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Perception of Host Plant Volatiles in *Hyalesthes obsoletus*: Behavior, Morphology, and Electrophysiology

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Abstract The Palearctic planthopper *Hyalesthes obsoletus* is the natural vector of the grapevine yellow disease Bois noir. Grapevine is an occasional host plant of this polyphagous planthopper. To deepen our knowledge of the role of plant volatile organic compounds for *H. obsoletus* host plant searching, we carried out behavioral, morphological, and electrophysiological studies. We tested the attraction of *H. obsoletus* to nettle, field bindweed, hedge bindweed, chaste tree, and grapevine by using a Y-shaped olfactometer. The results showed a significant attraction of male *H. obsoletus* to chaste tree, and of the females to nettle. Male *H. obsoletus* were repelled by odor from hedge bindweed. Ultrastructural studies of the antennae showed at least two types of olfactory sensilla at the antennal pedicel: plaque organs and trichoid sensilla. Volatile organic compounds from nettle and chaste tree were collected, and the extracts were analyzed by coupling gas-chromatography to both mass-spectrometry and electroantennography. The volatile organic compounds that elicited electrophysiological responses in male and female antennae were identified. These findings are discussed with respect to behavior of *H. obsoletus* males and females in the field.

Keywords Planthopper · Phytoplasma vector · Volatile organic compounds · Y-tube bioassays · Electrophysiology · Antenna functional anatomy

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Introduction

Plant volatile organic compounds (VOCs) have critical roles in the evolution of host plant use by phytophagous insects (Linn et al., 2003). These insects detect VOCs via olfactory sensilla that are located on the antennae, and these cues provide information about food, mates, and oviposition sites (Visser, 1983; Schoonhoven et al., 2005). The role of olfaction in planthopper host location is still under investigation. The electroantennogram (EAG) technique and antenna morphology studies have been applied to *Nilaparvata lugens* Stal (Hemiptera: Fulgoromorpha: Delphacidae), and these have revealed olfactory receptors on the antennae that are responsive to plant volatiles (Aljunid and Anderson, 1983; Youn, 2002).

The insect investigated in the present study, the Paleartic planthopper *Hyalesthes obsoletus* Signoret (Hemiptera: Cixiidae), is known to use very different plants in different areas of its distribution (Table 1). Olfactometer assays and the EAG technique have shown that the chaste tree is the most attractive plant to *H. obsoletus* adults in Israel (Sharon et al., 2005). In the Marche region (central-eastern Italy), *H. obsoletus* larvae develop only on the nettle root system, and adult dispersion in a vineyard agroecosystem depends mainly on the spatial distribution of field bindweed (Riolo et al., 2007). In Germany, the formation of *H. obsoletus* host plant races on nettle and field bindweed has

Table 1 Host plants associated with *Hyalesthes obsoletus* in European and Mediterranean countries

Host plants		Country	Life stage
Family	Species		
Amaranthaceae	*Amaranthus retroflexus* L.	Israel[1]	A
Apiaceae	*Falcaria vulgaris* Bernh.	Turkey[2]	L, A
Asteraceae	*Ambrosia artemisiifolia* L.	Italy[3]	A
	Artemisia vulgaris L.	Italy[4]	A
	Tanacetum vulgare L.	Italy[4]	A
	Taraxacum officinale Weber	Switzerland[5]	A
	Taraxacum sp.	Turkey[2]	L, A
Boraginaceae	*Onosma armenum* DC.	Turkey[2]	L, A
Brassicaceae	*Crambe orientalis* L.	Turkey[2]	L, A
	Isatis glauca Gilib.	Turkey[2]	L, A
	Lepidium draba L.	†France[6]	L, A
		Switzerland[5]	A
		Turkey[2]	L, A
Convolvulaceae	*Calystegia sepium* (L.) R. Br.	France[6]	A
		†Germany[7]	L, A
		Italy[3,8]	L, A
		Switzerland[5]	A
	Convolvulus arvensis L.	†France[6]	L, A
		†Germany[7]	L, A
		Israel[1]	A
		Italy[4,8,9]	L, A
		Slovenia[10]	A
		Switzerland[5]	A
		Turkey[2]	L, A
Fabaceae	*Medicago sativa* L.	†Turkey[2]	L, A
	Melilotus officinalis (L.) Pall.	Turkey[2]	L, A
	Onobrychis viciifolia Scop.	Turkey[2]	L, A
Geraniaceae	*Geranium tuberosum* L.	Turkey[2]	L, A
Lamiaceae	*Lamium orvala* L.	Italy[8]	L, A
	Lavandula angustifolia Mill.	†France[6]	L, A
	Lavandula hybrida Reverchon	France[6]	A
	Satureja montana L.	France[6]	A
	Vitex agnus-castus L.	†Israel[1]	L, A
Myrtaceae	*Myrtus communis* L.	Israel[1]	A
Oleaceae	*Olea europaea* L.	Israel[1]	A
Plantaginaceae	*Linaria striata* L.	France[6]	A
	Plantago cynops L.	France[6]	A
	Plantago lanceolata L.	Switzerland[5]	A
Polygonaceae	*Polygonum aviculare* L.	Switzerland[5]	A
	Rumex sp.	Turkey[2]	L, A
Ranunculaceae	*Ranunculus bulbosus* L.	†Germany[7]	L, A
	Clematis vitalba L.	Switzerland[5]	A
Rubiaceae	*Galium verum* L.	France[6]	A
Urticaceae	*Urtica dioica* L.	France[11]	L, A
		†Germany[7]	L, A
		Hungary[12]	A
		†Italy[4,8,9]	L, A
		Slovenia[10]	A
		†Switzerland[5]	L, A

L: larvae; A: adults

†main host plants; [1]Sharon et al., 2005; [2]Güclü and Ozbek, 1988; [3]Picciau et al., 2008; [4]Lessio et al., 2007; [5]Kessler et al., 2011; [6]Sforza et al., 1999; [7]Langer et al., 2003; [8]Forte et al., 2010; [9]Riolo et al., 2007; [10]Petrovic et al., 2003; [11]Bressan et al., 2007; [12]Palermo et al., 2004

been hypothesized (Johannesen et al., 2008; Imo et al., 2011), similarly to what has been reported for *Alebra* leafhoppers (Hemiptera: Cicadellidae) (Aguin-Pombo, 2002). However, the use of different host plants by *H. obsoletus* might also be influenced by the availability of the plants, the soil type (preference for soils with natural cavities, suitable for larval development), the cultivation practices (Weber and Maixner, 1998), and the presence of organic mulch (Howard and Oropeza, 1998). *Hyalesthes obsoletus* has an important role as a vector of the stolbur phytoplasma, which is associated with grapevine yellow, a disease that is known as Bois noir (Maixner et al., 1994). Grapevines represent only an occasional host for planthopper adults (Lessio et al., 2007) that can transmit the phytoplasma during their feeding probing. Vineyard inter-row grass cover and border vegetation represent potential phytoplasma reservoirs (Maixner et al., 1995; Riolo et al., 2007).

The aim of this study was to investigate the influence of plant odor on *H. obsoletus* host plant searching. We determined the attractiveness of odor from nettle, field bindweed, hedge bindweed, chaste tree, and grapevine to females and males of *H. obsoletus* associated to nettle by using a Y-tube olfactometer. Furthermore, we recorded EAG responses to volatile extracts from the plants that were most attractive to both sexes of *H. obsoletus*, and we identified the VOCs that elicited positive EAG responses. Finally, the functional anatomy of the *H. obsoletus* antenna pedicel was studied.

Methods and Materials

Insects Adult *H. obsoletus* were collected from nettle plants in the Ancona district (43°32′ N; 13°23′ E), in the region of Marche, in Italy. These were transferred to the laboratory, separated according to sex, and caged with fresh shoots of nettle. Twenty-four hours before the beginning of the bioassays, adult planthoppers were transferred to cages containing sucrose solution (5 % sucrose, 0.5 % sorbitol), to avoid any influence on the plants used in the subsequent experiments. Planthoppers were kept at 26±1 °C and 60 %±10 % relative humidity, under a natural photoperiod.

Plants Chaste tree and grapevine cv. Chardonnay were grown in plastic pots (diam., 20 cm; height, 20 cm) in a greenhouse (temperature, 26±1 °C; relative humidity, 60 % ±10 %; natural photoperiod). Nettle, field bindweed, and hedge bindweed were collected in the field (in the Ancona district).

Y-Tube Olfactometer Bioassays The responses of *H. obsoletus* adults to plant VOCs were investigated using a dual choice Y-tube olfactometer (stem, 25 cm; arm length, 20 cm, arm angle, 75°; internal diam, 4 cm). Each arm of the Y-tube was connected to a glass cylinder (9×18 cm); one chamber served as the control, and the other held the test material. An airflow was maintained from each cylinder through the olfactometer arms, using an air pump with the airflow adjusted with a flow meter to 1.0 $l min^{-1}$. The incoming air was passed through activated charcoal and humidified with double-distilled, deionized water. The glass Y-tube was positioned with a slope of 10° from the horizontal plane.

Single planthoppers were introduced individually into the olfactometer at the entrance of the stem, and they were observed until they had walked at least 13 cm up one of the arms, or until 5 min had elapsed. Planthoppers that did not choose a side arm within 5 min were recorded as 'no choice'. For each individual planthopper, their activation time, as the exit time from the release vial, and their choice time were recorded.

Samples were randomly assigned at the beginning of the bioassays, and they were reversed after having tested 5 planthopper adults in order to minimize any spatial effects on the choices of the planthoppers. Bioassays were conducted from ~15:00 to 19:00 hr. After each trial, the Y-tube was washed with detergent, rinsed with distilled water and absolute ethanol, and baked overnight at 200 °C. The responses of 60 planthoppers per sex were tested for each of the various treatments. Experiments were conducted in a laboratory at a temperature of 26±1 °C and a relative humidity of 60 %±10 %.

Volatiles Sources and Experiments Experiments were designed to investigate the adult *H. obsoletus* male and female responses to the volatiles from the different plant species. Parts of plants of nettle, field bindweed, hedge bindweed, chaste tree, and grapevine were collected 1 hr prior to the start of the bioassays. Fresh shoots were used (length, 10–15 cm; weight, ca. 5 g). The cut stem was wrapped in cotton wool and inserted into a 6-ml vial filled with distilled water. Cotton wool inserted into a 6-ml vial filled with distilled water was used as the blank. Vials were sealed with Teflon tape.

Headspace Collection Headspace collections were made from fresh shoots of nettle and chaste tree, which were the plants that elicited significant behavioral responses in the Y-olfactometer experiments (see Results section). Fifteen shoots (ca. 200 g) were placed in a 25×38 cm polyacetate bag (Toppits, Melitta, Sweden) for collection of the volatiles (Tasin et al., 2005; Faccoli et al., 2008). The ends of the shoots were placed into glass vials (4 ml) filled with water and sealed with parafilm. The air from the headspace of each bag was drawn out at 150 ml min^{-1} through an adsorbent cartridge trap (75 mg Super Q; Sigma-Aldrich, Milan, Italy) connected to a vacuum pump. Charcoal-filtered air was simultaneously pulled into the bag by the same pump, to maintain constant pressure. Collections

were carried out over 24 hr in a climatic chamber at a temperature of 25±2 °C, a relative humidity of 60 %±10 %, and a photoperiod of 16:8 (L:D), with 1,000 lx during the light period. Volatiles were eluted from the adsorbent cartridges by solvent desorption at room temperature, using 500 µl hexane (>99 % purity, Sigma-Aldrich). Three collections from different groups of each of the shoots were carried out. Some of these extracts were prepared for chemical quantification, and 0.5 µg heptyl acetate (≥99 % purity) was added as an internal standard (Bengtsson et al., 2001). The collected extracts were reduced to 50 µl using a slow stream of nitrogen, and then stored in 2-ml vials at −18 °C until use.

Gas Chromatography and Electroantennography Detection Two microliters of the concentrated plant extracts in hexane were injected into a Hewlett-Packard 5890 gas chromatography (GC) system. This used a polar Innowax column (30 m×0.32 mm; J & W Scientific, Folsom, CA, USA) programmed to increase from 60 °C (held for 3 min) at 8 °C min^{-1}, to 220 °C (held for 7 min), and was interfaced with the EAG apparatus (Arn et al., 1975). The outlet of the GC column was split in a 1:1 ratio between a flame ionization detector and an antenna of *H. obsoletus*.

We used an EAG technique that is similar to that described by Den Otter et al. (1996), using a standard EAG apparatus (Syntech, Hilversum, The Netherlands). A glass capillary indifferent electrode was filled with Kaissling solution (Kaissling, 1987), which contained 5.0 gl^{-1} polyvinylpyrrolidone K90 (Fluka Chemie, Buchs, Switzerland), and this was inserted into the head of the planthopper. The difference electrode was a similar glass capillary from which the tip was previously cut, and this was brought into contact with the distal end of an antenna. The GC-EAG responses of *H. obsoletus* antennae to nettle extracts were recorded for antenna activity. Compounds eluting from the capillary column were delivered to the antenna through a glass tube (12 cm×8 mm) in a charcoal-filtered and humidified airstream. The antenna signal and the flame ionization detector signal were amplified and recorded simultaneously using Syntech software.

Samples from both nettle and chaste tree extracts were tested on 5 different *H. obsoletus* males and females. A compound was considered electrophysiologically active when it elicited at least three antennal responses that were different from background noise (Zhang et al., 2001).

Chemical Analysis Three samples of each extract were analyzed by coupled GC and mass spectrometry (GC-MS). The analyses were performed on a Hewlett-Packard 5890 GC system, with a polar Innowax column (30 m×0.32 mm; J & W Scientific, Folsom, CA, USA) programmed to increase from 60 °C (held for 3 min) at 8 °C min^{-1}, to 220 °C (held for 7 min). This was interfaced with a Hewlett-Packard 5970B mass spectrometer that was operated using electron impact ionization (70 eV). The identities of most (84 %) of the compounds in the volatiles collections were verified by comparison with synthetic compounds, as indicated in Table 3. The compounds that did not elicit antennal responses and for which no standards were available, were tentatively identified using the Wiley mass spectra database. The identified compounds were quantified by comparing their peak areas to those of the internal standard.

Scanning Electron Microscopy Ten individuals of each sex were used for the scanning electron microscopy (SEM) observations. Planthoppers were anesthetized using CO_2 and kept at −18 °C until they died. Individual planthoppers then were dissected, to remove antennae from the head capsule. In some cases, the whole head was detached from the rest of the body, with the antennae in their natural positions. The specimens were dehydrated through a series of graded ethanol concentrations, from 50 to 99 %. After dehydration, the 99 % alcohol was substituted with pure hexamethyldisilazane (Sigma), and the specimens were left to dry under a chemical hood under room conditions. Five antennae were mounted on each aluminum stub, with care taken to position them with different orientations, to have a clear view of the ventral, dorsal, and both of the lateral sides. These mounted antennae were gold-sputtered using a Balzers Union SCD 040 unit. Observations were carried out using Philips XL 30 and Zeiss Supra scanning electron microscopes.

Transmission Electron Microscopy Ten individual planthoppers of each sex were anesthetized with CO_2 and immediately immersed in a solution of 5 % glutaraldehyde and 2.5 % paraformaldehyde in 0.1 M cacodylate buffer, 5 % sucrose, pH 7.2–7.3. For each antenna, the pedicel was separated from the scape and the flagellum to aid fixative penetration, and they were left at 4 °C for 2 hr. Samples were kept at 4 °C overnight in 0.1 M cacodylate buffer, 5 % sucrose, pH 7.2–7.3. Then, the antennae were post-fixed in 1 % OsO_4 (osmium tetroxide) for 1 hr at 4 °C, and rinsed in the same buffer. Dehydration through a graded ethanol series was followed by embedding in Epon-Araldite, with propylene oxide as the bridging solvent. Thin sections (90 nm) were cut with a diamond knife on a Nova LKB ultramicrotome, and mounted on formvar-coated 50-mesh grids. Finally, after staining with uranyl acetate (20 min, room temperature) and lead citrate (5 min, room temperature), the sections were examined with a Philips EM 208 electron microscope. Digital pictures were obtained (1,376×1,032 pixels, 8b, uncompressed grayscale Tiff files), using a high-resolution MegaViewIII (SIS) digital camera connected to the transmission electron microscope.

Statistical Analysis The Y-tube olfactometer data were analyzed by one-tailed binomial tests (H_0: insects do not prefer

the odor source compared with the blank). Differences between treatments (plants) were evaluated by contingency table analysis based on *Chi-square* (Zar, 1999). Percentages (arcsin transformed) of males and females leaving the release vials were tested by one-way ANOVA. The variabilities in the activation times and first choice times between the host plants and the sexes were determined by one-way ANOVA followed by least significant difference tests. The significance level of all of the statistical tests was set at $P<0.05$. These analyses were performed using Systat 11 (Systat Software Inc.). The individuals that did not make a choice were not included in the analyses.

Results

Bioassays The olfactometer assays showed that *H. obsoletus* males were significantly ($P<0.05$) attracted to the volatiles of chaste tree, whereas females were attracted to nettle, when these plant shoots were compared with blank (Figs. 1 and 2). Furthermore, the males were significantly ($P<0.05$) repelled by hedge bindweed when this plant was compared with blank (Fig. 1). However, no differences in *H. obsoletus* preferences were recorded between treatments (males: $\chi^2=6.878$, $df=4$, $P=0.142$; females: $\chi^2=5.025$, $df=4$, $P=0.285$).

The exit time from the release vial (i.e., the activation time) was significantly influenced by the sex of the planthoppers: males exited the release vial faster than the females (in average 15.7 sec for males vs. 23.9 sec for females) ($P<0.01$; Table 2). Similarly, overall, the males showed a shorter first choice time (mean 95.3 sec), while for females the first choice time was in average 119.8 sec ($P<0.001$; Table 2). In particular, when the bioassays were carried out with field bindweed, males showed a shorter activation time ($P<0.01$) and first choice time ($P<0.001$) than the females (Table 2). Furthermore, by comparing the different treatments, a higher mean choice time ($P<0.05$) was observed for males when nettle was tested vs. blank. Other differences were seen for male vs. female activation times when hedge bindweed ($P<0.01$) and grapevine ($P<0.05$) were assayed (Table 2). Moreover, the percentage of female planthoppers that made a choice between the arms of the olfactometer was lower than that of the male planthoppers (76 % females vs. 88 % males; $P<0.001$).

Chemical Analysis Compounds identified from the shoots of nettle and field bindweed are listed in Table 3. Hydrocarbons, alcohols, aldehydes, ketones, esters, aromatic compounds, monoterpenes, sesquiterpenes, and other terpenoids were identified from nettle, with a total of 41 compounds. Similarly for chaste tree, hydrocarbons, alcohols, aldehydes, esters, aromatic compounds, monoterpenes and sesquiterpenes were identified, with a total of 24 compounds.

The most abundant compound in the headspace collections for nettle was (*Z*)-3-hexenyl-acetate, with an average amount of 200 g of nettle shoots of 4.66±0.98 μg h^{-1} (74.3 %±15.1 % of the total ion abundance). The most abundant compound in the headspace collections for chaste tree was α-pinene, here with an average amount of 200 g of chaste tree shoots of 0.57±0.09 μg h^{-1} (44.1 %±13.20 % of the total ion abundance).

Gas Chromatography and Electroantennography Detection The GC-EAG analyses of the headspace collections from nettle shoots detected nine active compounds for the male *H.*

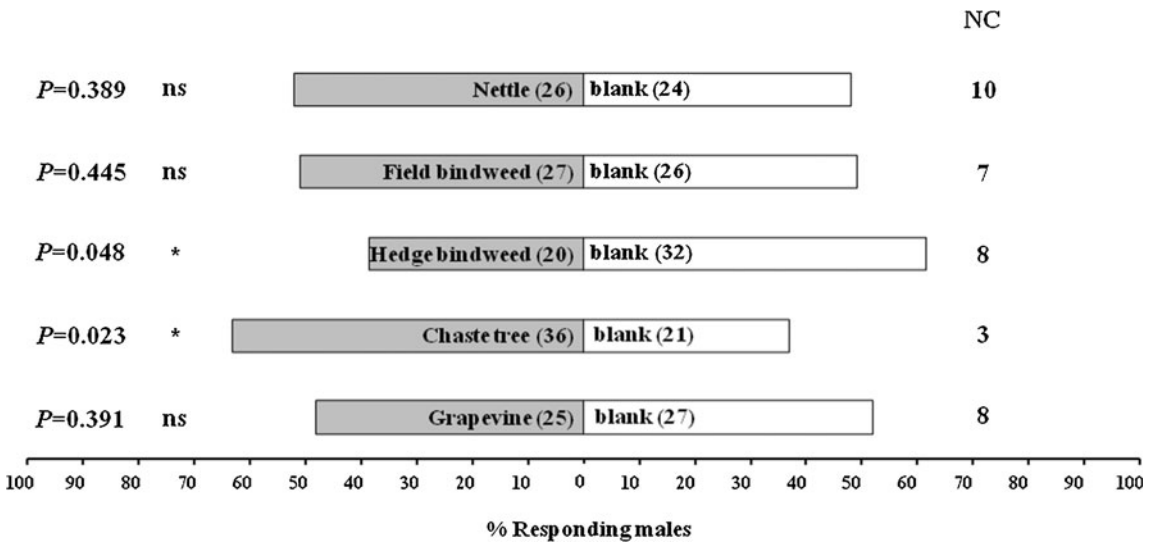

Fig. 1 Behavioral responses of *Hyalesthes obsoletus* males to the plants tested. The numbers in *brackets* indicate the number of insects that made a choice; the level of significance is indicated on the left (one-tailed binomial test: ns, non-significant; *$P<0.05$); NC, number of insects that did not complete a choice

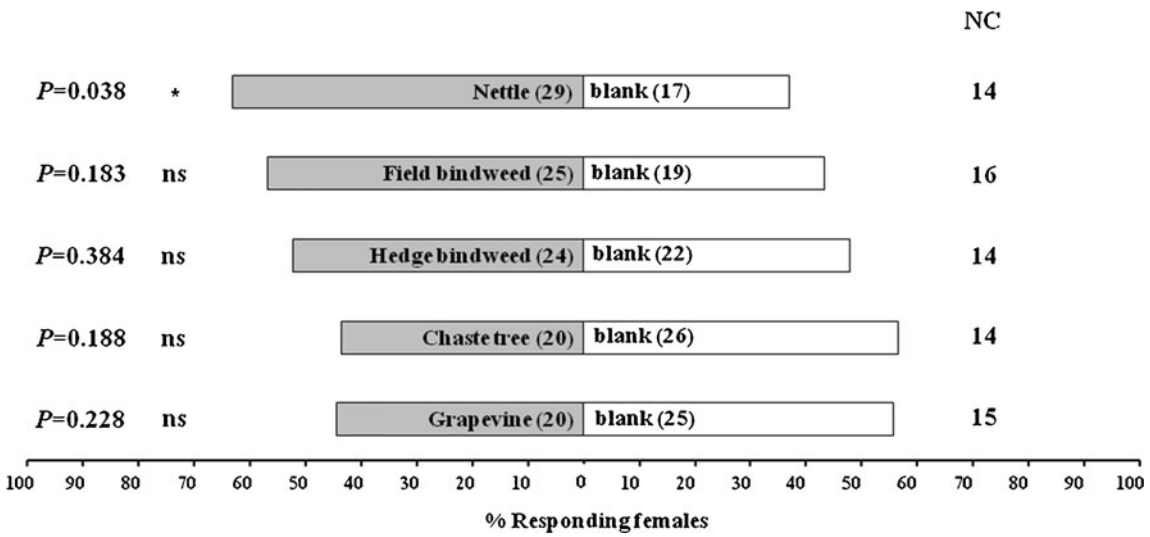

Fig. 2 Behavioral responses of *Hyalesthes obsoletus* females to the plants tested. As for legend to Fig. 1

obsoletus antennae, and 11 active compounds for the female *H. obsoletus* antennae (Table 3). The antennal responses of the males were elicited by (*Z*)-3-hexen-1-ol, (±)-linalool, (*E*)-β-caryophyllene, (*E*)-β-farnesene, (*Z*,*E*)-α-farnesene, (*E*,*E*)-α-farnesene, methyl salicylate, (*Z*)-jasmone, and benzothiazole, whereas the antennal responses of the females were elicited by 4,8-dimethyl-1,(*E*)-3,7-nonatriene, (*Z*)-3-hexenyl-acetate, (*Z*)-3-hexen-1-ol, (±)-linalool, (*E*)-β-caryophyllene, (*E*)-β-farnesene, (*Z*,*E*)-α-farnesene, (*E*,*E*)-α-farnesene, methyl salicylate, (*Z*)-jasmone, and benzothiazole (Table 3). *Hyalesthes obsoletus* males and females responded to the same eight volatile compounds collected from the chaste tree headspace (Table 3): 1,8-cineole, (±)-linalool, (*E*)-β-caryophyllene, methyl benzoate, (*E*)-β-farnesene, (*Z*,*E*)-α-farnesene, (*E*,*E*)-α-farnesene, and methyl salicylate.

Functional Anatomy In *H. obsoletus*, the antennae have three segments: the short, cylindrical scape that is devoid of sensilla, the pedicel, and the elongated flagellum. The sensory structures associated with the flagellum have previously been studied in detail (Romani et al., 2009). The pedicel is the largest antennomere, with 200 μm diam. and ca. 250 μm length (Fig. 3a, d). The surface of the pedicel has two types of multiporous sensilla: the sensilla placoidea, or 'plaque organs' (*sensu* Lewis and Marshall, 1970), and the sensilla trichoidea (Fig. 3d). No differences were found in the number and arrangement of these sensilla between these male and female planthoppers. The presence of abundant wax covering the antennal structures was difficult to prevent in all of the preparations.

Sensilla Placoidea These structures are present on most of the pedicel surface, with about 30 elements (Fig. 3a). Each sensillum appears as a rounded, flattened, 'rose-like' structure (diam ca. 35 μm) (Fig. 3b). The porous cuticle is folded several times, which gives rise to up to 15 elongated elements. The space between each of these structures is occupied by a cuticular spine (length, ca. 16 μm), which points

Table 2 Activation and first choice times of *Hyalesthes obsoletus* males and females

Treatment	Activation time [sec]		First choice time [sec]	
	Males	Females	Males	Females
Nettle vs. blank	23.6±5.8a	22.4±4.4a	124.7±10.8a	121.4±10.9a
Field bindweed vs. blank	13.3±3.1**a	28.5±3.3a	80.1±8.9***b	148.9±10.9a
Hedge bindweed vs. blank	8.9±1.9**a	27.2±5.8a	95.2±9.6b	109.8±11.9a
Chaste tree vs. blank	19.3±4.4a	15.1±2.1a	92.4±9.1b	108.4±10.9a
Grapevine vs. blank	13.6±4.2*a	26.3±4.5a	84.2±9.7b	110.9±10.2a
Overall means	15.7±3.9**	23.9±4.0	95.3±9.6***	119.8±11.0

Data are means ± SE

N=60 males or females for each comparison. Significance in male vs. female comparisons: *P<0.05; **P<0.01; ***P<0.001 (one-way ANOVA test). Identical letters within the same column indicate no difference between treatments (least significant difference test, P<0.05)

Table 3 Mean levels of volatile compounds [μg h^{-1}] collected in the headspace from shoots of nettle and chaste tree, and GC-EAG activity on *Hyalesthes obsoletus* male and female antennae

Compound[1,2]	Source of standards[4]	Plant shoots		GC-EAG activity			
		Nettle	Chaste tree	Nettle		Chaste tree	
				Male	Female	Male	Female
Hydrocarbons							
Dodecane	SA	–	0.65±0.15				
Tetradecane	F	–	0.45±0.07				
Pentadecane	F	0.16±0.07	0.31±0.09				
Alcohols							
3-hexanol	SA	0.46±0.11	0.03±0.01				
2-hexanol	SA	0.20±0.06		–			
(*Z*)-3-hexen-1-ol	SA	2.41±1.01	0.29±0.10	*	*		
(*E*)-3-hexen-1-ol	SA	0.18±0.04	–				
1-octen-3-ol	SA	0.69±0.17	–				
1-octanol	SA	0.03±0.01	–				
Aldehydes							
Hexanal	F	0.12±0.05	–				
Nonanal	SA	–	0.08±0.02				
Ketones							
3-hexanone	SA	0.38±0.13	–				
Acetophenone	SA	0.05±0.01	–				
4-ethyl-acetophenone[3]	–	0.05±0.02	–				
Esters							
2-hexyl-acetate[3]	–	0.08±0.03	–				
n-hexyl-acetate	SA	0.65±0.19	–				
(*Z*)-3-hexenyl-acetate	SA	74.29±15.06	0.03±0.02		*		
3-cyclohexenyl-acetate[3]	–	0.24±0.09	–				
Exobornyl-acetate[3]	–	0.06±0.04	–				
(*Z*)-3-hexenyl-benzoate	SA	0.11±0.03					
Aromatics							
Methyl benzoate	SA	0.02±0.01	0.04±0.02			*	*
Naphthalene	SA	0.04±0.03	–				
Methyl salicylate	SA	0.17±0.07	0.12±0.04	*	*	*	*
2-methyl-benzothiazole[3]	–	0.06±0.02	–				
Benzothiazole	SA	9.68±2.49	0.38±0.16	*	*		
Homoterpenes							
4,8-dimethyl-1,(*E*)-3,7-nonatriene	IS	0.55±0.21	–		*		
Irregular terpenoids							
(*Z*)-jasmone	SA	0.04±0.03	–	*	*		
Monoterpenes							
α-pinene	SA	0.48±0.18	44.07±13.20				
β-pinene	SA	0.05±0.01	–				
Myrcene	SA	0.02±0.01	5.37±1.92				
Ocimene	SA	0.12±0.03	0.29±0.07				
Limonene	SA	0.09±0.05	2.70±1.28				
Camphor[3]	–	0.10±0.06	–				
(±)-linalool	SA	0.02±0.01	0.02±0.01	*	*	*	*
1,8-cineole	F	–	20.00±4.07			*	*
α-terpinene	SA	–	0.61±0.19				
α-terpineol	SA	–	3.22±1.03				

Table 3 (continued)

Compound[1,2]	Source of standards[4]	Plant shoots		GC-EAG activity			
		Nettle	Chaste tree	Nettle		Chaste tree	
				Male	Female	Male	Female
Carene	SA	–	5.46±2.56				
p-cymene	SA	–	1.21±0.56				
Sesquiterpenes							
Longifolene[3]	–	0.03±0.02	–				
β-elemene[3]	–	0.08±0.01	–				
(*E*)-β-caryophyllene	SA	0.93±0.18	3.45±1.34	*	*	*	*
(*E*)-β-farnesene	B	0.24±0.09	1.27±0.34	*	*	*	*
α-humulene	SA	0.09±0.03	–				
Germacrene-D	AB	0.27±0.07	2.59±0.79				
(*Z*,*E*)-α-farnesene	F	0.34±0.18	0.46±0.12	*	*	*	*
(*E*,*E*)-α-farnesene	F	4.35±1.21	7.14±2.49	*	*	*	*
Farnesol	SA	0.08±0.03	–				
α-thujene	C	–	0.30±0.08				
Caryophyllene oxide	SA	0.11±0.05	–				

Data are means ± SE

N=3 for each plant species. Unit is μg h^{-1}

[1] Mean amount of (*Z*)-3-hexenyl-acetate collected from 200 g shoots of nettle was 4.66±0.98 μg h^{-1}

[2] Mean amount of α-pinene collected from 200 g shoots of chaste tree was 0.57±0.09 μg hr^{-1}

[3] Compounds tentatively identified with Wiley mass spectra database

[4] Standards were obtained from Sigma-Aldrich (Milan, Italy) (SA), Fluka Chemie (Buchs, Switzerland) (F), Bedoukian Research Inc. (Danbury, CT) (B), Firmenich (Geneva, Switzerland) (F), Ingve Stensrøm (Aas, Norway) (IS), Anna-Karin Borg-Karlson (Stockholm, Sweden) (AB)

* Compounds eliciting responses in *Hyalesthes obsoletus* male and female antennae for GC-EAG; a compound was considered electrophysiologically active when it elicited at least three antennal responses that were different from background noise (Zhang et al., 2001)

towards the center of the sensillum (Fig. 3b). A second series of shorter cuticular spines (length, ca. 10 μm) is arranged all around the sensillum. The TEM cross-sections revealed a substantial difference in the cuticular organization between the porous cuticle and the spines, with the cuticle being thin (150 nm) and pierced by numerous scattered pores, and the spines being thick (ca. 500 nm) and aporous (Fig. 4a–c). Each sensillum placoideum is innervated by a variable number of sensory neurons (from 45 to 80) that are arranged in units (Fig. 4d–f). Each unit is surrounded by a dendritic sheath, and is formed by two to 18 dendrites (Fig. 4e). At the level of the sensillum socket, each neuron gives rise to numerous dendritic branches that completely fill the space below the porous cuticle.

Sensilla Trichoidea These sensilla are grouped on a specific side of the pedicel, i.e., the side facing the compound eye, and there are ca. 140 of them (Fig. 3d). Each sensillum (base diam. ca. 1.6 μm; length, ca. 22 μm) is seen as a blunt-tipped hair, and they are slightly curved and inserted into the pedicel wall through an inflexible socket (Fig. 3e). The hair cuticle is densely covered with pores (Fig. 3f). The TEM shows that two sensory neurons innervate each sensillum (Fig. 5c, d). The dendrites have branches near the base of the sensillum which gives rise to some ramifications that extend up to the sensillum tip (Fig. 5a, b).

Discussion

Bioassay The bioassay results showed sex-specific responses by *H. obsoletus* to plant volatiles. Males responded positively to odor from chaste tree, whereas females were attracted by nettle volatiles, when these plant shoots were compared with blank. Males also showed a repellent response to the hedge bindweed volatiles. We observed shorter activation and first choice times for males, as compared to females, when field bindweed was assayed. Furthermore, comparing the different treatments, a higher mean choice time was observed for males when nettle was tested vs. blank.

The attraction of females to nettle volatiles was expected, as nettle is a primary host for *H. obsoletus* larval development. Previous studies have shown that *H. obsoletus* show plant affiliation with their primary hosts (Sharon et al.,

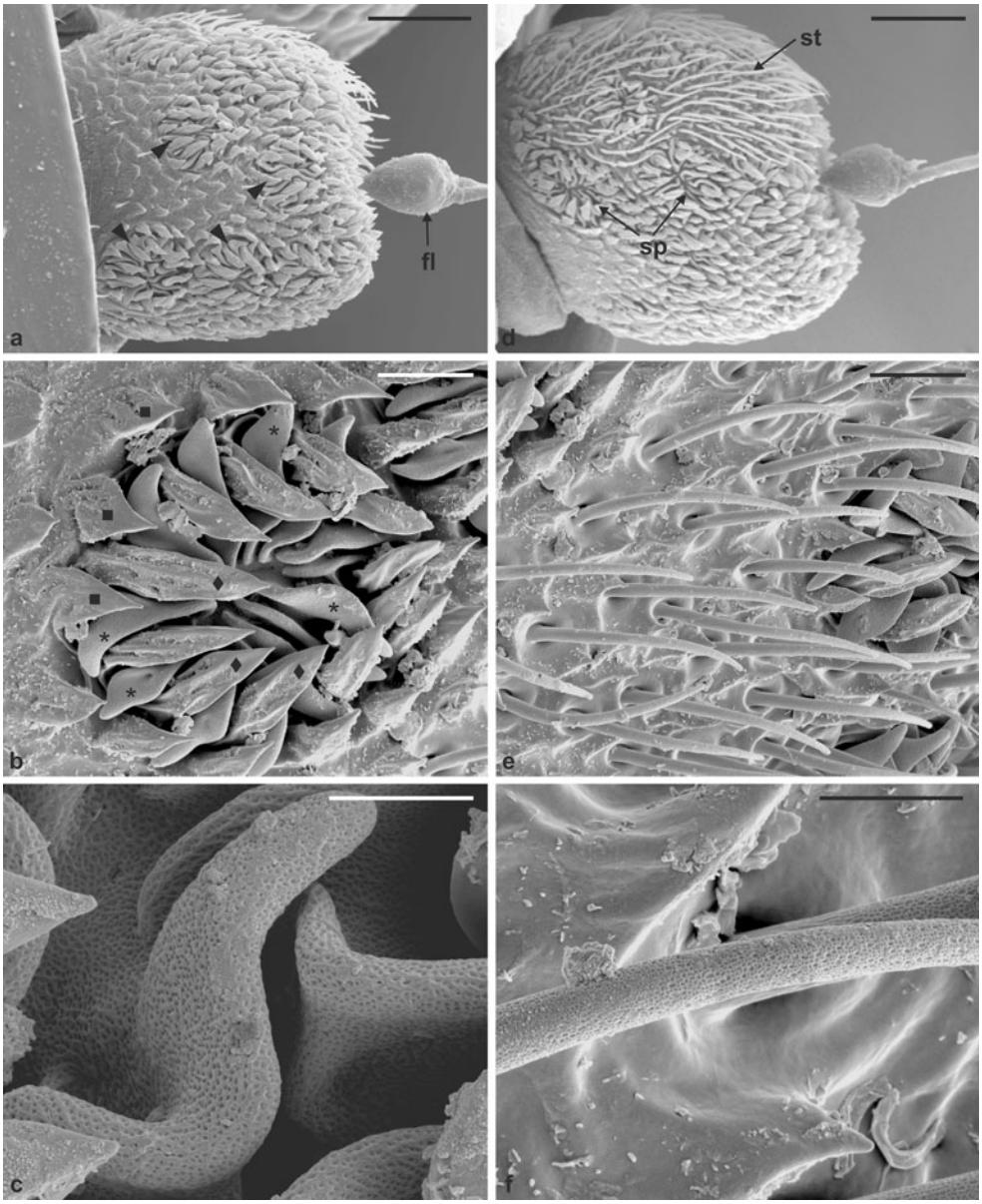

Fig. 3 SEM images from *Hyalesthes obsoletus*. **a** Lateral view of the pedicel, showing sensilla placoidea (*arrowheads*). FL, flagellum. **b** Close-up of a sensillum placoideum. The folded porous cuticle (*asterisk*), as well as the long cuticular spines (*black diamond suit*) and the short cuticular spines (*black square*), are clearly visible. **c** Detail of the porous cuticle. **d** Dorsal view of the pedicel showing the sensilla trichoidea (ST) and sensilla placoidea (SP). **e** Numerous sensilla trichoidea are visible. **f** High magnification detail of the sensilla trichoidea porous cuticle. Scale bars: a, d: 50 μm; b, e: 10 μm; c, f: 3 μm

2005; Maixner et al., 2009; Kessler et al., 2011) and that affiliation is established during its last larval instar (Kessler et al., 2011). The formation of sympatric host races in *H. obsoletus* also has been hypothesized (Imo et al., 2011). The attraction of *H. obsoletus* males to chaste tree (primary host in Israel) volatiles that was observed during this study is unexpected, as this plant is not found in the Italian agroecosystems. In olfactometer assays, Sharon et al. (2005) observed that chaste tree is the most attractive plant for both males and females when compared to bindweed and grapevine. Johannesen et al. (2008) reported that southern and western European populations of the planthopper have a common Levantine origin. This implies that the plant affiliation of *H. obsoletus* is not only based on associative learning of chemical and/or physical cues, but also determined by genetic factors (Papaj and Prokopy, 1989; Bernays, 2001).

Hyalesthes obsoletus males also show greater activity than the females in the field (Bressan et al., 2007). Field bindweed has been reported as the main primary host for *H. obsoletus* in France and Germany (Sforza et al., 1999; Langer et al., 2003). Moreover, the dispersion of this planthopper depends on the field bindweed spatial distribution when its primary host is unavailable (i.e., mowing) (Riolo et al., 2007). We suggest that in many Italian agroecosystems, field bindweed is not an *H. obsoletus* primary host because it grows in compacted loam soils that are not suitable for larva development. Indeed, despite the intrinsic genetic ability of *H. obsoletus* to change the rank order of its plant preference, the available host plants, the soil type (preference for soils with natural cavities), the cultivation practices (Weber and Maixner, 1998), and the presence of organic mulch (Howard and Oropeza, 1998) also have major roles in planthopper larval development.

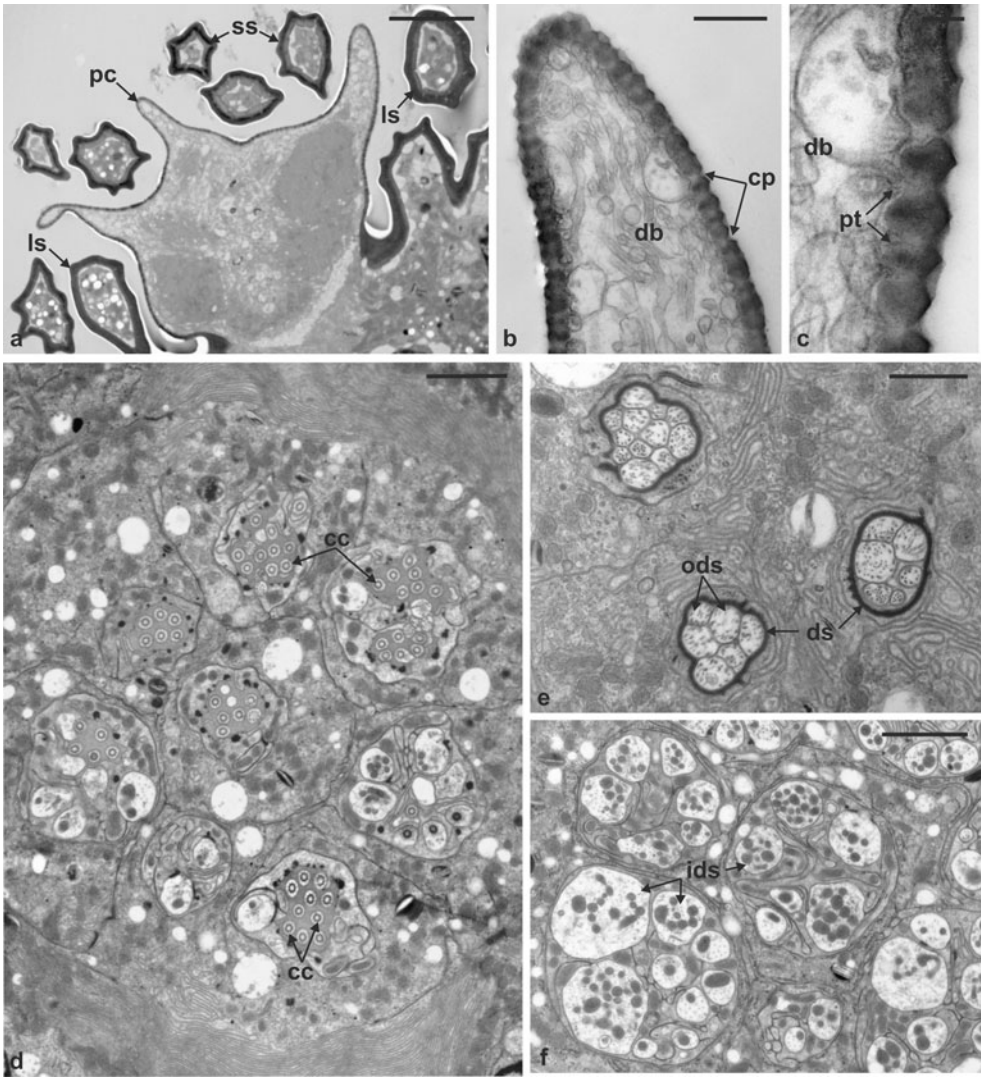

Fig. 4 TEM images of *Hyalesthes obsoletus* sensilla placoidea. **a** Longitudinal section of a sensilla placoidea. The porous cuticle (PC) develops into three arms, surrounded by the aporous long cuticular spines (LS) and short cuticular spines (SS). **b**, **c** Details of the porous cuticle at the level of the folded cuticle. A large number of dendritic branches (DB) completely fill the lumen of the process, running very close to the cuticular pores (CP), where pore tubules (PT) are found. **d** Cross-section of a sensillum placoideum below the sensillum socket, at the level of the sensory neuron ciliary constrictions (CC). The sensory neurons are grouped into eight different 'neuronal units'. Each unit is made up of two to 18 sensory neurons. **e** Cross-section of three neuronal units, showing the outer dendritic segments (ODS). Each bundle of the ODS is isolated from the others by a separated dendritic sheath (DS). **f** Cross-section showing three neuronal units at the inner dendritic segment (IDS) level. Scale bars: a: 5 μm; b: 500 nm; c: 100 nm; d, f: 2 μm; e: 1 μm

Sex-specific differences between behavioral responses of *H. obsoletus* to plant VOCs are reflected by the different ecological niches that males and females occupy. Indeed, *H. obsoletus* males are found in the canopy of nettle, probably for reasons of mate searching (Mazzoni et al., 2010). On the other hand, females lay their eggs in the soil near the roots (Sforza et al., 1999), and so they feed more frequently on basal stems and find hiding places in the organic mulch (Riolo personal observation). The *H. obsoletus* males might use nettle VOCs to avoid adverse effects of intraspecific and interspecific competition, as has been reported for other planthoppers that live and feed in the canopy layer (Ferrenberg and Denno, 2003; Matsumura and Suzuki, 2003).

Hedge bindweed has not been reported as an *H. obsoletus* plant host in Italian agroecosystems, where it frequently grows amongst nettle. Further GC-EAD and behavioral assays are needed to investigate the deterrent activity of hedge bindweed VOCs towards *H. obsoletus* males.

Gas Chromatography and Electroantennography Detection The odor bouquets emitted from the shoots of nettle and chaste tree are considerably different in their compositions and quantities, and in the ratios of the compounds released. Nevertheless, there were 17 VOCs that overlapped between the 41 and 26 compounds that we identified here in the headspace from nettle and chaste tree, respectively. Similarly, of the 11 and 8 compounds, respectively, that elicited GC-EAG responses in both *H. obsoletus* males and females, six were present in both the nettle and chaste tree extracts. This represents the first successful application of a GC-EAG experimental approach to a planthopper species.

This subset of six compounds that elicited antennal responses and were present in the headspace of both nettle and chaste tree included: one aromatic compound (methyl salicylate), one monoterpene (±)-linalool, and four sesquiterpenes [(*E*)-β-caryophyllene, (*E*)-β-farnesene, (*Z*,*E*)-α-farnesene, (*E*,*E*)-α-farnesene]. These are all ubiquitous plant

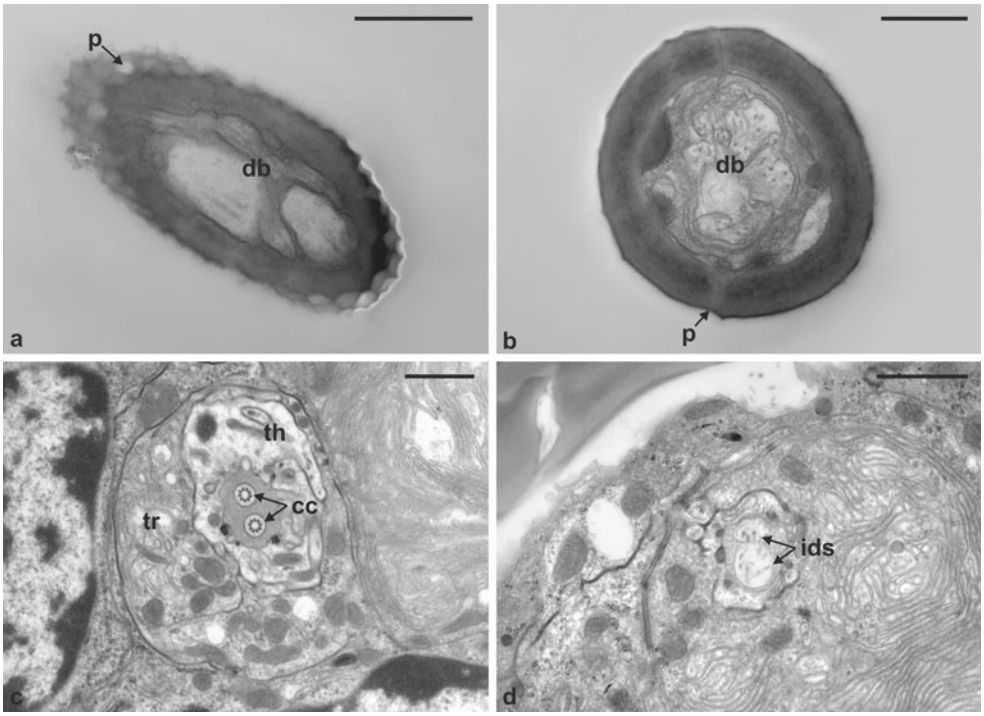

Fig. 5 TEM images of *Hyalesthes obsoletus* sensilla trichoidea. **a**, **b** Cross-section of the cuticular shaft of a sensillum trichoideum, showing the pores (P) and the dendritic branches (DB) within the sensillum lumen. **c**, **d** Cross-sections below the sensillum trichoideum socket: the two sensory neurons are pictured at the level of the ciliary constrictions (CC) and inner dendritic segment (IDS). TH, thecogen cell; TR, trichogen cell. Scale bars: a: 10 μm; b: 2 μm; c: 100 nm; d: 200 nm

volatiles that are known to mediate host recognition in several insect species (Visser, 1986; Bruce et al., 2005). With (±)-linalool, we note that it was not possible to distinguish between its enantiomers.

All of these compounds have also been detected in headspace volatiles from grapevine (Tasin et al., 2005; Cha et al., 2008). One of the mechanisms that mediates the shift in host range of an insect might be the sharing of a subset of compounds that are important for attraction (Tasin et al., 2010). Therefore, as for the majority of polyphagous herbivores, *H. obsoletus* might rely on the absolute and relative amounts of such ubiquitous plant volatiles during the process of finding a host plant (Bruce et al., 2005; Anfora et al., 2009; Cha et al., 2011). They might, thus, be driven towards grapevine by the same group of compounds, and transmit the stolbur phytoplasma during their feeding probing.

On the other hand, among the EAG-active compounds, the terpenoids 4,8-dimethyl-1,(*E*)-3,7-nonatriene and (*Z*)-jasmone were extracted only from the nettle headspace, while the monoterpene 1,8-cineole was present only in chaste tree. In contrast, (*Z*)-3-hexen-1-ol, benzothiazole, and (*Z*)-3-hexenyl-acetate were identified in both nettle and chaste tree extracts, but elicited antenna responses only in the case of nettle; these compounds were, thus, potentially under the *H. obsoletus* perception threshold with chaste tree. A reversed result was seen for methyl benzoate. (*Z*)-3-hexen-1-ol, (±)-linalool, and methyl benzoate have been shown to elicit antennal responses in another planthopper species, the brown rice planthopper *N. lugens*, where the females were more responsive than the males (Youn, 2002). Furthermore, as host plants are often selected by gravid females, it is particularly intriguing that only the females show sensitivity to (*Z*)-3-hexenyl acetate and 4,8-dimethyl-1, (*E*)-3,7-nonatriene, which were abundant in the nettle. Variations in the sensitivities of the antennal receptors and the specificities between males and females might explain this sex-biased plant-volatile attraction in some insects (Fraser et al., 2003).

The GC-EAG technique was chosen as a suitable and powerful tool for the identification of active compounds in a complex odor blend, where it can often reveal the biological importance of secondary compounds in a mixture (Riffell et al., 2009). Compounds that elicited electrophysiological responses in *H. obsoletus* antennae are likely to have roles in the behavior of this insect. However, further studies need to investigate whether the EAG active compounds indeed affect *H. obsoletus* behavior and also play a role in the field.

Functional Anatomy Despite differences in the responses to these plant volatiles shown by *H. obsoletus* male and female individuals, the antennal structures investigated did not show any sexual dimorphism. Similar results have been reported for the carrot psyllid, *Trioza apicalis* Foerster (Hemiptera: Triozidae); the male and female antennae of this psyllid have the same sensilla, but they respond to different volatiles (Kristoffersen et al., 2006, 2008). In *H. obsoletus*, we found two different types of olfactory sensilla. The first type are the sensilla placoidea, and these have been reported to be common (although with some structural variations) within the Fulgoromorpha (Lewis and Marshall, 1970; Marshall and Lewis, 1971; Aljunid and Anderson,

1983; Bourgoin and Deiss, 1994). In *H. obsoletus*, we found ca. 30 sensilla placoidea scattered on the pedicel surface. Within the Fulgoromorpha, the number of sensilla placoidea ranges between 17 with *N. lugens* (Aljunid and Anderson, 1983) and 200 with *P. candelaria* (Lewis and Marshall, 1970). The organization of the porous cuticle varies from simple, flat plates (as in Tettigometridae; Bourgoin, 1985) to elaborate structures (Marshall and Lewis, 1971; Bourgoin and Deiss, 1994). This large variation appears not to be common, and it has been reported almost exclusively for this insect group, together with most of these sensilla being at the pedicel level.

Sensilla placoidea in *H. obsoletus* are innervated by up to 80 sensory neurons, which makes them one of the highest innervated olfactory sensilla described to date. Other exceptionally innervated sensilla have been reported by Slifer and Sekhon (1961), Behan and Ryan (1978), Aljunid and Anderson (1983) and Isidoro et al. (2001).

Another relevant feature is the presence of bundles of dendrites that are separated by their own dendritic sheath. Within the Fulgoromorpha, TEM-based ultrastructural details of the olfactory sensilla are known only in *P. candelaria* (Marshall and Lewis, 1971) and *N. lugens* (Aljunid and Anderson, 1983). In both of these species, groups of neurons forming 'neuronal units' (*sensu* Jez and McIver, 1980) were described, although in *N. lugens* there was no dendritic sheath. In more recent studies, the occurrence of neuronal units has also been seen in other hemipterans, belonging to Homoptera (Rossi Stacconi and Romani, 2011) and Heteroptera (Romani and Rossi Stacconi, 2009). The occurrence of dendritic bundles within a single sensillum that is related to the potential fusion of earlier separated structures into a more complex sensory element has been hypothesized in Fulgoromorpha (Lewis and Marshall, 1970; Aljunid and Anderson, 1983).

The second type of antennal sensilla in *H. obsoletus* is represented by the sensilla trichoidea, which show structural features that are linked to an olfactory function. Aljunid and Anderson (1983) reported that in *N. lugens* there are three different types of sensilla trichoidea, one of which has a putative olfactory function.

The occurrence of at least two different types of olfactory sensilla in *H. obsoletus* promotes the hypothesis that they can be tuned to different volatiles coming from either the host plant or from conspecifics, although there is yet no evidence of sex pheromones available for these planthoppers. In a relatively recent study, Kristoffersen et al. (2008) showed that the primary olfactory centers in Homoptera are not organized into distinct glomeruli, as has been seen in neuroanatomy studies carried out in psyllids and aphids (Hemiptera: Sternorrhyncha). We hypothesize that the antennal lobe organization in *H. obsoletus* in particular, and in the Fulgoromorpha in general, is characterized by a glomerular structure, which would reflect the large number of olfactory receptor neurons on the antennae.

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