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Effect of epicuticular waxes from triticale on the feeding behaviour and mortality of the grain aphid, *Sitobion avenae* (Fabricius) (Hemiptera: Aphididae)

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Abstract: Surface waxes from wax-covered triticale plants (RAH 122) were sprayed on plants of the waxless genotype RAH 366 or the surface waxes were used to make artificial diet preparations. The results were significant increases in the mortality of apterous adults of the grain aphid *Sitobion avenae* (Fabricius) (Hemiptera: Aphididae) at all concentrations tested in comparison with those aphids which fed on the control plants or aphids which were reared on the diets. In the choice tests, most aphids settled on plants without surface waxes or on diet preparations which did not have surface waxes (the controls). When the concentration of the surface waxes was increased on one of the plants or surface waxes were increased in the diet preparation, the number of wandering aphids increased. Those aphids which did not wander were mainly on the waxless control plants or on the waxless diet preparations. Aphids did settle on those plants or on the diet preparations which had 100 and 1,000 $\mu g \cdot g^{-1}$ of surface wax. The aphids rarely settled on the diet preparations containing 10,000 $\mu g \cdot g^{-1}$ of surface waxes. From these observations it appears that surface waxes can act as a feeding deterrent. Since aphids on plants with surface waxes, or aphids which settled on diet preparations with surface waxes, started to die earlier than aphids fed only the control plants or the control diet preparations, it is possible that the surface waxes had a toxic effect that led to early mortality. Thus, it can be said that the surface waxes caused feeding deterrence and had a toxic effect on the aphids.

Key words: feeding deterrent, waxes, winter triticale, *Sitobion avenae*

Introduction

In an agroecosystem, the physical and chemical characteristics of plants play important roles in controlling pests and protecting natural enemies (Sas-Piotrowska *et al*. 2005; Wilkaniec *et al*. 2015). The aerial, primary cuticles of terrestrial plants are covered with a mixture of lipophilic compounds typically termed plant surface waxes (Eigenbrode *et al*. 2000). These waxes are embedded in the polymer matrix (intracuticular) and also deposited on the surface (epicuticular waxes) (Buschhaus and Jetter 2011). The epicuticular wax layer is the interface between the atmosphere and primary plant tissues. For many plant systems, this layer differs in chemical composition from the bulk cuticular wax (Wiśniewska *et al*. 2003). Micromorphologies of epicuticular waxes range from thin films to thick crusts on which three-dimensional wax crystals are often superimposed (Buschhaus and Jetter 2011). In addition to providing a barrier against water loss, plant surface waxes have other ecological effects, including influencing insect-plant interaction (Eigenbrode *et al*. 2000). 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; Städler and Reifenrath 2009; Supapvanich *et al*. 2011; Haliński *et al*. 2012; Razeq *et al*. 2014). Secondary substances include phenolics; especially o-dihydroxyphenols, glucosinolates, alkaloids, and furanocumarins, which are known as plant protection agents against various species of insects (Wójcicka 2010a). Wax constituents act as allelochemicals by influencing insect development and insect behaviour (Wójcicka 2014).

The population parameters such as mortality and feeding behaviour of aphids and other insects are the most important parameters in classifying the resistance of triticale varieties. In this paper, it is reported that surface waxes from triticale cause feeding deterrence and may be toxic to aphids.

Materials and Methods

Plant material

The plants used for aphid colony maintenance and used in the experiments, were grown in pots (9 cm in diameter) in

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a growth chamber at 21±1°C with a photoperiod of 16 : 8 h (L : D), and 70% relative humidity (RH). Plants of waxy triticale (RAH 122) and waxless triticale (RAH 366) were used for investigating the aphid and surface-wax interactions. The studied triticale plants were obtained from the Institute of Plant Breeding and Acclimatisation, located in the village of Radzików, (Błonie district), near Warsaw, Poland.

Aphids

The grain aphid, *Sitobion avenae* (Fabricius), used in the experiments, came from a stock culture maintained at the University of Natural Sciences and Humanities, Siedlce, Poland. The colony was maintained on Tonacja (susceptible) wheat at 21±1°C, 16 : 8 h (L : D) photoperiod, and 70% RH. Adult apterous aphids were used for the experiments.

Extraction of epicuticular wax

Surface waxes were removed from plants of the waxy genotype RAH 122. The removal was done by immersing the flag leaves and 20-day old seedlings in 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. When the solvent turned green it meant 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 μg · g^{-1}) of dry extract were prepared by dissolving the dry extract in a suitable solvent.

Agarose-sucrose diet

The effect of the extracts of the surface waxes on the mortality and feeding-behaviour of the grain-aphid was also investigated *in vitro*, using an agarose-sucrose diet. The diets were prepared by adding 1.25% agarose (Sigma A-0169) to a 30% sucrose solution and then adding one of the extracts of surface waxes to the obtained concentrations of 0 (the control), 100, 1,000 and 10,000 μg · g^{-1}. The control treatment included only sucrose and agarose.

Effect of the surface waxes on aphids

The presence of feeding deterrents/stimuli in surface extracts of the waxy triticale RAH 122 were conducted on the ethanol extracts. The plants of the waxless genotype RAH 366 and the diets preparations were sprayed with a definite solution. Apterous, partenogenetic, grain-aphid adults were placed on flag leaves, and on 20-day old seedlings, and on the diet substance, at a rate of one aphid per plant or diet substance. A fine-haired brush was used to move the aphids. Isolated plants and the diet substances were placed in plastic cylinders (30 × 15 × 15 cm) in an environmental chamber (21±1°C, 70% RH, 16 h photoperiod). Entomological observations were carried out on these isolated plants and diets. The experiments were run for 72 h for 20 aphids on 20 different plants or diets substances.

Mortality of aphids feeding on plants or diets with different concentrations of surface waxes

In three independent experiments, the mortality of aphids was recorded after 24, 48, and 72 h intervals. The first experiment involved spraying the flag leaves of the waxless genotype RAH 366 with the previously obtained extracts of RAH 122 and related to the control flag leaves sprayed only with the used solvent. The second test was done on young seedlings of the waxless genotype RAH 366. The third test was done on diet preparations.

Feeding behaviour and mortality of aphids exposed to diet preparations or plants with and without surface waxes

To determine if the surface waxes acted as a feeding deterrent, the feeding behaviour of aphids was examined in a series of tests. The aphids were given a choice between two plants or two diets, one without surface waxes and other with or without (the control experiment). The first experiment was conducted on flag leaves of the waxless genotype RAH 366. The experiment involved the following: 100 μg · g^{-1} of surface waxes *vs*. control flag leaves; 1,000 μg · g^{-1} surface waxes *vs*. control flag leaves; 10,000 μg · g^{-1} surface waxes *vs*. control flag leaves. The second test was done on young seedlings of the waxless genotype RAH 366. The third test was done on the diet substances. The feeding behaviour and mortality was followed for 72 h.

Statistics

Differences in aphid mortality after being on the treated and untreated triticale plants or diet substances, were subjected to the two-tailed unpaired Student's t-test.

Results

The entomological tests showed that plants of the waxless genotype RAH 366 treated with the extract of surface waxes, shortened the survival of aphids. Treating plants with an ethanol extract of the waxy triticale RAH 122, clearly affected the values of mortality of those aphids reared on the 100, 1,000 and 10,000 μg · g^{-1} of surface waxes. Using surface waxes on the flag leaves resulted in significant increases in the mortality of aphids at all concentrations tested, when compared with those aphids which fed on the control plants (Table 1). A similar, clear trend was observed for the seedlings. These results were significantly lower than the results obtained for apterous adults exposed to the control treatment (Table 2). The insecticidal activity of the above-mentioned extract was also tested on the artificial diet preparations. Entomological tests indicated that the addition of the extract of surface waxes to the agarose-sucrose diet clearly affected the mortality of *S. avenae* (Table 3). Differences in aphid mortality for aphids fed the plants or diets with and without wax, were clear and significant. Compared to the control, waxes at all concentrations shortened the survival of the aphids after 24, 48, and 72 h. The choice tests showed that the tested extract of surface waxes from the waxy RAH 122 plant, was a feeding deterrent. In the choice tests, when plants (Figs. 1 and 2)

Table 1. Mortality (%) of aphids feeding on flag leaves with different concentrations of ethanol surface waxes ($\mu g \cdot g^{-1}$)

Time [h]	Flag leaves of the waxless genotype RAH 366 sprayed with the C_2H_5OH extract from RAH 122			
	the control	100	1,000	10,000
24	5.0±0.00	26.0±0.10*	36.0±0.20*	52.0±0.17*
48	10.0±0.11	35.0±0.18*	42.0±0.00*	66.0±0.10*
72	10.0±0.00	48.0±0.21*	54.0±0.00*	74.0±0.00*

Asterisked values differ significantly from the control values: *$p < 0.001$ (Student's t-test)

Table 2. Mortality (%) of aphids feeding on seedlings with different concentrations of ethanol surface waxes ($\mu g \cdot g^{-1}$)

Time [h]	Seedlings of the waxless genotype RAH 366 sprayed with the C_2H_5OH extract from RAH 122			
	the control	100	1,000	10,000
24	10.0±0.16	32.0±0.00*	46.0±0.28*	65.0±0.00*
48	20.0±0.23	40.0±0.11*	48.0±0.18*	70.0±0.16*
72	20.0±0.00	46.0±0.00*	64.0±0.23*	92.0±0.17*

Asterisked values differ significantly from the control values: *$p < 0.001$ (Student's t-test)

Table 3. Mortality (%) of aphids feeding on artificial diets with different concentrations of ethanol surface waxes ($\mu g \cdot g^{-1}$)

Time [h]	Concentrations of the surface waxes			
	the control	100	1,000	10,000
24	10.0±0.21	35.0±0.14*	50.2±0.27*	60.0±0.00*
48	20.0±0.00	50.4±0.14*	64.3±0.00*	72.0±0.00*
72	20.0±0.11	56.2±0.00*	78.0±0.16*	90.0±0.23*

Asterisked values differ significantly from the control values: *$p < 0.001$ (Student's t-test)

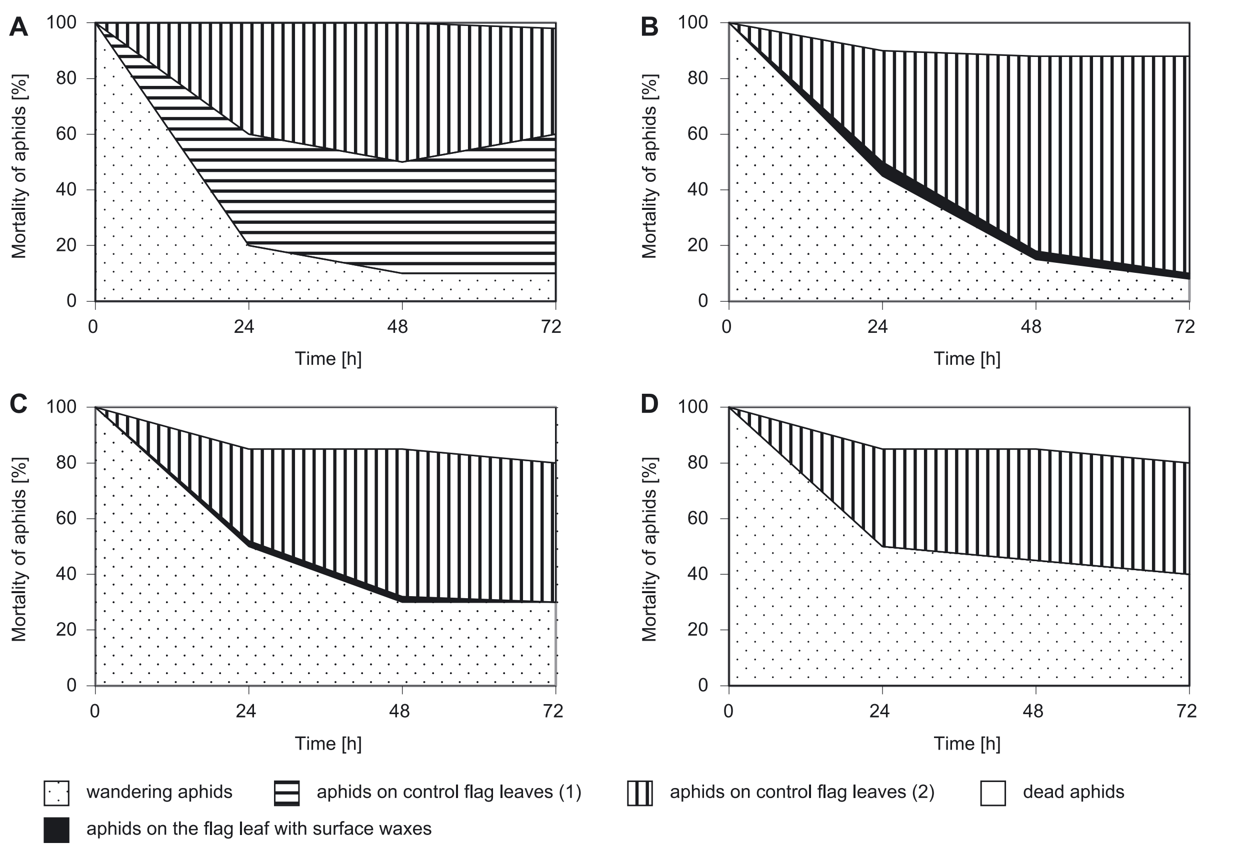

Fig. 1. Feeding behaviour and mortality of aphids exposed to the alternative of flag leaves with and without surface waxes from waxy RAH 122 plants: A – the control plant *vs*. the control plant; B – 100 $\mu g \cdot g^{-1}$ surface waxes *vs*. the control plant; C – 1,000 $\mu g \cdot g^{-1}$ surface waxes *vs*. the control plant; D – 10,000 $\mu g \cdot g^{-1}$ surface waxes *vs*. the control plant

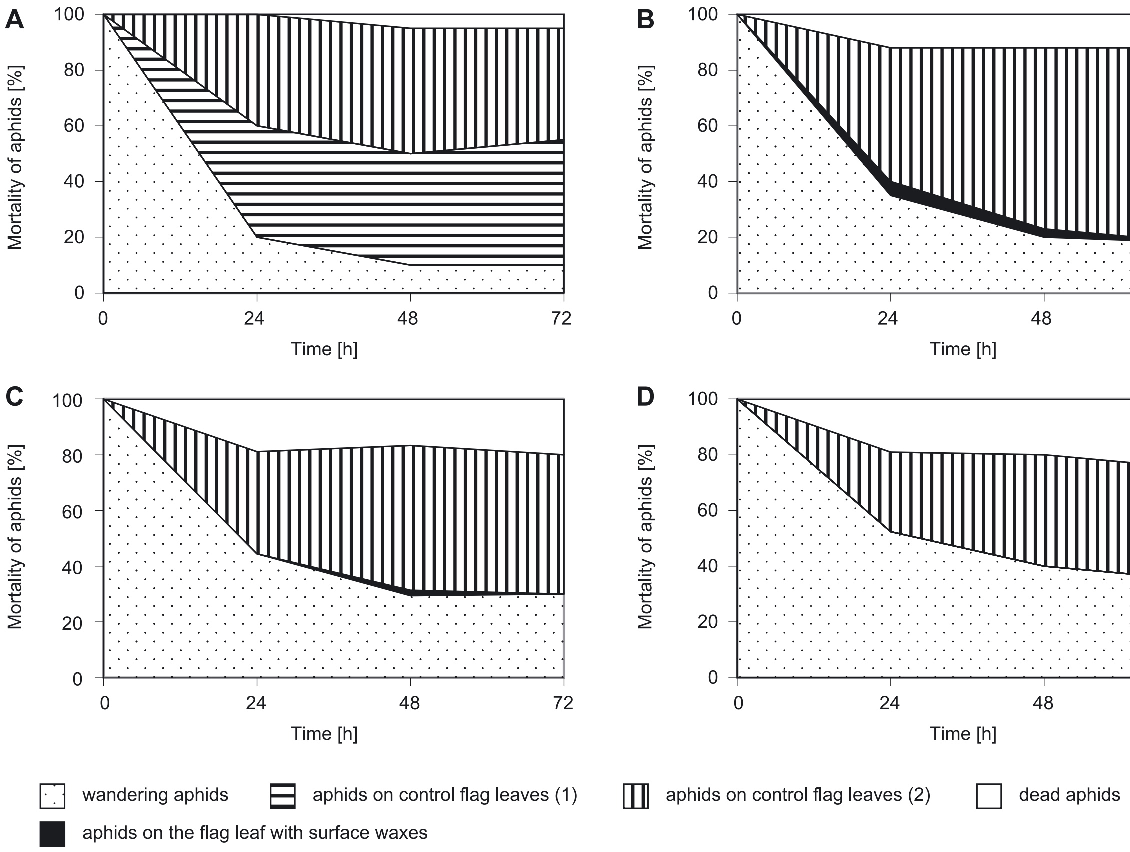

Fig. 2. Feeding behaviour and mortality of aphids exposed to the alternative of seedlings with and without surface waxes from waxy RAH 122 plants: A – the control plant *vs*. the control plant; B – 100 μg · g^{-1} surface waxes *vs*. the control plant; C – 1,000 μg · g^{-1} surface waxes *vs*. the control plant; D – 10,000 μg · g^{-1} surface waxes *vs*. the control plant

or diet substances (Fig. 3) were controls (viz., without surface waxes) most aphids settled on them. When the concentration of surface waxes was increased in one of the plants or diet preparations, the number of wandering aphids increased. Those aphids which did not wander were mainly settled on the control plants or diet preparations. Aphids did settle on the diet substances which had 100 and 1,000 μg · g^{-1} of surface waxes, but rarely settled on the plant or diet substances containing 10,000 μg · g^{-1} of surface waxes (Figs. 1, 2, and 3).

Discussion

In nature, plants use many strategies to protect themselves against insects. Various cues on the surface of plants, such as the epicuticular wax structure and chemical composition, can influence aphid behaviour and physiology (Powell *et al*. 1999; Wójcicka 2013). Experiments with grain aphids on the waxy and waxless genotype showed that wax significantly affected the mortality and feeding behaviour of *S. avenae*. The epicuticular wax of plants has been reported to affect a variety of insect herbivores, including both chewing and piercing-sucking insects. There are many studies on *Brassica*, corn (*Zea mays* L.), and other crop plant epicuticular waxes, and their interactions with insect pests (Espelie *et al*. 1991; Bodnaryk 1992; Yang *et al*. 1993; Eigenbrode and Espelie 1995; Ni *et al*. 1998). Differences in wax chemistry may modulate ecological interactions (Rostás *et al*. 2008; Yin *et al*. 2011). The differences in wax chemistry seriously affect aphid behaviour, physiology, and metabolism and as a result reduce aphid populations on resistant plants, and also negatively influence aphid development, prolong aphid growth, reduce fecundity, and lower the intrinsic rate of natural increase in insect populations (Wójcicka 2013). The performed experiments demonstrated that surface waxes caused feeding deterrence and were toxic to aphids. The current studies suggest that surface waxes may have the potential to be triticale resistance factors toward the grain aphid. A number of studies provided evidence that in some cases the insect-plant interaction might be influenced by the presence or absence of surface waxes (Eigenbrode and Espelie 1995). Most studies on plant-insect interactions have focused on the chemical composition of surface waxes (Sarkar *et al*. 2013). The layer of the epicuticular waxes may contain aliphatic components, sugars, and amino acids (Eigenbrode and Espelie 1995; Athukorala and Mazza 2010; Yin *et al*. 2011; Haliński *et al*. 2012) as well as secondary metabolites with such properties as: toxicity, growth regulation, antifeedance, and deterrence, which influence behaviour and reduce insect fecundity (Ji and Jetter 2008; Städler and Reifenrath 2009; Supapvanich *et al*. 2011). There are many examples of negative associations between the surface waxes and insects. Wójcicka *et al*. (2010b) found a negative influence of surface waxes on the grain aphid population. Moreover, extracts from the RAH 122 genotype applied on the waxless genotype RAH 366, significantly decreased the number of alate

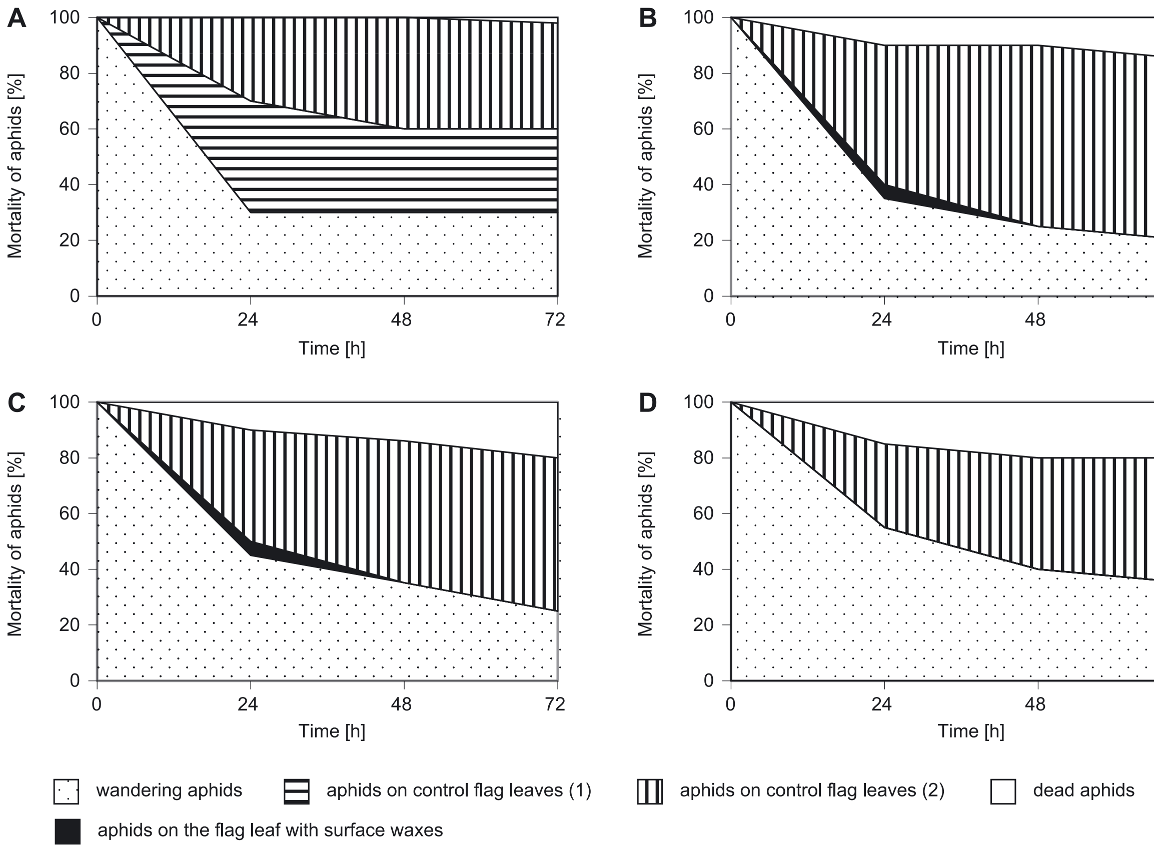

Fig. 3. Feeding behaviour and mortality of aphids exposed to the alternative of diets with and without surface waxes from waxy RAH 122 plants: A – the control diet *vs*. the control diet; B – 100 μg · g^{-1} surface waxes *vs*. the control diet; C – 1,000 μg · g^{-1} surface waxes *vs*. the control diet; D – 10,000 μg · g^{-1} surface waxes *vs*. the control diet

forms, and the percentage of plants infested with cereal aphids significantly decreased (Wójcicka 2010c). Steinbauer *et al*. (2004) have suggested that waxes provide host assessment/acceptance cues for female *Mnesampela privata* (Guenee, 1858). When female *M. privata* mistakenly oviposit on *Eucalyptus melliodora* (A. Cunn. ex Schauer) or *E. sideroxylon* (A. Cunn. ex Woolls) (both of which produce waxy leaves), the *M. privata* larvae suffer high neonate mortality. Steinbauer and Matsuki (2004) suggested that the high neonate mortality of these two species when on trees, was because of the high concentrations of sideroxylonal (a toxic plant secondary metabolite). Hence, the deterrent effects of the epicuticular waxes are reflected by longer non-probing periods (Wójcicka 2015a). Wójcicka (2015b) also showed that the surface waxes from the waxy genotype (RAH 122) used in the artificial diets, completely stopped salivation (pattern E1) as well as passive and active ingestion (pattern E2 and pattern G). The onset of stylet activity (EPG pattern C) was also delayed.

From these observations it appears that surface waxes can act as a feeding deterrent. Since aphids settling on plants or diet substances with surface waxes, started to die earlier than aphids fed control plants or diet preparations only, it is possible that the surface waxes had a toxic effect that led to early mortality. Thus, it appears that surface waxes cause feeding deterrence and have a toxic effect on aphids.

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