult individuals and immature stages on infested plants, probably mediated by intra- and inter-specific semiochemicals (Guarino et al. 2008; Reed et al. 2013). Given that the single individual can produce more damage than other pentatomid species, as demonstrated above, infestations of *B. hilaris* may cause heavy plant damage and crop losses. However, the small size of *B. hilaris* adults compared to *N. viridula* might reduce damage at the ecophysiological level.

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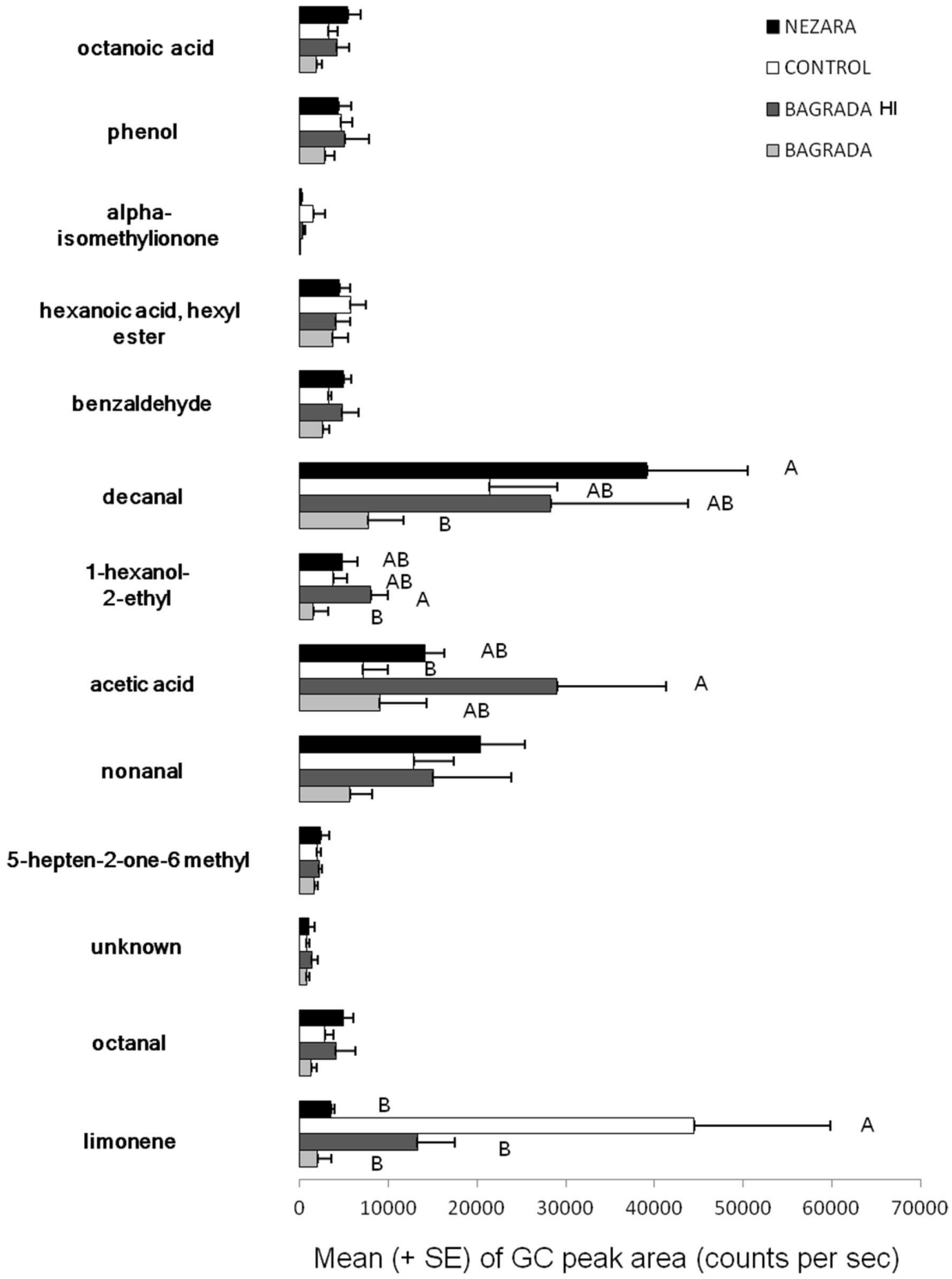

Fig. 2 Volatile (VOC) blend composition of the *B. oleracea* var botrytis leaves after a 24-h long exposure to *B. hilaris* (15 adults) (BAGRADA), *B. hilaris* (40 adults) (BAGRADA HI), *N. viridula* (15 adults) (NEZARA), or healthy plants (CONTROL). *Bars* indicate the mean amount ± SE of each compound. *Different letters* indicate significant ($P < 0.05$) differences as evaluated by ANOVA and LSD test

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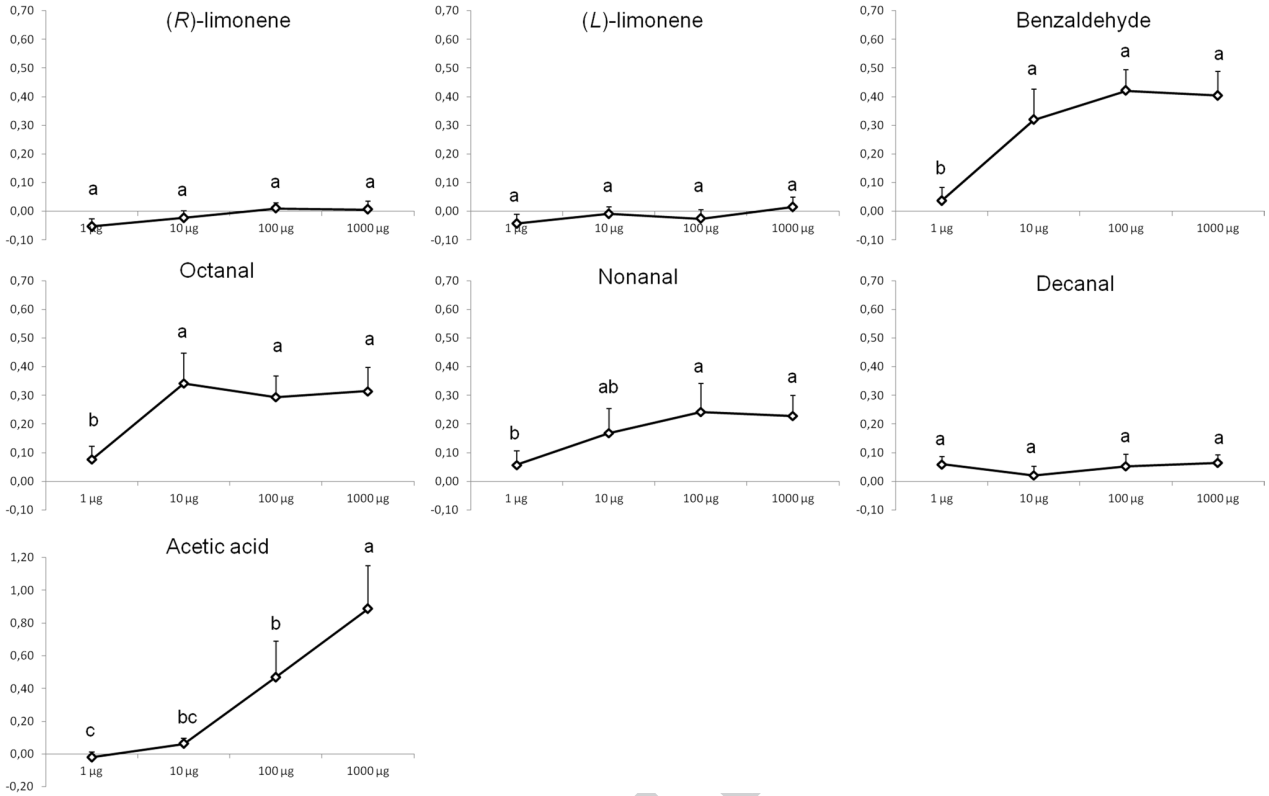

Fig. 3 EAG dose–response curves of *B. hilaris* adults to VOC of *B. oleracea* var botrytis leaves. EAG amplitudes were adjusted to a control stimulus (hexane). *Different letters* indicate that values differ statistically at $P < 0.05$ (Repeated measures ANOVA, followed by Fisher LSD test)

Huang et al. (2014) observed a strong reduction of chlorophyll content, and a reduction of leaf growth, in brassica plants infested by *B. hilaris* adults. Velikova et al. (2010) showed a significant reduction of photosynthesis in cabbage plants infested by *M. histrionica* and in broad bean plants infested by *N. viridula*. The same study showed that the permanent reduction of photosynthesis was due to the damage of photochemical reactions associated to chlorophyll loss. Photosynthesis might be solely limited by photochemical reactions also in *B. oleracea* infested by *B. hilaris*, as indicated by discolored areas associated to chlorophyll degradation. Stomatal closure, albeit not limiting photosynthesis, might indicate down-regulation of gas exchange, which leads to reduced water loss by crop transpiration, an issue that should be carefully investigated.

Infestation by *B. hilaris* and *N. viridula* adults resulted in some quantitative changes of VOC emission as compared to emission by healthy *B. oleracea* plants. However, as also stated by other authors, the changes of VOC emission in response to piercing-sucking insect infestations can be low and at times undetectable (Du et al. 1998; Turlings et al. 1998). In the present work, the emission of limonene, representing the main component of the VOC blend emitted by healthy plants, was significantly reduced in infested plants. Monoterpenes, such as limonene, generally accumulate in specific plant tissues where they have constitutive defensive functions (Gershenzon and Croteau 1991). Temporary pools of monoterpenes that are not associated with reservoirs, such as those found in *B. oleracea* plants, may also play a protective role, e.g., against heat stress (Loreto et al. 1998) or oxidative stress (Loreto et al. 2004). Monoterpene synthesis is induced by mammal herbivore attack (Litvak and Monson 1998), insect damage (Paré and Tumlinson 1997), and other biotic and abiotic causes of wounding (Lewinsohn et al. 1991). The reduction of limonene synthesis observed in our experiment is therefore somehow surprising and difficult to explain. Monoterpenes are formed by carbon branching directly from photosynthesis in the chloroplast (Loreto et al. 1996). Therefore, impairment of photosynthesis induced by *B. hilaris* infestation might also have reduced the carbon available for the synthesis of the main monoterpenes. Among the other VOC, emission of acetic acid was largely increased in plants strongly infested by *B. hilaris* adults. Release of oxygenated VOC is attributed to enzymatic oxidation of foliar fatty acids by lipoxygenases (Hatanaka 1993). This is generally induced by wounding, and limited to C6 oxygenated compounds, collectively called green leaf volatiles (GLV) (Loreto

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et al. 2006). However, especially under anoxic conditions, or after light–dark transitions, release of C2 compounds (methanol, acetic acid) may also occur (Jardine et al. 2012). Acetic acid emission could also be independent of plant metabolism, rather being associated with the higher presence of microorganism on plants heavily infested by *B. hilaris* as observed on other plants infested by other piercesucking insect species (Franke et al. 1999). Among GLV, only 2-ethyl-1-hexanol was found in higher amount in plants highly infested with *B. hilaris*. As briefly explained above, the increase of this fatty alcohol, common in other *Brassica* species (Barros et al. 2014), could indicate a damage occurring at plant membrane lipids. For example, GLV are induced by biotic stress factors such as herbivores (Mattiacci et al. 1994; Ruther et al. 2002; Williams et al. 2005). However, we note that 2-ethyl-1-hexanol is the only GLV induced by *B. hilaris* feeding. Remarkably, GLV that are produced in the early steps of fatty acid degradation (e.g., hexanol and hexenol) were not found in the blend emitted by infested plants. Similar results were obtained with other piercing-sucking insects feeding on different plant species. For example, broad bean plants infested by the pea aphid, *Acyrthosiphon pisum* (Harris), and maize plants infested by *Rhopalosiphum maidis* (Fitch) did not produce higher levels of GLV, even when infestation levels were heavy (Du et al. 1998; Turlings et al. 1998). The scarce induction of GLV in *B. oleracea* plants infested by *B. hilaris* and *N. viridula* infestation could be explained by a low mechanical damage associated with the feeding strategy of these insects, insufficient to activate the lipoxygenase pathway.

Some of the VOC identified in this study were perceived by the *B. hilaris* antennae in EAG bioassays, such as benzaldehyde, octanal, nonanal, and acetic acid. In particular, these compounds elicited increasing EAG response at increasing doses. A higher sensitivity was found for benzaldehyde and octanal, as these compounds were detected by the insect at the dose of 10 µg, while nonanal and acetic acid were only detected at 100 µg. The sensitivity of *B. hilaris* to benzaldehyde, octanal, nonanal, and acetic acid could indicate a role of these compounds in host detection. In fact, these molecules have been reported in the VOC profile of several *B. hilaris* host plants such as mustard *Brassica juncea* (Coss.) (Zhao et al. 2007), oliseed rape *Brassica napus* L. (Müller et al. 2002), and caper bush *C. spinosa* (Romeo et al. 2007). The use of plant volatiles for host location and recognition depends on insect ability to process olfactory signals. This is a difficult task in a natural environment, where insects are exposed to many different volatile chemicals, at different concentrations and in different combinations (Bruce et al. 2005; Schroeder and Hilker 2008). Finally, the lack of EAG response to limonene suggests that this monoterpene is not involved in plant–insect communication. Although monoterpenes serve primarily as chemical defenses against insects and diseases, these compounds may be also degraded and recycled back into primary compounds when there is a demand (Gershenzon 1993). Limonene might have been catabolized providing a carbon source for other processes, including production of VOC directly involved in plant response to insect attack.

To summarize, our results suggest that photosynthesis of *B. oleracea* plants is sensitive to infestation by numerous individuals of *B. hilaris*, which may impair leaf functionality and crop productivity. This is accompanied by a change of the constitutive emission of several VOC, some of which are perceived by insect antennae, suggesting a role in host location.

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