Surface Waxes as a Plant Defense Barrier Towards Grain Aphid

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The electrical penetration graph (EPG) method was used to quantify the effect of surface waxes on probing behaviour of the grain aphid *Sitobion avenae* F. (Hemiptera: Aphididae). The experiments showed that wax removal significantly affected probing behaviour of *S. avenae*. Generally, the aphids feeding on the plants without wax had a shortened non-probing (EPG-pattern np) and prolonged penetration of peripheral tissues – epidermis and mesophyll (EPG-pattern C). The EPG tests also showed that the three tested extracts of surface waxes from waxy plants RAH 122 were active as aphicides against the grain aphid.

Key words: Epicuticular wax, peripheral tissues, epidermis, mesophyll, triticale, *Sitobion avenae*, EPG, artificial diet

INTRODUCTION

The surface of primary aerial parts of terrestrial plants is covered by a cuticle, which has crucial autecological functions, but also serves as an important interface in trophic interactions (Rostàs et al., 2008; Yin et al., 2011; Wójcicka, 2013). A cuticle covers all aerial parts of higher plants, i.e., stems, leaves, petals and fruits, with the exception of stems that have undergone secondary growth. It is a continuous layer where the only gaps are the pores of the stomata. The thickness of a cuticle varies widely among different plant species and different organs of the same plant (0.02 – 200 µm). However, fossil plants which have cuticles as thick as 50 – 500 µm are known (Wiśniewska et al., 2003; Wisuthiphaet et al., 2014). To understand different ecological functions of the cuticle it is important to know its chemical and physical nature (Müller and Riederer, 2005; Sarkar et al., 2013). A plant cuticle is composed of waxes dispersed on the surface of (epicuticular) and within (intracuticular) a lipophylic polymer, often composed of cutin and/or cutan and polysaccharides such as cellulose and pectins, with the latter layer often called the cuticular matrix (Buschhaus and Jetter, 2011). Epicuticular waxes are the major components of a plant cuticle and play an important role in protecting aerial organs from damage caused by biotic and abiotic stresses (Zhang et al., 2007; Wójcicka, 2014). These "epicuticular waxes" (EW) generally form a thin, continuous film but can also be decorated with protruding microscopic crystals occurring as filaments, rods, platelets, tubes or complex dendritic structures (Buschhaus and Jetter, 2011). Plant epicuticular waxes are complex mixtures of long chain aliphatic and cyclic components including fatty acids, hydrocarbons, alcohols, aldehydes, ketones, β-iketones and esters, as well as low levels of terpenoids, sterols, flavonoids, and phenolic substances. This layer may also contain sugars, amino acids and secondary plant substances such as glucosinolates, furanocumarins and alkaloids (Eigenbrode and Espelie, 1995; Schoonhoven et al., 2005; Städler and Reifenrath, 2009; Haliński et al., 2012; Supapvanich et al., 2011). The morphology as well as the composition of EW vary widely between species or cultivars and are also affected by the plant age and certain environmental factors, such as heat, humidity and irradiance levels. This film gives the cuticle its hydrophobic character that determines the extent of wettability of the plant surface. Thus, the epicuticular wax layer prevents formation of stable, macroscopic water phases and, hence, germination of the spores of many plant pathogens. It is also a protective barrier against water loss and loss of organic

and inorganic compounds by leaching from the interior of the plant tissues. The external layer of EW may have other ecological functions including shielding of UV-B, protection against pathogen invasion (bacteria and fungi) and influencing insect behaviour by functioning as allelochemicals (Müller and Riederer, 2005; Städler and Reifenrath, 2009; Yin et al., 2011; Niemietz et al., 2009).

Feeding behaviour of piercing-sucking phytophagous insects is difficult to observe visually. Therefore, electrical monitoring, i.e. the use of the electrical penetration graph (EPG) technique forms a good and well-established alternative to visual observation. The EPG technique has been used extensively in studies of aphid feeding behaviour. The present paper reports on effects of surface waxes on the probing behaviour of *S. avenae* during 1h of EPG (electrical penetration graph) recordings.

MATERIALS AND METHODS

PLANT MATERIAL

The plants used in aphid colony maintenance and the experiments were grown in pots (9 cm in diameter) in a growth chamber at $21\pm1°C$ with a photoperiod of L16:D 8, and 70% RH. Plants of waxy triticale (RAH 122) and waxless triticale plants (RAH 366) were used for investigating the aphid-surface wax interactions. The studied triticale plants were obtained from the Institute of Plant Breeding and Acclimatization at Radzików/Błonie near Warsaw (Poland).

APHIDS

The grain aphids *Sitobion avenae* Fabricius (Hemiptera: Aphididae) used in the experiments came from a stock culture maintained at the University of Natural Sciences and Humanities in Siedlce. The colony was maintained on Tonacja (susceptible) wheat at $21\pm1°C$, L16:D 8 photoperiod, and 70% RH. Adult apterous aphids were used in the studies. The aphids were selected and starved for approximately 1 h before the beginning of EPG recording.

EXTRACTION OF EPICUTICULAR WAX

Surface waxes were isolated from 20-day old seedlings of waxy genotype RAH 122. The extraction methods differed in duration and in solvent polarity. The seedlings were immersed in dichloromethane for 5 s, chloroform for 10 s, or ethanol for 20 s. The extraction time depended on the length of time the leaves could be immersed in solvent before the solvent began to turn green, indicating that the internal leaf components, including chlorophyll, were being extracted. The obtained extracts were evaporated to dryness. Three concentrations (100, 1,000 and 10,000 $\mu g \cdot g^{-1}$) of each dry extract were prepared by dissolving the dry extract in a suitable solvent.

AGAROSE-SUCROSE DIET

The effect of extracts of surface waxes on grain aphid feeding behaviour was also investigated in vitro, using an agarose-sucrose diet. The diets were prepared by incorporating 1.25% agarose (Sigma A-0169) into a 30% sucrose solution and then adding one of the extracts of surface waxes to the obtained concentrations of 0 (control), 100, 1,000 and 10,000 $\mu g \cdot g^{-1}$. After the mixtures had been stirred, they were heated in a water bath (75°C for 30 min) and then poured into plastic rings (10 mm high and 15 mm diameter) covered with a stretched Parafilm M$^{®}$ membrane. Transparent gels formed after 1–2 min and were offered to the aphids for probing. The control treatment included only sucrose and agarose. A fresh diet was prepared just before the start of EPG recording.

ELECTRICAL PENETRATION GRAPH TESTS

Electrical penetration graphs (DC EPG) were used to monitor probing/feeding behaviour of the grain aphid, *Sitobion avenae* F. on triticale plants and agarose-sucrose gels. EPG recordings were performed inside a Faraday cage under laboratory conditions ($25\pm1°C$). Apterous adult aphids were connected to a DC electrical penetration graph (EPG) amplifier (type Giga-4) with a 2–3 cm gold wire (20 μm in diameter), and attached to the conductive silver point (Demetron, L 2027, Dermstadt, Germany). The other electrode was introduced into the soil or diet. The collected insects were starved for 1 h and then placed on the tested plants or diets. The experiments were run during 1h for 10 aphids on 10 different plants or diets. The recorded EPGs from ten aphids probing were stored on a computer hard disc, using STYLET 2.2 software and analysed in terms of number and duration of the EPG waveforms, as classified by Tjallingii (1994): np pattern (non-probing, aphids cannot start probing), C pattern (pathway phase; penetration of peripheral tissues – epidermis and mesophyll), E1 pattern (salivation into sieve elements), E2 pattern (ingestion of phloem sap) and G pattern (ingestion of xylem sap). The EPG patterns generated by the aphids feeding on plants were used to interpret the patterns generated on the diets (Sauvion et al., 2004; Cid and Fereres, 2010). In waveform np, the aphids stylet is outside the diet (analogous to the stylet being outside the plant). Waveform C indicates stylet activity in the diet (analogous to the stylet penetrating the epidermis and mesophyll). Waveform E1 indicates sali-

TABLE 1. The feeding response of apterous adult *S. avenae* was tested with and without removal of surface components.

Aphid activity (min)	Control	Plants of the wax genotype RAH 122		
		5s immersion in CH_2Cl_2	10s immersion in CH_2Cl_3	20s immersion in C_2H_5OH
Non-probing time	56.43 ± 3.09a	41.51 ± 2.30b	43.73 ± 3.54b	45.36 ± 4.19b
Time until first probing	51.44 ± 2.84a	18.75 ± 1.03c	28.21 ± 1.55bc	32.11 ± 2.00b
Time of first probing	2.59 ± 0.25c	6.83 ± 2.13b	13.02 ± 1.06a	9.66 ± 0.10ab
Number of probes	0.7 ± 0.04c	2.2 ± 0.12a	1.4 ± 0.06b	1.7 ± 0.04b
Total penetration time	3.57 ± 0.18b	18.49 ± 1.02a	16.27 ± 0.95a	14.64 ± 0.70a
Average time of probing	2.81 ± 0.16c	8.95 ± 0.37ab	14.63 ± 0.03a	11.82 ± 0.55a

Values in rows (mean ± SE) not followed by the same letters are significantly different at P≤0.05 (Kruskal-Wallis test).

vation into the diet (analogous to secreting saliva). Waveforms E2 and G represent ingesting the diet (analogous to ingestion of phloem and xylem sap).

PROBING BEHAVIOUR OF *S. AVENAE* ON TRITICALE PLANTS AND DIETS

In this experimental setup, aphid and plant or diet are made parts of an electric circuit, which is completed when the aphid inserts its stylets into the plant or diet. Weak voltage is supplied in the circuit and all changing electric properties are recorded as EPG waveforms that can be correlated with the aphids' activities and stylet position in the plant tissues or diets. The aphids were starved for 1 h prior to the experiment. Each aphid was given access to a freshly prepared plant or diet. All experiments started at 10–11 a.m.

Three independent experiments were conducted with this device. Presence of feeding deterrents/stimuli in surface extracts of the waxy triticale RAH 122 were conducted on the dichloromethane, chloroform and ethanol extracts.
1. The first experiment was conducted on RAH 122 seedlings with chemically removed surface compounds in comparison to the control (without extraction) seedlings.
2. The second test was done on young seedlings of the wax-less genotype RAH 366 which were sprayed with the previously obtained extracts of the RAH 122 and related to the control seedlings sprayed only with the used solvents.
3. The third test was done on diets with tested extracts of surface waxes from waxy plants RAH 122 (control diets contained only sucrose and agarose).

STATISTICAL ANALYSIS

The values of the EPG parameters (non-probing time, time until the first probing, time of the first probing, number of probes, total penetration time, average time of probing) were analyzed with the Kruskal-Wallis test followed by post hoc multiple comparisons of mean ranks for all groups.

RESULTS

The experiments with grain aphids *Sitobion avenae* (F.) on the waxy genotype RAH 122 showed that wax removal significantly affected probing behaviour of *S. avenae* (Table 1). Generally, the aphids feeding on the plants without wax had a shortened pattern np (non-probing, aphids cannot start probing) and prolonged pattern C (pathway phase; penetration of peripheral tissues – epidermis and mesophyll). The average time of probing also tended to be longer with removal of surface waxes and the effect was statistically significant. Compared to the control, the plants without wax also prolonged the timing of the first probe and increased the number of probes (Table 1). The plants without wax also delayed the time to the first probe. Differences in aphid probing among the plants with and without wax were clear and significant (Table 1).

The EPG tests also showed that the three tested extracts of surface waxes from waxy plants RAH 122 were active as aphicides against the grain aphid. Treating plants with dichloromethane (Table 2), chloroform (Table 3) and ethanol (Table 4) extracts of the waxy triticale RAH 122 clearly affected the values of all the probing parameters. The plants of the waxless genotype RAH 366 with tested extracts at all concentrations prolonged pattern np (non-probing, aphids cannot start probing) and shortened pattern C (pathway phase; penetration of peripheral tissues – epidermis and mesophyll). Moreover, all the extracts at all concentrations reduced the timing of the first probe and number of probes. The average time of probing and time of the first probing also tended to be shorter with tested extracts. These results were significantly lower than these obtained in apterous adults exposed to the control. The effect

TABLE 2. Effect of methylene chloride surface waxes ($\mu g \cdot g^{-1}$) on probing behaviour of *S. avenae*.

Aphid activity (min)	Control	Plants of the waxless genotype RAH 366 sprayed CH_2Cl_2 extract from RAH 122		
		100	1,000	10,000
Non-probing time	28.75 ± 1.58d	35.62 ± 2.07c	47.34 ± 1.13b	60.00 ± 0.00a
Time until first probing	8.32 ± 0.60c	12.77 ± 0.80b	31.97 ± 1.29a	0.00 ± 0.00d
Time of first probing	4.54 ± 0.30a	3.95 ± 0.12a	2.05 ± 0.06b	0.00 ± 0.00c
Number of probes	4.50 ± 0.35a	3.1 ± 0.10b	2.90 ± 0.05b	0.00 ± 0.00c
Total penetration time	31.25 ± 4.73a	24.38 ± 1.05b	12.66 ± 1.13c	0.00 ± 0.00d
Average time of probing	9.44 ± 0.82a	8.13 ± 0.25a	3.83 ± 0.21b	0.00 ± 0.00c

Values in rows (mean ± SE) not followed by the same letters are significantly different at P≤0.05 (Kruskal-Wallis test).

TABLE 3. Effect of chloroform surface waxes ($\mu g \cdot g^{-1}$) on probing behaviour of *S. avenae*.

Aphid activity (min)	Control	Plants of the waxless genotype RAH 366 sprayed CH_2Cl_3 extract from RAH 122		
		100	1,000	10,000
Non-probing time	27.85 ± 1.04d	38.47 ± 2.1cd	47.8 ± 2.04b	60.00 ± 0.00a
Time until first probing	5.20 ± 0.13b	16.50 ± 1.01a	21.62 ± 1.10a	00.00 ± 0.00c
Time of first probing	7.50 ± 0.31a	4.50 ± 0.15b	3.07 ± 0.16b	00.00 ± 0.00c
Number of probes	4.00 ± 0.25a	2.30 ± 0.02b	2.30 ± 0.03b	00.00 ± 0.00c
Total penetration time	32.15 ± 1.03a	18.75 ± 1.04b	12.20 ± 0.60b	00.00 ± 0.00c
Average time of probing	8.04 ± 0.24b	7.37 ± 0.32a	5.29 ± 0.56b	00.00 ± 0.00c

Values in rows (mean ± SE) not followed by the same letters are significantly different at P<0.05 (Kruskal-Wallis test).

TABLE 4. Effect of ethanol surface waxes ($\mu g \cdot g^{-1}$) on probing behaviour of *S. avenae*.

Aphid activity (min)	Control	Plants of the waxless genotype RAH 366 sprayed C_2H_5OH extract from RAH 122		
		100	1,000	10,000
Non-probing time	28.13 ± 1.43c	39.06 ± 2.95bc	41.23 ± 3.04b	60.00 ± 0.00a
Time until first probing	5.28 ± 0.10c	13.65 ± 1.24b	26.80 ± 1.85a	00.00 ± 0.00d
Time of first probing	5.80 ± 0.22a	4.39 ± 0.13a	0.32 ± 0.01b	00.00 ± 0.00c
Number of probes	6.00 ± 0.18a	3.90 ± 0.27b	5.00 ± 0.17ab	00.00 ± 0.00c
Total penetration time	31.87 ± 1.72a	20.93 ± 1.64b	18.77 ± 1.03b	00.00 ± 0.00c
Average time of probing	5.31 ± 0.40ab	4.80 ± 0.30a	3.75 ± 0.11b	00.00 ± 0.00c

Values in rows (mean ± SE) not followed by the same letters are significantly different at P<0.05 (Kruskal-Wallis test).

of waxes on probing behaviour increased with waxes concentration (Tabs 2, 3, 4).

The insecticidal activity of the above mentioned extracts was also tested in vitro. EPG recordings indicated that addition of the extracts of surface waxes to the agarose-sucrose diet clearly affected the probing and feeding behaviour of *S. avenae*. The duration of the waveform patterns was affected by the concentration of waxes in the diet (Tabs 5, 6, 7). Generally, the aphids feeding on the diets with a waxes concentration had a prolonged pattern np (corresponding to the stylet being outside the plant). These concentrations of waxes reduced the time spent on penetration of the diet (pattern C). Waxes at all concentrations reduced the average time of probing and number of diet penetrations. Compared to the control, waxes also delayed the timing of the first probe and increased the average time per probe. The effect of waxes on probing behaviour increased with waxes concentration. The values of

TABLE 5. Effect of methylene chloride surface waxes ($\mu g \cdot g^{-1}$) on *S. avenae* activity on an artificial diet during 1h of EPG recordings.

Aphid activity (min)	Control	Surface waxes concentrations		
		100	1,000	10,000
Non-probing time	33.69 ± 1.98b	45.03 ± 3.15ab	56.27 ± 4.29a	60.00 ± 0.00a
Time until first probing	3.58 ± 0.04b	8.70 ± 0.65a	6.73 ± 1.76a	00.00 ± 0.00c
Time of first probing	5.09 ± 0.30a	2.30 ± 0.00b	1.21 ± 0.09c	00.00 ± 0.00d
Number of probes	9.01 ± 0.68a	4.00 ± 0.26b	2.50 ± 0.05bc	00.00 ± 0.00c
Total penetration time	26.31 ± 0.90a	14.97 ± 0.89b	3.73 ± 0.10c	00.00 ± 0.00d
Average time of probing	3.11 ± 0.14a	1.74 ± 0.20b	0.69 ± 0.03c	00.00 ± 0.00d

Values in rows (mean ± SE) not followed by the same letters are significantly different at P≤0.05 (Kruskal-Wallis test).

TABLE 6. Effect of chloroform surface waxes ($\mu g \cdot g^{-1}$) on *S. avenae* activity on an artificial diet during 1h of EPG recordings.

Aphid activity (min)	Control	Surface waxes concentrations		
		100	1,000	10,000
Non-probing time	32.00 ± 1.02c	44.15 ± 4.17b	55.54 ± 3.86a	59.12 ± 5.24a
Time until first probing	3.67 ± 0.30c	26.88 ± 1.25a	5.54 ± 0.82c	9.14 ± 0.00b
Time of first probing	4.65 ± 0.08a	0.62 ± 0.01b	1.08 ± 0.02b	0.63 ± 0.01b
Number of probes	10.10 ± 1.07a	6.00 ± 0.23b	2.25 ± 0.03c	1.20 ± 0.05d
Total penetration time	28.00 ± 1.32a	15.85 ± 0.52b	5.72 ± 0.18c	0.87 ± 0.02d
Average time of probing	3.02 ± 0.60a	2.64 ± 0.05b	1.58 ± 0.04c	0.34 ± 0.01d

Values in rows (mean ± SE) not followed by the same letters are significantly different at P≤0.05 (Kruskal-Wallis test).

TABLE 7. Effect of ethanol surface waxes ($\mu g \cdot g^{-1}$) on *S. avenae* activity on an artificial diet during 1h of EPG recordings.

Aphid activity (min)	Control	Surface waxes concentrations		
		100 ppm	1,000 ppm	10,000 ppm
Non-probing time	34.11 ± 3.17c	41.83 ± 3.15b	59.10 ± 4.05a	59.90 ± 3.10a
Time until first probing	4.05 ±0.20ab	2.29 ± 0.03b	6.10 ± 0.28a	5.00 ± 0.00a
Time of first probing	5.13 ± 0.19a	3.33 ± 0.01b	0.12 ± 0.01c	0.10 ± 0.00c
Number of probes	9.30 ± 0.99a	3.00 ± 0.00b	0.70 ± 0.05c	0.60 ± 0.05c
Total penetration time	25.89 ± 1.17a	18.17 ± 1.30b	0.90 ± 0.03c	0.10 ± 0.01d
Average time of probing	2.97 ± 0.96a	1.03 ± 0.04b	0.40 ± 0.02bc	0.23 ± 0.03c

Values in rows (mean v SE) not followed by the same letters are significantly different at P≤0.05 (Kruskal-Wallis test).

all the studied parameters (without non-probing time and time to the first probing) were reduced by higher concentrations of waxes (Tabs 5, 6, 7).

DISCUSSION

Almost all land ecosystems have been strongly shaped by interactions between plants and insects. Plants are attacked by many different herbivores. Some will consume whole leaves or roots, while others will attack specific types of tissue. For example, piercing-sucking herbivores may feed on sap of xylem, phloem or other plant cells (Shepherd et al., 1999; Bonnemain, 2010). After an aphid lands on a plant, various cues on the surface of plants, such as epicuticular wax structure and chemical composition, can influence aphid behaviour (Powell et al., 1999; Wójcicka, 2013). The experiments with grain aphids on the waxy genotype RAH 122 showed that wax removal significantly affected probing behaviour of *S. avenae*. Although since the 1960s epicuticular wax has been reported to affect insect-plant interactions (Thompson, 1963; Way and Murdie, 1965), the problem has not been investigated thoroughly until recently (Espelie at al., 1991; Eigenbrode and

Espelie, 1995; Nam and Hardie, 2012; Mukhtar at al., 2014). Previous reports on epicuticular wax-aphid interactions have shown that the leaf waxiness had either positive, negative or no effects on aphid biology. Glossy genotypes of *Brassica* are more susceptible to the green peach aphid, *Myzus persicae* (Sulzer), but more resistant to the cabbage aphid, *Brevicoryne brassicae* (L.) when compared to waxy *Brassica* genotypes (Thompson, 1963; Way and Murdie, 1965; Stoner, 1990). While epicuticular waxes of the wheat are attractive to adult oviposition of the Hessian fly *Mayetiola destructor* (Say) (Foster and Harris, 1992), leaf epicuticular waxes negatively affected both the neonate larval movement and development of the fall armyworm *Spodoptera frugiperda* (J. E. Smith) on the corn *Zea mays* L. (Östrand et al., 2008). Moreover, examples of epicuticular wax – herbivore interactions have been identified and characterized in *Eucalyptus globulus* Labill (Brennan and Weinbaum, 2001), *Hordeum vulgare* L. (Tsumuki et al., 1989), *Sorghum bicolor* (L.) Moench (Nwanze et al., 1992) and *Triticum aestivum* L. (Lowe et al., 1985). Most studies on plant-insect interactions have focused on chemical composition of surface waxes (Sarkar et al., 2013). There are many examples of negative associations between surface waxes and insects. Increased surface wax levels have been correlated with resistance of the cabbage (*Brassica oleracea* L.) to the aphid *Bravicoryne brassicae* L., of the sorghum (*Sorghum bicolor* L.) to the green bug *Schizaphis graminum* (Rondani), of the winter wheat (*Triticum aestivum* L.) to the English grain aphid *Sitobion avenae* (F.) (Shepherd et al., 1999). For example, epicuticular waxes have been well studied in *Brassica* crops, and the evidence has shown that wax blooms on glaucous surfaces reduce adult and larval feeding by some herbivores (Eigenbrode and Espelie, 1995; Eigenbrode et al., 2000). However, cereal resistance to *D. noxia* was minimally influenced by the removal of cereal leaf epicuticular wax with other aphid-resistant traits of the plants (e.g., allelochemicals and leaf surtoughness). Therefore, the issue needs to be further investigated (Ni et al., 1998).

The EPG tests showed that the aphids feeding on the plants without wax had a shortened pattern np and prolonged pattern C. The degree of acceptance of the studied genotypes was strongly related to the epicuticular wax layer of the plants. The results were similar as those reported by others. For example, removal of the surface waxes with chloroform from seedlings of *Sorghum bicolor* (L.) caused their acceptance by nymphs of *Locusta migratoria* L. (Woodhead, 1983). Hexane extracts of surface lipids from resistant rice cultivars deterred feeding of the brown planthopper, *Nilaparvata lungens* (Stäl) (Woodhead and Padgham, 1988). Shepherd et al. (1999) reported that the preference of raspberry

aphids (*Amphorophora idaei* Börner) for older leaves of raspberry genotype Jawel may be related to lower wax coverage on these leaves relative to the younger emerging leaves. This type of preference has previously been shown by spotted alfalfa aphids, *Therioaphis maculata* (Buckton), in the foliar canopy of the alfalfa (*Medicago sativa* L.). Host acceptance by sucking insects starts with the first labial contact with the plant surface, followed by stylet penetration through successive tissue layers between the epidermis and the vascular tissues and finally feeding (Lei et al., 2001). The 'time to the first probe' can be considered as the insects' evaluation of subepidermal tissues (Troncoso et al., 2005). This is important, as epicuticular wax composition and thickness of the epidermal cuticle can influence the host plant's acceptance (Lei et al., 2001). A longer period of time spent on a plant before probing suggests adverse effects of the plant exterior on the insect (Lei et al., 1997; Gabryś and Tjallingii, 2002; Sandanayaka et al., 2013). Hence, the deterrent effects of the epicuticular waxes are reflected by longer non-probing periods. The acceptance or rejection of plant species for insect feeding is one assessment of host range (Troncoso et al., 2005) and for sap-sucking insects stylet penetration is a key factor (Prado and Tjallingii, 1997). The parameters describing aphid behaviour during probing and feeding, such as total time of probing, number of probes, average time of probing and duration of the first probing are good indicators of plant suitability or interference of probing by chemical or physical factors in a particular plant surface. However, of the different EPG parameters, the time to the first probe and duration of the first probing measured the initial response to the plant. Brennan and Weinbaum (2001) showed that the epicuticular wax on juvenile leaves reduced stylet probing by *Ctenarytaina spatulata* and *C. brimblecombei*. Moreover, epicuticular wax on juvenile leaves of *Eucalyptus globulus* plays a primary role in resistance to *C. spatulata* and *C. brimblecombei*, because these species survived longer and settled more often on 'de-waxed' than on 'waxy' juvenile leaves. Therefore, the scarcity of *C. spatulata* and *C. brimblecombei* stylet tracks in 'waxy' juvenile leaves suggests that their relatively poor survival may have been due to starvation (Brennan and Weinbaum, 2001). Ni et al., (1998) showed that aphids produced significantly greater number of probes on oat than barley leaves, irrespective of wax removal.

The layer of the epicuticular waxes may contain aliphatic components, sugars and amino acids (Eigenbrode and Espelie, 1995; Niemietz et al., 2009; Yin et al., 2011; Haliński et al., 2012), as well as secondary metabolites (Schoonhoven et al., 2005; Städler and Reifenrath, 2009; Supapvanich et al., 2011). The roles of primary and secondary com-

pounds on aphid feeding and probing behaviour have been studied for decades. For instance, it has been reported that aphid feeding and probing behaviour can be affected by primary compounds such as sucrose (Pescod et al., 2007), and secondary compounds such as glycosides (Takemura et al., 2006), glucosinolates (Kim and Jander, 2007; Nam and Hardie, 2012) and phenolic substances (Wójcicka, 2010; Lahtinen et al., 2006). Jones et al. (2002) and Rapley et al. (2004) suggested that benzyl *n*-tetracosanoate in the epicuticular waxes of *E. globulus* was a biologically active component responsible for the repellence of oviposition by female *M. privata*. It should be noted that aromatics and triterpenoids may also function as anti-feedants to smaller organisms or as chemical signalling compounds for those herbivores that probe into the plant surface (Eigenbrode and Espelie, 1995; Buschhaus and Jetter, 2011). Differences in wax chemistry may modulate ecological interactions (Rostás et al., 2008; Yin et al., 2011). Feeding and reproduction follow selection of a suitable host. In the absence of the appropriate stimuli, the sequence may be interrupted at any stage, and characteristic behaviour on non-host plants includes increased periods of walking relative to probing, and ultimately the departure of the insect (Shepherd et al., 1999; Polletier and Giguère, 2009; Nam and Hardie, 2012). Plant acceptability can be accessed from probing (physical penetration of the plant) and sap-ingesting periods (Tjallingii, 1993). However, several recent studies on the reproduction of *Acyrthosiphon pisum* and *Aphis fabae* (Caillaud and Via, 2000; Powell and Hardie, 2001; Del Campo et al., 2003) indicate that the chemicals used as parturition stimulant by aphids may be detected in peripheral plant tissues before the contact with the phloem, and lead to initiation of reproduction before sustained ingestion of the phloem. It suggests that host acceptance by aphids may be independent of phloem feeding (Nam and Hardie, 2012; Sandanayka et al., 2013). Nam and Hardie (2012) indicated that, for winged morphs of *R. padi*, the decision to reproduce on host plants may be made in the early stages of the probing process before sustained phloem contact. Caillaud and Via (2000) demonstrated that after a brief probe of the plant tissues, two biotypes of *A. pisum*, which utilize different plants as hosts, abandon non-host plants or feed and settle on their host plant. Tosh et al. (2002) also showed that winged virginoparae and gynoparae of *A. fabae* initiated larviposition before sustained phloem contact on their host plants. All these results show that the chemical cues used as parturition stimulants or sign stimuli indicating a suitable host may be located in the surface waxes and peripheral tissues rather than in phloem sieve elements, and detected early in aphid host-selection process and affect the aphid host-acceptance behaviour.

Frazier and Chyb (1995) suggested that insect feeding can by inhibited by three kinds of effects that occur at different stages of the insect-plant interactions: preingestional, ingestional and postingestional effects. Because aphid-probing behaviour cannot be observed directly, the parameters from EPG recordings are used to quantify the effect of the surface waxes on the probing behaviour of the grain aphid *S. avenae*. In this study, waxes deterred aphid probing and feeding. Overal, these data suggest that waxy surface acts as an antifeedant.

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