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Leaves of Salix spp. (Salicaceae)*

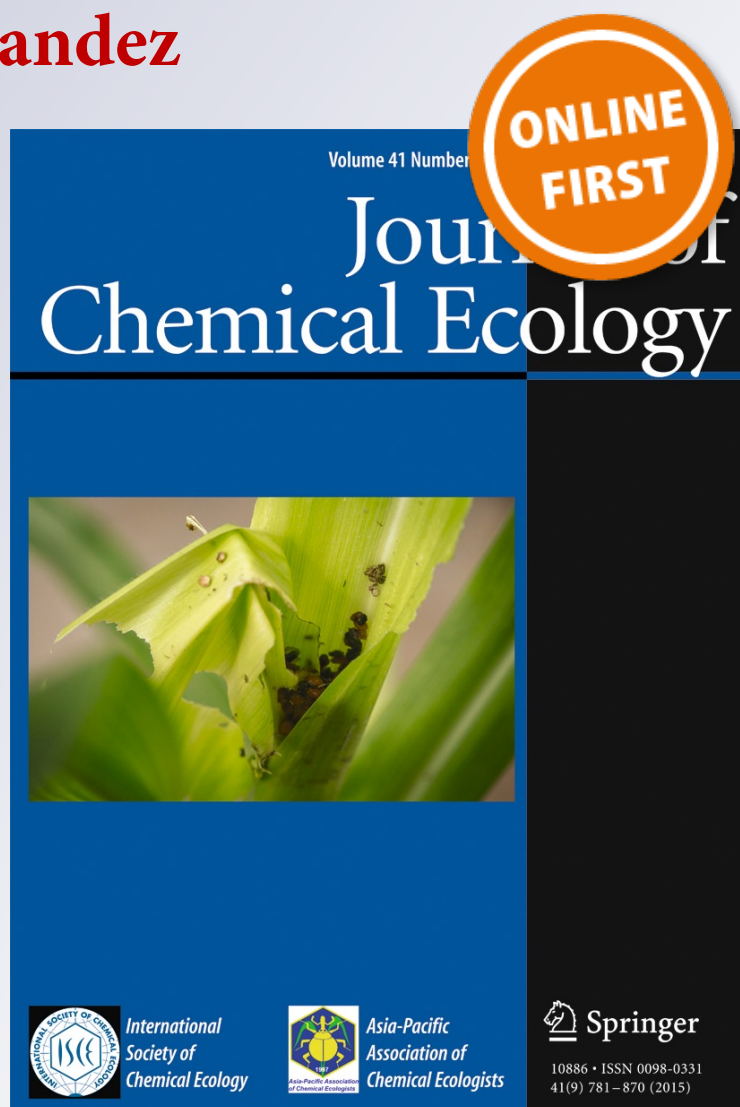
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Journal of Chemical Ecology

ISSN 0098-0331

J Chem Ecol

DOI 10.1007/s10886-015-0637-z



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Both Volatiles and Cuticular Plant Compounds Determine Oviposition of the Willow Sawfly *Nematus oligospilus* on Leaves of *Salix* spp. (Salicaceae)

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Received: 15 June 2015 / Revised: 14 September 2015 / Accepted: 21 September 2015
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Abstract Plant volatile organic compounds play a role in selection of host plants by herbivorous insects. Once the insect reaches the plant, contact cues determine host acceptance. Although the willow sawfly *Nematus oligospilus* (Hymenoptera: Tenthredinidae) can differentiate among willow genotypes, no knowledge is available on the cues used by this insect to seek and accept the host plant. In this study, we recorded behavioral orientation in a Y-tube olfactometer of willow sawfly females to volatiles of the highly preferred genotype *Salix nigra* and the non-preferred genotype *S. viminalis*. The volatiles released by undamaged willows of each genotype were analyzed by coupled gas chromatography-mass spectrometry. Contact cues were evaluated first by oviposition preference bioassays after selective leaf wax removal, and then by studying the micromorphology of abaxial and adaxial leaf surfaces and their chemical composition. Willow sawfly females oriented preferentially to *S. nigra* volatiles, which contained more than 3 times the

amount of volatiles than that collected from *S. viminalis*. Analysis of volatiles showed significant differences in amounts of (*Z*) and (*E*)- β -ocimene, undecane, decanal, and β -caryophyllene. The adaxial leaf surface of *S. nigra* was less preferred after wax removal, suggesting a role of cuticular waxes for oviposition acceptance. No differences were found among the micromorphology of leaf surfaces between preferred and non-preferred genotypes. The chemical analysis of cuticular waxes showed that the abaxial leaf surface of *S. viminalis*, which is completely avoided for oviposition, possessed 97 % of alkanes. The accepted leaf surfaces contained a more diverse wax profile including alcohols, acids, and esters. Thus, non-alkane wax compounds might be related to oviposition. In sum, our study suggests that several cues act in concert to provide oviposition cues for the sawfly *N. oligospilus*: females are attracted to volatiles from a distance, and once alighting on the plant, they seek specific chemical contact cues in order to lay eggs.

Peter E. Teal is deceased.

Electronic supplementary material The online version of this article (doi:10.1007/s10886-015-0637-z) contains supplementary material, which is available to authorized users.

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Keywords Oviposition cues · Tenthredinidae · Insect-plant interaction · Host plant location · Volatiles · Cuticular wax · Olfactometer

Introduction

Herbivorous insects rely on plant cues for successful colonization of a suitable host plant. Not only refined perceptive abilities are necessary to locate a plant but also enormous integrative skills to recognize its suitability. Several cues from different sensory modalities converge to orient herbivorous insects towards their host plants. Cues can be visual, olfactory, and/or contact and require the integrative capacity of herbivores to identify them and perform a choice (Renwick and Chew 1994; Schoonhoven et al. 2005). An interplay between positive and negative stimuli is likely to affect the final choice whether to accept or reject a particular plant, and requirements for specific physical characteristics may enlarge the complexity of interactions (Renwick 1989). It is widely accepted that orientation of herbivorous insects towards their host plant is chemically mediated by plant volatiles, whereas non-volatile contact stimuli largely are responsible for triggering oviposition (Renwick 1989; Schoonhoven et al. 2005).

Plant volatiles, primarily green leaf volatiles and terpenes, usually are used as long to medium range cues although other classes of compounds can be important. The recognition of a host plant can occur by using species-specific volatile compounds, as indicated by the attraction of the cabbage seed weevil (*Ceutorhynchus assimilis* Payk.) to isothiocyanates released from oilseed rape, *Brassica napus* L. (Blight 1995). However, most studies favor the idea that plant odor specificity is achieved by a particular ratio among ubiquitous volatile constituents (Bruce et al. 2005). Once the plant has been located, the outermost leaf surface constitutes the zone of initial contact. The structural properties and/or chemical composition of this surface layer may determine the insect's acceptance of this plant, and also provide a preference cue for specific localities such as the abaxial or adaxial leaf surfaces (Kanno and Harris 2000; Müller and Hilker 2001). It is highly adaptive for an herbivore to recognize a suitable plant by the epicuticular waxes or secondary compounds present on the surface without wasting energy and time on a non-host (Riederer and Müller 2006). There are several studies that show that the chemical composition of cuticular waxes plays a role as oviposition stimulant for lepidopterans (Brooks et al. 1996; Li and Ishikawa 2006), dipterans (Degen and Städler 1997; Morris et al. 2000), and coleopterans (Müller and Hilker 2001). In addition, the microstructure of this layer may affect oviposition behavior. It has been suggested that the presence of wax films, crystals, or pubescence may impede or deter egg laying (Calatayud et al. 2008; Scott Brown and Simmonds 2006).

Nematus oligospilus Förster (Hymenoptera: Tenthredinidae) is an external leaf feeder that develops exclusively on species of *Salix* L. (Salicaceae) and has been reported worldwide (Charles and Allan 2000; Dapoto and Giganti 1994; Smith 2003; Urban and Eardley 1995). Females of this willow sawfly must find and recognize their host plant when they emerge as adults after pupating in the soil. Previous studies on *N. oligospilus* described differential oviposition preference depending upon willow genotype (Braccini et al. 2013; Charles et al. 1998). Orientation towards host plant volatiles has been established for a number of sawflies. In free feeding sawflies, attraction to volatile compounds including green leaf volatiles, terpenes, and isothiocyanates has been documented (Barker et al. 2006; Martini et al. 2010; Piesik et al. 2008). Additionally, in the leaf-galling sawfly (*Pontania proxima* Lepeletier), host plant location by volatiles has been hypothesized (Kehl et al. 2010). However, in the case of *N. oligospilus*, it remains unclear what plant cues are sought by newly emerged females to find suitable hosts.

Recently, Braccini et al. (2013) found that *N. oligospilus* deposits eggs indiscriminately on both leaf surfaces of the preferred genotype *S. nigra* Marshall, but lays only a few on the adaxial surface of the non-preferred *S. viminalis* L. Moreover, females never lay eggs on the abaxial surface of *S. viminalis*, suggesting rejection cues or the absence of oviposition cues at all. Different compounds, including cuticular waxes and phenolic glycosides, have been reported as oviposition stimulants or deterrents of willow sawflies (Craig et al. 1988; Roininen et al. 1999). In *N. oligospilus*, causes of differential oviposition preference according to leaf surface characteristics are uncertain. Thus, a thorough study combining behavioral aspects as well as chemical and micromorphological leaf properties should be carried out in order to understand the differences reported in egg laying of *N. oligospilus*.

This study focused on the olfactory (i.e., volatile) and contact surface cues (i.e., plant cuticular waxes and micromorphology) that influence the host plant recognition process in the willow sawfly *N. oligospilus*. Specifically, we first determined adult colonization and oviposition, and larval damage on either *S. nigra* or *S. viminalis* under field and laboratory conditions. To explain the mechanisms of detection and discrimination of the host by *N. oligospilus* adults we: 1) evaluated female orientation to preferred and non-preferred willow genotypes by volatile cues; 2) determined chemical differences in volatile cues; 3) assessed the importance of cuticular waxes of adaxial leaf surfaces for oviposition; and 4) evaluated differences in the micromorphology and chemistry of the cuticular wax layer on adaxial and abaxial surfaces of preferred and non-preferred willow genotypes. The results suggest that *N. oligospilus* females use volatile cues to approach preferred willow genotypes. Once alighting on the plant, they seek specific chemical contact cues from

the cuticular waxes in order to lay eggs. Neither evidence of oviposition deterrents nor effects of physical contact cues were found.

Methods and Materials

Natural Colonization and Leaf Damage Feeding damage by *N. oligospilus* larvae was assessed in two field nurseries by estimating defoliation in natural colonized one year old plants. Both sites were located in areas of homogeneous characteristics of soil, light, and water irrigation. Plants corresponded to a first nursery of 114 trees of *S. nigra* genotype (60 trees *S. nigra* x free pollination and 54 trees *S. nigra* x *S. matsudana*) and a second nursery of 80 trees of *S. viminalis* genotype (20 trees of each of the following varieties: “Amarillo”, “Verde”, “Manes” and “Guri”). They were planted in rows of 10 or 20 and spaced by 1 m × 0.5 m, with a total of 194 plants. Sawfly activity was monitored weekly from December 2007 to April 2008. Since similar patterns of damage were recorded for *S. nigra* and *S. viminalis* during the entire season, we showed records of the last week of January 2008. The level of damage was estimated after having seen larval feeding by using an arbitrary scale of 6 levels: L0 = no defoliation, L1 ≤ 5 % defoliation, L2 = 5–25 %, L3 = 25–50 %, L4 = 50–75 %, L5 = 75–100 %.

Insects Adult *N. oligospilus* females from a laboratory population were used in all bioassays. In spring 2013, the population was started from larvae and pupae collected in a field with a history of sawfly infestation in the lower Delta of Paraná river (34° 10' 23.08" S, 58° 45' 57.67" W). Larvae were reared until pupation on fresh cut twigs of a genotype from *Salix alba* L. × *Salix alba* in transparent plastic boxes (46 × 30 × 32 cm) in a controlled environment chamber at 25 °C and L16:D8 h photoperiod. Pupae were removed from the boxes and held in separate plastic jars until emergence of adult females. During the season, the laboratory population was renewed several times with field-collected material to minimize selection for laboratory-adapted insects.

Plants Material from the National Program of Genetic Improvement for Salicaceae (willow genetic improvement area from Instituto Nacional de Tecnología Agropecuaria, INTA, Argentina) was used in this study. Based on marked oviposition differences in previous findings (Braccini et al. 2013), two willow genotypes were chosen: *Salix nigra* (‘Alonzo nigra 4 INTA’) and *Salix viminalis* (‘Macollado amarillo’).

In late winter of 2011, 15 cuttings (30 cm length) of each genotype were planted in consecutive rows (i.e., nursery) in an experimental station in INTA Castelar (Buenos Aires, Argentina) in order to provide fresh twigs and leaves for

bioassays and maintenance of the insect laboratory population. In October 2012, cuttings of *S. nigra* and *S. viminalis* were planted individually in 5-l pots and kept outdoors under natural conditions. After 3 mo, potted plants were subjected to volatile collection (see below). Both nursery-willows as potted plants were protected from insect damage (i.e., leaf-cutting ants) by placing ant baits in their surroundings.

Oviposition Preference Bioassays To investigate oviposition preference, dual-choice bioassays were carried out. Two willow genotypes (i.e., *S. nigra* and *S. viminalis*) were simultaneously offered to individual female sawflies. Bioassays were conducted inside the controlled environment chamber previously mentioned. Transparent plastic boxes (33 × 23 × 14 cm) containing a two-leaf fresh cut twig of each genotype in tap water were employed. A newly emerged or a 1-d-old female was released in the center of the box and was allowed to lay eggs until death (normally up to 3 d). The number of eggs laid per genotype was recorded.

Olfactometer Bioassays The attractiveness of volatile compounds from two willow genotypes on *N. oligospilus* females was evaluated in olfactory choice tests using a Y-tube glass olfactometer. The system consisted of a central and two lateral arms (15 cm long, 3 cm internal diam each of them). Each lateral arm was connected via Teflon (PTFE) tubing to a cylindrical glass chamber (32 cm long, 4.5 cm internal diam) holding an odor source. A vacuum pump (Air Cadet Vacuum/Pressure Pump, Barnat Company, USA) was used to push air through the olfactometer’s two lateral arms. The air stream was humidified by passing through distilled water, and then filtered by using charcoal filters prior to entering the olfactometer. Two flowmeters (Bruno Schilling SA, model MB60V-B, Argentina) kept air flows constant at 0.2 l per min. As odor sources, bouquets of three freshly cut twigs (25 cm long) were employed. Cut ends were wrapped in Parafilm. The olfactometer was set on a white table inside the controlled environment chamber previously mentioned. A 30 cm-high white cardboard was placed on each side of the table in order to avoid possible visual effects. For the same purpose, the cylindrical glass chambers containing the odor sources were covered with white paper. Equipment was cleaned by rinsing the parts with boiling water, acetone, and ethanol every time an odor source was changed. On a given day, one odor source connected to the same arm of the olfactometer was evaluated. Y-tubes were changed for every replicate, and in that case, they were cleaned by rinsing with ethanol and placed in a drying oven.

To perform bioassays, the Y-tube was marked into different zones. The zone between the opening of the central arm and the junction area was referred as the “base”. The triangle determined in the junction area was referred as the “bifurcation”.

Finally, the “arms” were considered from the bifurcation to the end of each glass tube.

To start a measurement, one freshly hatched female was transferred gently through a glass tube to the bottom of the base. The vacuum pump was turned on, and the test began once the female started walking and tapping the surface with the antennae, which usually took only a few seconds. The time the female spent in each zone of the olfactometer was recorded in real time during 10 min by using JWatcher V1.0 (Blumstein and Daniel 2007). The first choice of each female also was recorded.

We evaluated the relative attractiveness of *S. nigra* and *S. viminalis* by comparing odor sources against each other. The location of the odor sources (i.e., right or left arm of the olfactometer) was alternated to avoid position effects.

Volatile Collection and Chemical Analysis Intact potted plants from *S. nigra* and *S. viminalis* were employed to collect volatile compounds. For this purpose, plants were moved early in the morning from outdoors to the controlled environment chamber previously mentioned. Each 3-mo-old plant was employed only once. Headspace samples were taken by enclosing the upper part of the plant (approximately 50 cm) into a polyethylene (PET) bag (Toppits oven bags, Minden, Germany). Charcoal-filtered air was pushed into the PET bag with an aquarium air pump (Atman, model AT-703, China) and then pulled by a suction pump (Tuff, model Standard, Bedford, UK) at a constant rate of 0.9 L per min. Air leaving the bag through an outlet passed through a volatile collection trap made of 30 mg HayeSep Q adsorbant (Grace, Deerfield, IL, USA) where volatiles were collected for analysis. After a sampling period of 6 h (between 10:00 and 16:00 h), the volatile collection traps were wrapped in Teflon tape and foil and stored in the freezer until elution. Empty PET bags were used as blanks.

Volatile compounds were eluted from the filters with 150 μ l of dichloromethane containing 5 ng of tetradecane as internal standard. Volatile samples were analyzed by coupled gas chromatography-mass spectrometry (Agilent 6890 instrument coupled to Agilent 5973 selective mass detector). A J&W 30-m HP1-MS capillary column was used (0.25-mm i.d., film thickness 0.25 μ m). Samples (1 μ l) were injected at 240 °C in a splitless mode. Helium was used as carrier gas at 0.7 ml per min (inlet pressure: 20.48 kPa). The column temperature was held at 35 °C for 1 min, and then increased at a rate of 10 °C per min until it reached 230 °C. Finally, the temperature was held at 230 °C for 15 min. Compounds were identified by comparing mass spectra to those provided by NIST (National Institute of Standards and Technology) and natural product mass spectral libraries developed by the Chemistry Research Unit CMAVE-USDA, and by comparison of retention times with authentic standards. Authentic standards from Sigma- Aldrich including 2-hexanone,

hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenol, α -pinene, limonene, salicylaldehyde, (*Z*) and (*E*)- β -ocimene, β -caryophyllene, (*E*-*E*)- α -farnesene were from the compound library at CMAVE-USDA. Compounds were quantified by relating their peak areas with those of the internal standard and expressed as mass equivalent of the internal standard (ng/ μ l). Previously, blank values were subtracted from the samples.

Contact Bioassays To evaluate the role of epicuticular waxes as oviposition cues, contact bioassays were carried out. Four-leaf fresh cut twigs of each genotype (i.e., *S. viminalis* and *S. nigra*) were offered to an individual female sawfly. In all cases, only the adaxial leaf surfaces were exposed (the abaxial surfaces were covered with paper tape). Two out of the four adaxial leaf surfaces were treated with a polymer film of commercial gum arabic in order to mechanically remove the epicuticular wax layer. A 50 % w/w aqueous solution of gum arabic was applied onto the entire adaxial leaf surface using a small paintbrush. After 1 h, the solution was dry and a thin polymer film could be gently peeled off in pieces, leaving the leaves physically intact (without damaging the epidermal tissue) (Jetter and Schäffer 2001). Thus, two adaxial leaf surfaces were devoid of epicuticular waxes and two remained intact. Bioassays were conducted in transparent plastic boxes (33 \times 23 \times 14 cm) located inside the controlled environment chamber previously mentioned. A newly emerged or a 1-d-old female was released in the center of the box and was allowed to lay eggs during 24 h. The number of eggs laid per leaf was recorded. Particularly, females exposed to *S. viminalis* then were exposed to a second *Salix* genotype (*S. alba* \times *S. alba*) for another 24 h. This procedure was performed to assure the capability of female sawflies to lay eggs normally, as they usually avoid oviposition on *S. viminalis*.

Scanning Electron Microscopy (SEM) Studies In late spring, fully-expanded mature leaves were collected from the mid-section of branches of *S. nigra* and *S. viminalis* plants. Segments of the middle portion of air-dried leaf blades were mounted on stubs, coated with a gold-palladium (40–60 %) alloy by a Thermo VG Scientific (West Sussex, England), and then observed using a Philips XL 30 Scanning Electron Microscope (Philips, Eindhoven, The Netherlands) at the Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”, Argentina. Microphotographs from both leaf sides of each genotype were obtained.

Leaf Waxes Extraction and Chemical Analysis Leaves were collected in January 2013 from the willow plantation grown in the experimental station of INTA Castelar (Buenos Aires, Argentina) and stored in plastic bags in the freezer until use. Selective extraction of waxes from either the adaxial or abaxial leaf surface was achieved by

placing the intact leaf onto a flexible rubber mat, gently pressing a glass cylinder (10 mm internal diam) onto the exposed surface and filling the cylinder with approximately 1 ml of dichloromethane (Jetter et al. 2000). The solvent was agitated for 30 s by pumping with a Pasteur pipette, and removed. When any solvent leaked between the cylinder and the leaf, the sample was discarded. Each sample consisted of an extract obtained from 75 individual leaves. The samples were dried with Na₂SO₄, filtered, and then the solvent was evaporated under a stream of N₂ until dryness, weighed, and redissolved in methylene chloride using 25 µl of solvent per mg of extract. They were analyzed by coupled gas chromatography-mass spectrometry using an Agilent Technologies 5977 A selective mass detector (quadrupole) coupled to an Agilent Technologies 7890 A instrument fitted with an HP-5MS J&W column (5 % phenylmethylsiloxane, 30 m × 0.25 mm i.d. × 0.25 µm film thickness). Samples (1 µl) were injected in a splitless mode. The oven was programmed as follows: an initial temperature of 60 °C was increased to 200 °C at a rate of 10 °C per min, then 4 °C per min to 300 °C, and held at 300 °C for 15 min. Identification of the individual components was based on: computer matching with commercial mass spectra libraries (NBS 75 K; NIST 1999; McLafferty and Stauffer 1994), mass spectra reported in the literature (Hietala et al. 1995), and co-injection with authentic samples of palmitic acid, pentacosane, heptacosane, nonacosane, and triacontane from the compound library at INQUINOA-CONICET. Mass spectra of compounds from which we lacked authentic standards are shown in Supplementary Fig. 1. Quantification of compounds was accomplished by relating their peak areas with those of an internal standard of eicosane and dodecane and expressed as mass equivalent of the internal standard (µg/µl).

Statistical Analyses To compare the level of damage by defoliation in the field between *S. nigra* and *S. viminalis* a χ^2 test for association was performed (Dhytam 2011).

Results from the oviposition preference bioassay for both, genotype comparison and gum arabic treatment, were subjected to *Wilcoxon matched pairs test* to evaluate differences in the number of eggs laid.

Regarding the olfactometer bioassays, the ratio of the time spent in each arm over the total time spent in both arms of the olfactometer were compared in order to detect preference for one odor source or the other. Data were analyzed by a *Wilcoxon matched pairs test*. The female's first choice was evaluated by a χ^2 test.

The volatile collection data were subjected to *Mann Whitney U test* to determine differences between individual compounds and total volatiles emitted by *S. nigra* and *S. viminalis*.

All aforementioned analyses were conducted with the software package R 2.15.1 for Windows (R Core Team 2012).

Differences in the chemistry of cuticular waxes among willow genotypes were tested by means of discriminant analysis (Software XLSTAT 2015.2.02.17945) with willow genotype and leaf surface as independent factors and chemical concentrations as dependent factors.

Results

The survey from our field experiment showed that *S. nigra* genotypes were more damaged by larvae of *N. oligospilus* than *S. viminalis* genotypes (χ^2 test, $N = 114$ and $N = 80$ for *S. nigra* and *S. viminalis*, respectively, $\chi^2 = 164.97$, $df = 5$, $P < 0.05$, Fig. 1a). Similarly, the dual choice bioassay in the laboratory showed that *N. oligospilus* females strongly preferred *S. nigra* as a host plant for oviposition (*Wilcoxon matched pairs test*, $N = 23$, $P < 0.05$; Fig. 1b).

Host plants volatiles may explain the orientation of sawflies towards *S. nigra*. To assess the role of olfactory cues in the discrimination between *S. viminalis* and *S. nigra*, Y-tube olfactometer bioassays were performed. When evaluating the relative attractiveness of *S. nigra* compared to *S. viminalis*, females spent more time in the arm connected to *S. nigra* (*Wilcoxon matched pairs test*, $N = 23$, $P < 0.05$; Fig. 2a). A similar pattern was observed in the first choice of females: significantly more females showed a first choice towards *S. nigra* over *S. viminalis* (χ^2 test, $N = 23$, $P < 0.05$; Fig. 2b).

A total of 16 and 14 compounds were collected from volatiles released by *S. nigra* ($N = 8$) and *S. viminalis* ($N = 10$) trees, respectively. The amount of total volatiles collected from *S. nigra* was more than 3 times the amount of total volatiles collected from *S. viminalis* (*Mann Whitney U Test*, $P < 0.05$). Among others, the volatile profile included mono and sesquiterpenes like α -pinene, β -pinene, limonene, (*Z*)- β -ocimene, (*E*)- β -ocimene, β -caryophyllene, and (*E*)- α -farnesene, and green leaf volatiles (GLV) like hexanal, (*E*)-2-hexenal, and (*Z*)-3-hexenol. Amounts of (*Z*)- β -ocimene, undecane, and decanal were detected only in *S. nigra*, and β -caryophyllene was detected only in volatiles from *S. viminalis*. Besides, (*E*)- β -ocimene was present in significantly higher amount in *S. nigra* (*Mann Whitney U Test*, $P < 0.05$). Even if there is a trend of a higher quantity of (*E*)- α -farnesene in *S. nigra*, we did not detect differences among genotypes, maybe due to high variability among data (Table 1).

Leaf waxes may play a role as oviposition stimulants for *N. oligospilus* adults once they reach the plant. Thus, to assess the role of contact cues in the egg laying decision, epicuticular waxes were mechanically removed from the adaxial leaf surface both in *S. nigra* and *S. viminalis* by treatment with gum

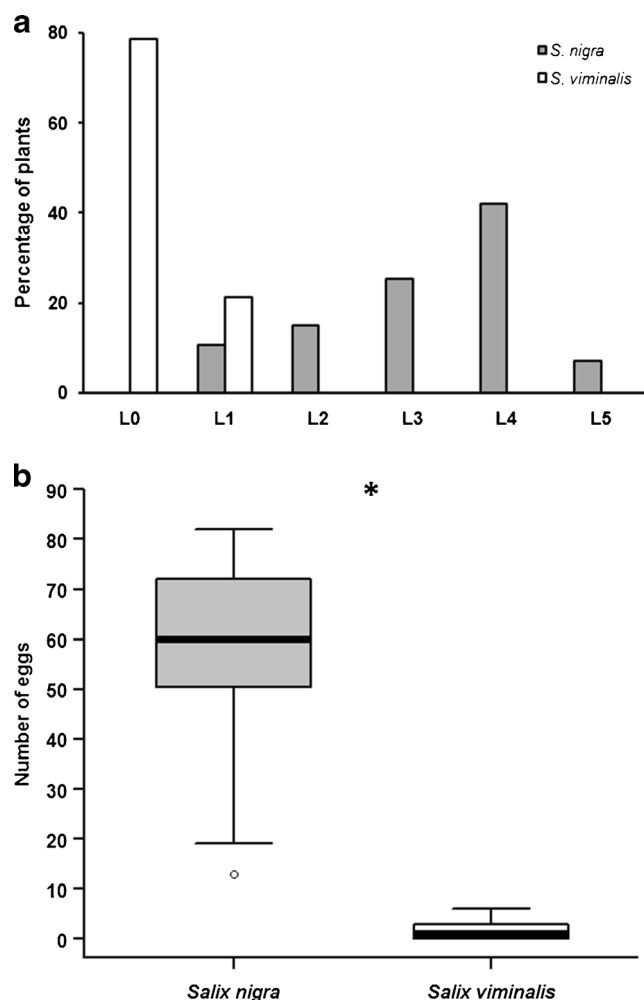


Fig. 1 **a** Percentage of *Salix nigra* and *Salix viminalis* plants that exhibit a level of damage by defoliation of L0–L5. Level of damage was estimated by using the following scale: L0 = no defoliation, L1 ≤ 5 % defoliation, L2 = 5–25 %, L3 = 25–50 %, L4 = 50–75 %, L5 = 75–100 %. χ^2 test, $N = 114$ and $N = 80$ for *S. nigra* and *S. viminalis*, respectively. **b** Number of eggs laid by adult females according to willow genotype in a dual choice bioassay. *Wilcoxon matched pairs test*, $N = 23$. * indicates significant difference at $P < 0.05$

arabic. Significantly fewer eggs were deposited in the treated surface of *S. nigra* (i.e., the surface without epicuticular waxes). Even if there may be a tendency, no differences were found in *S. viminalis* (Fig. 3, *Wilcoxon matched pairs test*, $N = 18$, $P < 0.05$, and $N = 24$, $P = 0.058$ respectively).

To evaluate the role of physical cues, SEM micromorphological studies were performed. Microphotographs showed a homogeneous, thin wax layer covering the abaxial (Fig. 4a–d) and adaxial leaf surface (Fig. 4e–h) in both genotypes. Wax scales were observed on the abaxial surface of *S. viminalis* (Fig. 4c–d), and wax tubes on the adaxial surface of *S. nigra* (Fig. 4e–f).

Chemical contact cues were analyzed after selective wax extractions according to leaf surface from *S. nigra* and *S. viminalis*. Results revealed differences in

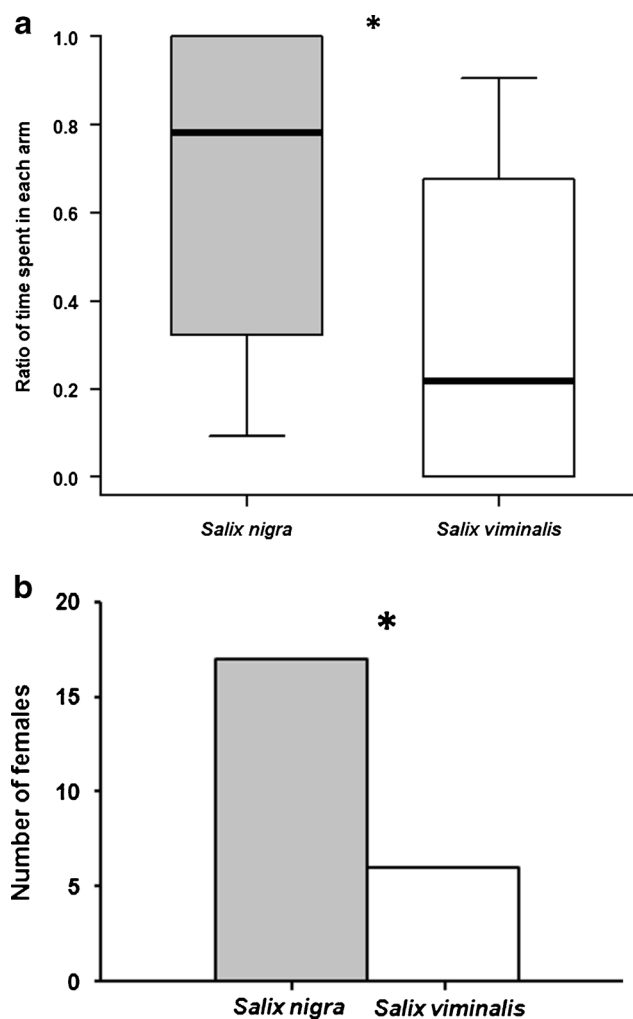


Fig. 2 **a** Ratio of time spent by females in each arm of the olfactometer over the total time spent in both arms. *Wilcoxon matched pairs test*, $N = 23$. * indicates significant difference at $P < 0.05$ **b** Number of females that first chose *Salix nigra* arm or *Salix viminalis* arm. χ^2 test, $N = 23$. * indicates significant difference at $P < 0.05$

composition between both genotypes and also between leaf surfaces of the same genotype (Table 2). *Salix nigra*, the preferred genotype, showed a complex profile in both leaf surfaces, including acids, alcohols, esters, sterols, and β -amyryn. In contrast, the wax composition of *S. viminalis*, the non-preferred genotype, was less compound rich. Odd-chained aliphatic alkanes, heptacosane, and nonacosane, were the major components, representing approximately 84 % and 90 % for adaxial and abaxial surfaces, respectively. Interestingly, the abaxial surface of *S. viminalis*, where *N. oligospilus* never lays eggs, possessed the least diverse wax composition. The quantitative data from the wax analysis were subjected to a discriminant analysis as a multivariate technique for discrimination of the genotypes. Data showed that not only the genotypes were separated, but also the leaf surfaces could be

Table 1 Volatile compounds recorded in the headspace of *Salix nigra* and *Salix viminalis* trees

Retention time	Compound	<i>Salix nigra</i>		<i>Salix viminalis</i>		<i>P</i>
		Mean	SE	Mean	SE	
5.06	1,8 nonadienol	0.02	0.02	0.04	0.02	n.s.
5.11	2-hexanone	0.08	0.04	0.05	0.02	n.s.
5.25	Hexanal	0.19	0.08	0.14	0.03	n.s.
5.81	4-hydroxy-4-methyl-2-pentanone	0.08	0.03	0.08	0.02	n.s.
6.01	(<i>E</i>)-2-hexenal	0.52	0.27	0.37	0.16	n.s.
6.21	(<i>Z</i>)-3-hexenol	0.05	0.03	0.06	0.03	n.s.
6.70	2-heptanone	0.03	0.02	0.02	0.01	n.s.
7.77	α pinene	0.37	0.13	0.53	0.19	n.s.
8.43	β pinene	0.17	0.06	0.30	0.11	n.s.
9.10	Salicylaldehyde	0.09	0.04	0.04	0.02	n.s.
9.25	Limonene	0.23	0.11	0.19	0.07	n.s.
9.31	(<i>Z</i>)- β -ocimene	0.17	0.06	–	–	*
9.50	(<i>E</i>)- β -ocimene	4.71	2.01	0.15	0.06	*
10.47	Undecane	0.07	0.06	–	–	*
11.77	Decanal	0.03	0.01	–	–	*
15.09	β -caryophyllene	–	–	0.03	0.01	*
15.97	α -farnesene	1.07	0.50	0.22	0.05	n.s.
	Total volatiles	7.88	3.45	2.23	0.80	*

Retention time of each compound is given in min

Quantity of volatile compounds emitted from the top 50 cm of *S. nigra* ($N = 8$) and *S. viminalis* ($N = 10$) trees for 6 h is expressed as ng/ μ l. * indicates significant difference at $P < 0.05$, n.s. indicates non-significant difference (*Mann Whitney U test*)

discriminated by the cuticular waxes. The first and second factors explained 97.87 % of the variation (Fig. 5). The

corresponding eigenvalues were 35.1 and 7.9 for factor 1 and 2, respectively.

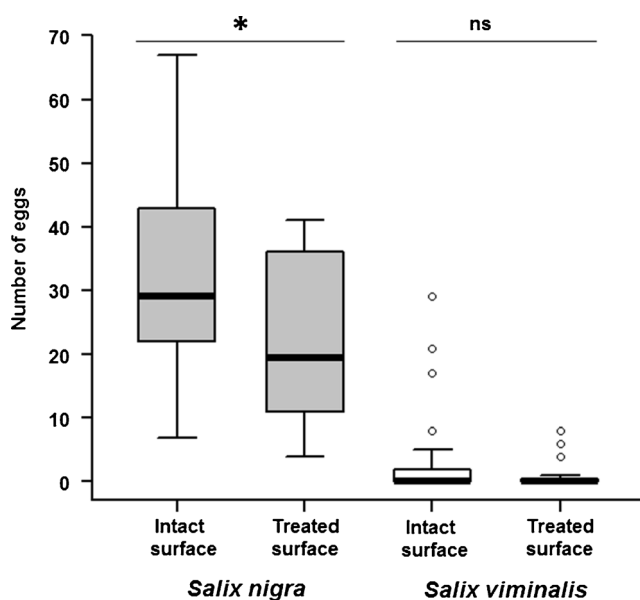


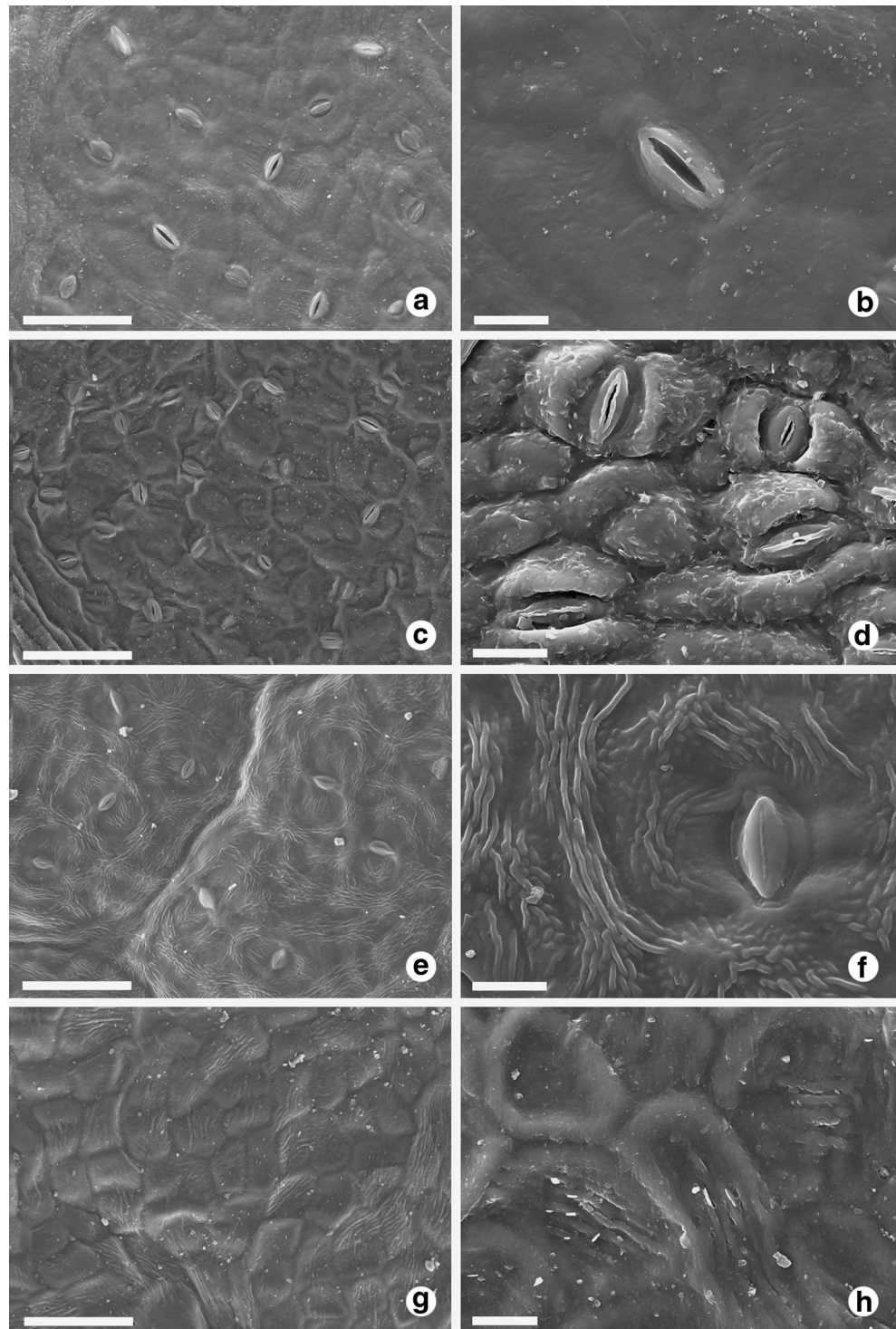
Fig. 3 Number of eggs laid by adult females on intact and treated adaxial leaf surfaces of *Salix nigra* and *Salix viminalis*. Epicuticular waxes were mechanically removed by treatment with gum arabic. Wilcoxon matched pairs test, $N = 18$ for *S. nigra* and $N = 24$ for *S. viminalis*. * indicates significant difference at $P < 0.05$, ns indicates non-significant difference

Discussion

Field surveys and laboratory experiments showed that there is a marked preference for *S. nigra* genotypes for oviposition by *N. oligospilus* females, and it becomes highly defoliated by the offspring. Our study suggests that several cues act in concert to attract females to the host plants and stimulate oviposition. Furthermore, after eclosing as adults, females are attracted to volatiles from *S. nigra*, and once alighting on the plant, they may seek specific chemical contact cues from cuticular waxes, in order to lay eggs.

Salix nigra, the most preferred genotype for oviposition, emits 3 times more volatiles than *S. viminalis*. Moreover, (*Z*) and (*E*)- β -ocimene, undecane, and decanal were significantly higher in *S. nigra*, while β -caryophyllene was more abundant in *S. viminalis*. While β -ocimene dominated the volatile blend (about 62 %) of *S. nigra*, this compound was present in only 6 % of the *S. viminalis* blend. By studying behavioral orientation, Weaver et al. (2009) suggest that (*Z*) and (*E*)- β -ocimene determine the suitability for oviposition of the

Fig. 4 SEM photographs of the leaf epidermis. **a–d** abaxial surface; **e–h** adaxial surface. **a, c, e, g**, general view. **b, d, f, h**, detail. **a, b, e, f, Salix nigra**; **c, d, g, h, Salix viminalis**. Scale bars: **a, c, e, g**, 50 μm ; **b, d, f, h**, 10,000 nm



free feeder wheat stem sawfly (*Cephus cinctus* Norton). However, the most abundant compound may not be necessary as an orientation cue: a particular ratio of compounds might be essential as previously mentioned (Bruce et al. 2005). Other volatile compounds found in our study have been identified as electroantennogram-active in sawflies. The galling sawfly (*Pontania proxima*)

responded to β -caryophyllene and α -farnesene in coupled gas chromatography-electroantennographic detection (GC-EAD) recordings (Kehl et al. 2010). Behavioral assays with individual compounds, as well as electroantennogram recordings, are now required to fully understand which of the volatile compounds are involved in the orientation of the willow sawfly.

Table 2 Compounds found in the CH₂Cl₂ - soluble fraction of the cuticular wax of *Salix nigra* and *Salix viminalis* according to leaf surface

Retention time	Compound	<i>Salix nigra</i>				<i>Salix viminalis</i>			
		Adaxial		Abaxial		Adaxial		Abaxial	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
7.80	Salicylic alcohol	tr		tr		–	–	–	–
9.96	<i>Trans</i> -cinnamic acid	tr		tr		–	–	–	–
16.20	Palmitic acid	3.31	1.90	1.68	0.94	0.63	0.37	tr	
17.19	Stearic acid	tr		tr		tr		–	–
19.05	Eicosanoic acid	tr		tr		–	–	–	–
24.60	Pentacosane	0.51	0.21	1.23	0.38	1.07	0.40	4.88	0.80
28.05	Heptacosane	13.83	2.69	27.64	1.79	30.03	0.72	50.01	1.90
31.38	Nonacosane	24.09	3.00	43.16	2.60	53.77	2.10	39.27	2.25
33.53	Probable alquene	0.50	0.24	1.92	0.67	1.62	0.94	tr	
34.50	Triacotane	1.61	0.76	7.50	3.09	4.88	0.86	1.99	0.59
35.11	Terminal alcohol	6.74	3.33	3.94	2.20	–	–	tr	
37.86	Stigmasterol/sitosterol	3.57	2.42	5.55	2.39	tr		–	–
38.46	β-amyrin	14.72	2.91	3.16	1.19	4.27	1.40	tr	
41.79	Long chain aliphatic ester	28.58	1.48	3.21	1.18	3.02	1.17	tr	

Retention time of each compound is given in min

Quantity of compounds found in the CH₂Cl₂ - soluble fraction of adaxial ($N = 7$) and abaxial ($N = 6$) cuticular waxes of *S. nigra* and *S. viminalis* is expressed as μg/μl. tr indicates traces

Once females of *N. oligospilus* guided by volatile compounds choose a plant, contact cues may play a role for

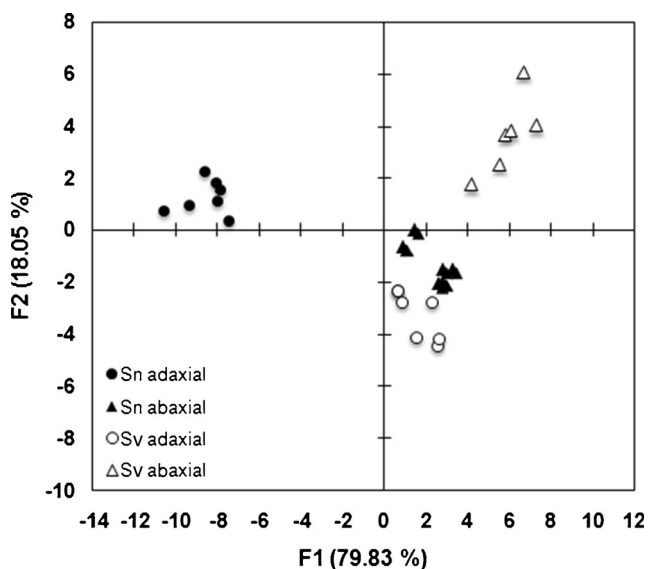


Fig. 5 Factors 1 and 2 from the discriminant analysis showing separation of genotypes and leaf surface according to cuticular compounds. Selective extraction of waxes was achieved by filling a glass cylinder with dichloromethane and pumping it. Percentage of variance explained by each axis is shown between parentheses. The data represents the datasets of both leaf surfaces of two willow genotypes: *Salix nigra* adaxial, black dots ($N = 7$), *Salix nigra* abaxial, black triangles ($N = 6$), *S. viminalis* adaxial, white dots ($N = 7$), *S. viminalis* abaxial, white triangles ($N = 6$)

oviposition. In a previous study (Braccini et al. 2013), we observed that before egg deposition, a *N. oligospilus* female walks along the leaf tapping several times over its surface with antennae. Occasionally she stops, bends her abdomen into a comma shape, touches the leaf surface, and waddles over the leaf. The female does not seem to injure tissue, so this stage of selection for egg laying may be based on her perception of plant metabolites present on the leaf surface. When she finds a suitable location, she faces down and lowers the ovipositor to lay the egg. The egg is laid inside the epidermis or between the epidermis and the adjacent chlorenchyma showing a tight association with the leaf, which reveals an intimate physical contact. This sequence of behavior reinforces the importance of contact cues to trigger egg laying behavior in *N. oligospilus*.

In our study, micromorphological analyses by SEM showed that patterns of epicuticular wax deposition were similar between leaf surfaces (adaxial/abaxial) and even between genotypes. In other studied species of *Salix*, like *S. babylonica* L., *S. babylonica* × *S. alba*, *S. matsudana* Koidz. × *S. alba*, and (*S. babylonica* × *S. humboldtiana* Willd.) × *S. matsudana*, both epidermis surfaces were differentiated clearly. In the mentioned species, conspicuous amounts of crystalline wax deposits were detected only on the abaxial leaf surface, responsible for their glaucous aspect, and trichomes were absent (Fernandez et al. 2012). Presence of conspicuous epicuticular waxes and trichomes also were observed in other species of *Salix* (Cameron et al. 2002; Szafranek et al. 2008). The

characteristics observed in other *Salix* species contrasted with a thin and smooth deposition of epicuticular waxes and absence of trichomes in mature leaf blades of *S. nigra* and *S. viminalis*, indicating that the enormous differences observed in oviposition preferences among genotypes are not related to physical barriers. Conversely, the results of this study suggest that oviposition preference may be linked to contact chemical traits, as has been proposed for other sawflies (Kolehmainen et al. 1994; Roininen et al. 1999).

Salix nigra leaves have a higher chemical diversity, including esters, alcohols, triterpenoids, acids, and plant sterols than *S. viminalis* leaves that possess less than 11 % of non-alkanes in composition. One or more of these compounds could play a role in host recognition. Long-chained alcohols, free fatty acids, and triterpenoids can be feeding stimulants for insects (Adati and Matsuda 1993; Sarkar and Barik 2015; Tamura et al. 2004), including those consuming Salicaceae (Coyle et al. 2003; Lin et al. 1998). It is noteworthy that the eggs found in *S. viminalis* are always laid on the adaxial surface, where a higher diversity of chemical compounds was detected. Moreover, contact bioassays removing epicuticular waxes showed the importance of chemicals as contact cues for oviposition. Wax surface removal by gum arabic treatment did not increase the number of eggs laid on this surface, suggesting the absence of a repellent compound. The fact that no eggs are laid on the abaxial surface of *S. viminalis* (Braccini et al. 2013) might indicate a complete lack of cues as suggested by Price et al. (2011). However, we cannot rule out the presence of deterrents since they may be more or less polar and we extracted the surface only with dichloromethane.

Cuticular lipids together with polar components may constitute the chemical signature influencing host plant recognition (Müller and Riederer 2005), and they may have a synergistic effect with other secondary metabolites to stimulate oviposition in many insect species (Morris et al. 2000; Roessingh et al. 1992; Spencer et al. 1999;). In a previous study, Braccini et al. (2013) described an association between salicin (salicylates) content and the number of eggs laid. The highest levels of salicin, a breakdown product of higher molecular weight salicylates (Lehrman et al. 2012; Ruuhola et al. 2003), was found in *S. nigra* leaves (Braccini et al. 2013). Thus, we hypothesize that cuticular wax compounds might be critical for oviposition in *N. oligospilus*, and that salicylates may play a role to determine oviposition preference, as has been found already in specialist *Euura* spp. sawflies (Kolehmainen et al. 1994; Roininen et al. 1999). However, the potential role of salicylates needs to be tested in further bioassays.

Understanding insect-plant interactions is of interest not only from an ecological and evolutionary perspective but also for the development of novel crop protection strategies. *Nematus oligospilus* is a serious pest in a region where willows are the primary agro-economic activity in the Delta

del Paraná, the most important wetland in Argentina. The population of this pest is normally monitored by yellow sticky traps and chemically controlled by synthetic pesticides. However, because of the fragility of the ecosystem, alternative management practices are needed. Strategies such as trap cropping and management based on semiochemicals have shown promise as methods of crop protection for sawflies (Morrill et al. 2001; Weaver et al. 2004). Detailed knowledge of the quantitative and qualitative properties of kairomones in plant species and cultivars is essential to define their roles in insect attraction and oviposition in a scientifically designed trap crop system (Hokkanen 1991). Thus, our study can help to improve monitoring and control of the willow sawfly by means of host kairomones. Knowledge of oviposition contact cues may help to identify susceptible genotypes. However, the possibility of managing pest insects by altering cuticular waxes must be taken with caution. Altering just this characteristic through traditional breeding or genetic engineering is possible, but it might not have the desired economic benefit because of the variety of cues important in the host acceptance process (Riederer and Müller 2006).

Acknowledgments This work is dedicated to the memory of Peter E. A. Teal, an extraordinary mentor with a contagious enthusiasm. We thank Dr. Diego Segura for his help with the Y olfactometer. We appreciate the valuable technical help of Ing. Agr. Lucas Landi during the entire study. This work was funded by means of Préstamo BID-PICT 247 from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), PNFOR 1104072 from Instituto Nacional de Tecnología Agropecuaria and SaFo S108 from Unidad para el Cambio Rural, Ministerio de Agricultura, Ganadería y Pesca de la República Argentina. We are also grateful to two anonymous reviewers who improved a previous version of this manuscript.

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