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ENANTIOSPECIFIC EFFECT OF LIMONENE AND LIMONENE-DERIVED BICYCLIC LACTONES ON SETTLING AND PROBING BEHAVIOUR OF THE PEACH POTATO APHID *MYZUS PERSICAE* (SULZ.)

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Abstract: The deterrent effect of limonene enentiomers and limonene derivative: (+)-(1S,4S,6S)-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one, on *Myzus persicae* was investigated. Only S-enantiomers of limonene and its derivative showed feeding deterrent properties. S-limonene inhibited phloem sap ingestion and reduced the number of phloem sap ingestion phases during aphid stylet penetration of plant tissues. Aphids spent twice less time on leaves painted with (+)-1S,4S,6S-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one than on control and S-limonene-treated leaves and the probes were shorter than 2 minutes on these leaves. Our studies confirmed that the chiral centre configuration of the lactones was important in expression of feeding deterrent activity.

Key words: antifeedants, aphid-plant relationships, EPG, aphid probing behaviour, plant protection

INTRODUCTION

Plants infested by aphids may be affected directly, mainly because of the fluid and nutrient removal, and indirectly, by virus transmission. The average yearly world crop loss due to aphids is estimated at least 2% of all losses on account of insect feeding (Wellings et al. 1989). The peach-potato aphid *Myzus persicae* (Sulz.) is

one of the most noxious species. According to Blackman and Eastop (1985), *M. persicae* can infest plants of over 40 different families including many economically important ones world wide and it is able to transmit over 100 plant viruses.

In the present day, the most important means for crop protection against insect feeding are broad-spectrum chemical pesticides. Considering various negative effects of their application there is an increasing demand for more specific, indirectly acting crop protection agents, such as repellents, insect-growth regulators, oviposition inhibitors, and antifeedants, which might, at least in part, replace conventional insecticides (Schoonhoven 1982; van Beek and de Groot 1986; Ley and Toogood 1990). Of the behaviour-controlling chemicals, insect antifeedants have attracted a lot of research in the recent years, and the most interesting discoveries included the terpenoids of plant origin: ajugarin, azadirachtin, and polygodial. The sesquiterpenoid polygodial was successfully applied in the field against *Rhopalosiphum padi*. The use of this antifeedant gave similar results to those obtained with the broad-spectrum pyrethroid cypermethrin (Pickett et al. 1994). In the laboratory, apterous adult *M. persicae* walked off the leaf treated with polygodial (Powell et al. 1993). From the practical point of view, the use of plant-derived antifeedants on a large scale is not cost-effective. Synthetic analogues of natural compounds are more accessible for application.

Usually, an antifeedant is expected to inhibit insect feeding through the contact with its taste receptors. In this respect aphids differ from chewing insects because their mouthparts lack external contact chemoreceptors (Wensler and Filshie 1969) and the ingestion of sap from sieve elements is crucial for the recognition and acceptance of the host plant (Harrewijn 1990). The phloem elements are a major source of food for aphids. However, during stylet penetration towards the vascular tissue, aphids ingest small sap samples from parenchyma cells for gustatory purposes (Martin et al. 1997). Contact chemoreceptors on the tips of antennae may also play a role in host plant recognition: these receptors were found responsible for the detection of polygodial on plant surface by *M. persicae* (Powell et al. 1995). Due to their specific way of feeding aphids are good tools for studying the tissular localisation of deterrent factors (Gabryś and Pawluk 1999). Thus, it is possible to evaluate the capability of artificially applied substances to enter parenchyma and vascular tissues through plant surface. An antifeedant, especially the one targeted at aphids, should possess these qualities (Chapman 1974).

In our laboratory, we synthesised a number of terpenoid lactones and those with S-limonene system had antifeedant activity against storage pest insects comparable to that of azadirachtin. We found that the chiral centre configuration of the lactones was important in expression of biological activity and the effect of different enentiomers was species-specific. One of these lactones, 1S,4S,6S – enantiomer of the bicyclic lactone (+)-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one (1a, Fig. 1) had deterrent properties towards *M. persicae*. Its enantiomer 1R,4R,6R (1b, Fig. 1) did not show such activity although it was a potent antifeedant to storage pests (Wawrzeńczyk et al. 1998; Paruch et al. 2001).

In the present study we investigated the effect of chiral structure of limonene, the naturally occurring precursor of (–)-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]

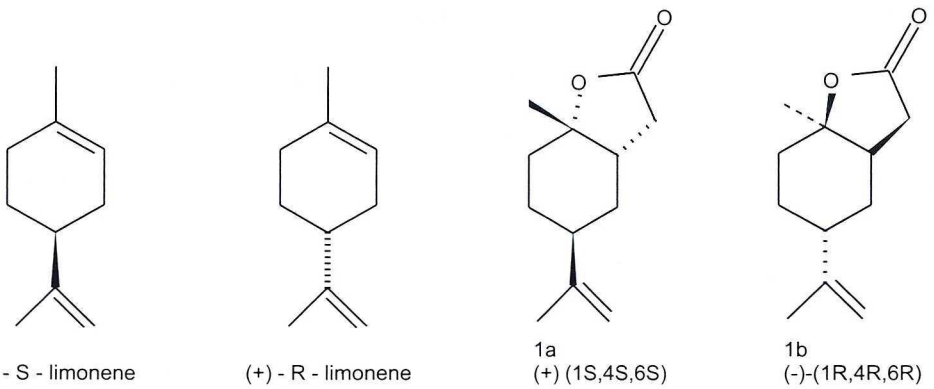

Fig. 1. Chemical structures of S-limonene, R-limonene, (+)-(1S,4S,6S) – enantiomer of the bicyclic lactone 4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one (**1a**), and (-)-1R,4R,6R – enantiomer of the bicyclic lactone 4-(1-methylethenyl)-9-oxabicyclo [4.3.0] nonan-8-one (1b)

nonan-8-one on aphid settling. We also studied behavioural responses of *M. persicae* to limonene and (–)-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one to reveal the biological background of their deterrent activity.

MATERIALS AND METHODS

Compounds: Enantiomeric lactones (+)-1S,4S,6S-4-(1-methylethenyl)-9-oxabicyclo [4.3.0] nonan–8-one and (–)-1R,4R,6R-4-(1-methylethenyl)-9-oxabicyclo [4.3.0] nonan-8-one were obtained in four-step synthesis from *S* and *R* isomers of perillyl alcohol, respectively (Paruch et al. 2000). Both starting compounds were purchased from Fluka. Two steps of these syntheses in which new chiral centres are formed: Claisen rearrangement and iodolactonization proceeded with high (over 97% according to GC) stereoselectivity. The configuration of the new-formed chiral centres in (+)–1S,4S,6S-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one were proved by X-ray structure of the corresponding iodolactone. S – limonene and R – limonene were obtained from SIGMA.

Aphids and Plants: The laboratory clone of *M. persicae* was reared on Chinese cabbage (*Brassica pekinensis*) in laboratory (±18°C; L16:D8). Young (2–3 days old) apterae were selected for experiments.

Application of the compounds: The tested compounds as 0.1% ethanolic solutions were applied on the upper (adaxial) side of Chinese cabbage leaves (at 0.01ml/cm^2). Two control treatments were designed: one – leaves treated with solvent only, second – leaves treated with water, to investigate the effect of ethanol on aphid behaviour. Aphids were placed on the leaves 1 hour after the application of the tested compounds.

Aphid settling: In this experiment we used the procedure of a "half-leaf" test in which the tested compound is applied to one half of a leaf (divided by the main vein) while on the other half only the solvent is applied. The compounds (S-limonene and R-limonene) were applied as described above. Adult apterous aphids

were placed on the main vein and the number of aphids settled on each half of the leaf was recorded after 15, 30, 60, and 120 minutes and after 24 hours. The experiment was replicated 8 times and each replicate included 20 aphids. The results were examined using analysis of variance at $p=0.05$.

Behaviour of freely moving aphids: The behaviour of freely moving aphids was observed for 15 min after their contact with treated leaf surface. Time spent on the leaf and the duration of probing were recorded basing on the relationship between antennal and body movements and penetration of the stylets, as described by Hardie et al. (1992). The position of antennae parallel to the abdomen and the cessation of body movements were associated with stylet penetration. Aphid behaviour was recorded on leaves treated with water, ethanol, S-limonene, and (+)-1S,4S,6S-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one. There were 20 replicates for each treatment. The following parameters were derived from data obtained in this experiment: total time of aphid presence on the leaf, total time of probing, number of probes, and mean duration of a probe. The results were examined using analysis of variance at $p=0.05$.

Electronic recording of aphid probing (EPG): Aphid behaviour during probing was monitored using the Electrical Penetration Graph Technique (EPG). This technique is commonly applied in insect-plant relationship studies. In this experimental set-up, an aphid and the plant are made parts of an electric circuit, which is completed when the aphid inserts its stylets into the plant. Weak voltage is supplied in the circuit, and all changing electric properties are recorded as EPG waveforms that can be correlated with aphid activities and stylet position in plant tissues (Tjallingii 1995). The values of parameters derived from EPG recordings, e. g., the duration of probing, duration of phloem sap ingestion, number of probes, etc., reflect the level of suitability of a food source to aphids (Mayoral et al. 1996).

In the present study, the probing behaviour of apterous adult aphids was recorded on leaves treated with S-limonene, (+)-1S,4S,6S-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan–8-one, water, and ethanol for 8 hours continuously. There were 14 replicates for each treatment. The results were examined using analysis of variance at $p=0.05$.

RESULTS

Aphid settling: S-limonene deterred aphid settling from the very beginning of the experiment, i.e. 15 minutes after that aphids had had access to the leaf and there was no change in deterrent activity until the end of experiment. The deterrent effect of R-limonene was observed 30 minutes after the beginning of the experiment. After 24 hours, aphid settling was deterred only by S-limonene. The deterrent effect of S-limonene was stronger and longer lasting than that of R-limonene (Fig. 2).

Behaviour of freely moving aphids: There were no significant differences in aphid behaviour between the two types of control (water and ethanol controls, respectively) (Tab. 1). Aphid behaviour was similar on control and S-limonene – treated leaves in respect to all calculated parameters during the 15-minute experiment. Aphids spent twice less time on leaves painted with (+)-1S,4S,6S-4-(1-me-

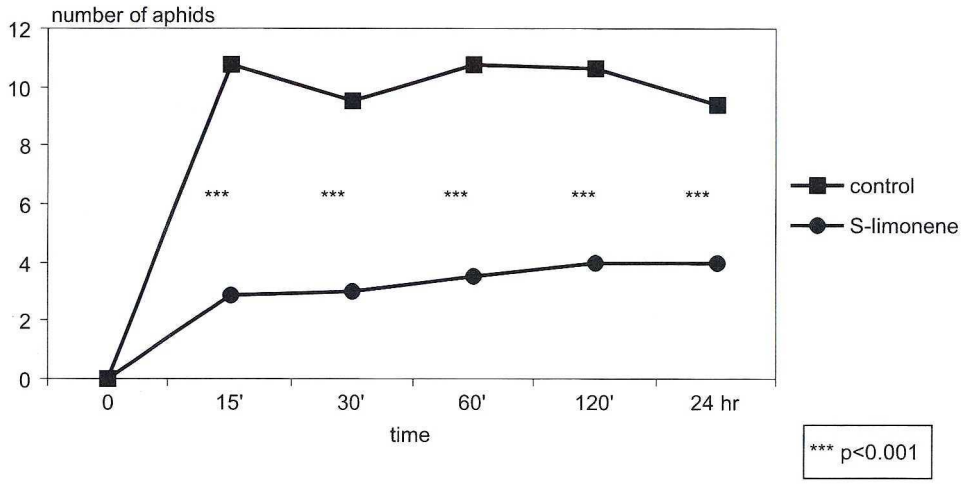

Fig. 2. Effect of S-limonene and R-limonene on settling of *Myzus persicae*. Asterisks show significant differences

thylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one than on leaves in other treatments. When aphids were on control and S-limonene-treated leaves they spent over 85% of that time on probing while on lactone-treated leaves – 44%. Total probing time was about 30% of the duration of that activity on control and S-limonene – treated leaves. The mean number of probes was similar in all treatments but the probes on lactone – treated leaves were 2.6–3.8 times shorter than on leaves in other treatments (Tab. 1).

EPG recorded probing behaviour: Stylet penetration activities (probing) occupied 66–75% of the experimental time and the total duration of these activities did not differ significantly between the treatments (Tab. 2). There were no significant differences in aphid behaviour before the first contact with phloem elements: the time from the first probe to the first phloem phase, the duration of pathway before phloem phase within the probe, and number of probes before the first phloem phase were similar in all treatments. Significant differences occurred in respect to activities related to contacts with phloem elements, mainly the ingestion of phloem

Table 1. Behaviour of free-moving *Myzus persicae* in response to S-limonene and on (+)-1S,4S,6S-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one (lactone) on cabbage leaves (n=20; standard deviation in parentheses; different letters in rows show significant differences; Kruskal-Wallis test)

Parameters	Compounds				
	Water	Ethanol	S-limonene	Lactone	Significance level
Total time of presence on the leaf (min.)	13.8 (±1.9)b	15.0 (±0.0)c	14.3 (±1.6)bc	8.6 (±4.7)a	0.0000
Total time of probing (min.)	11.6 (±3.3)b	13.0 (±1.1)b	11.4 (±3.6)b	3.8 (±4.5)a	0.0000
Mean number of probes	2.2 (±1.1)a	2.5 (±1.4)a	3.2 (±1.5)a	2.9 (±2.7)a	0.2540
Mean duration of probing (min.)	6.5 (±4.3)b	7.3 (±4.4)b	5.0 (±4.3)b	1.9 (±3.0)a	0.0000

Table 2. EPG recorded probing behaviour of *Myzus persicae* on S-limonene and on (+)-1S,4S,6S-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one (lactone) – treated cabbage leaves. E1 – phloem salivation. E2 – phloem sap ingestion. (8-hour observations; n=14; standard deviation in parentheses; different letters in rows show significant differences; Kruskal-Wallis test. p=0.05)

Parameters	Compounds				
	Water	Ethanol	S-limonene	Lactone	Significance level
Total penetration time (hr)	6.0 (±1.4)a	5.7 (±2.4)a	5.6 (±1.5)a	5.3 (±0.8 a	0.5555
Total pathway time (hr)	3.4 (±1.9)a	3.7 (±1.9)a	5.0 (±1.4)a	3.9 (±0.7 a	0.0931
Total duration of E1 and E2 (hr)	2.6 (±2.6)b	2.0 (±1.9)b	0.6 (±1.3)a	1.4 (±1.6)ab	0.0150
Total duration of E2 (hr)	2.5 (±2.6)b	2.0 (±1.9)b	0.6 (±1.3)a	1.3 (±0.5)ab	0.0274
Number of probes	33.4 (±20.4)a	28.4 (±18.9)a	30.7 (±10.8)a	31.1 (±18.5)a	0.8585
Number of E phases	4.2 (±3.6)b	5.1 (±4.1)b	1.3 (±1.4)a	2.3 (±3.5)a	0.0066
Number of E2 phases	3.5 (±3.4)bc	4.2 (±3.4)c	1.1 (±1.4)a	1.9 (±3.0)ab	0.0155
Time from 1st probe to E (hr)	1.3 (±1.7)a	1.8 (±2.0)a	2.4 (±2.7)a	1.9 (±1.9)a	0.9204
Pathway to E within a probe (hr)	0.7 (±0.5)b	0.5 (±0.4)b	0.3 (±0.5)a	0.3 (±0.5)a	0.0085
number of probes before E	7.1 (±10.8)a	7.4 (±7.3 a	9.9 (±12.7)a	9.7 (±7.0)a	0.7717
Duration of 1st E (hr)	1.3 (±2.4)a	0.5 (±0.6)a	0.3 (±0.9)a	0.7 (±1.3)a	0.0834
Duration of 1st E2 (hr)	1.4 (±2.4) b	0.6 (±0.6) b	0.3 (±0.9) a	0.7 (±1.3)ab	0.0334

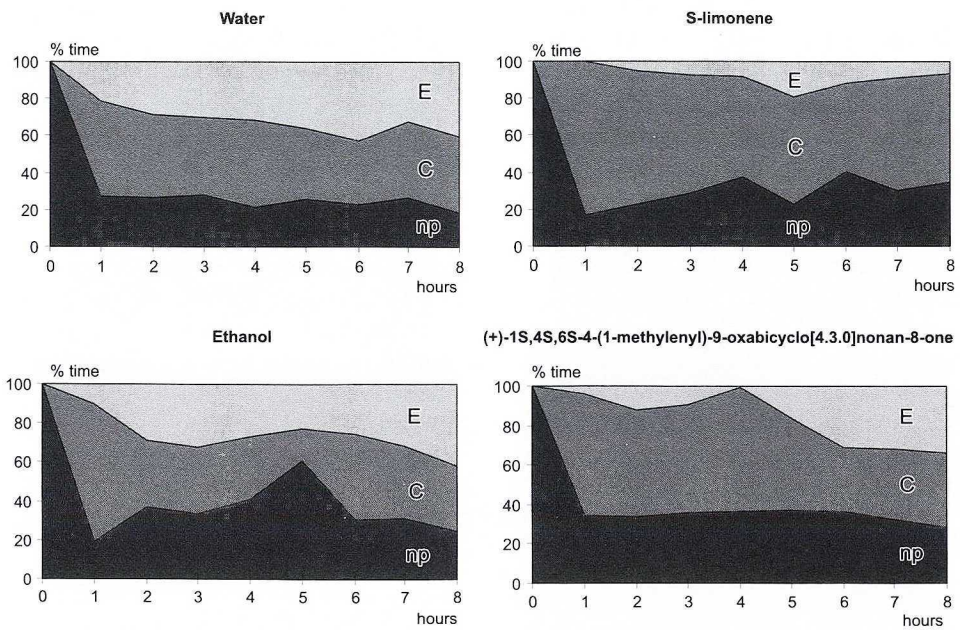

Fig. 3. EPG-recorded aphid probing behaviour during 8-hour experiment. np – non penetration, C – pathway activities, E – phloem salivation and sap ingestion

sap (E2). The shortest time spent on feeding on phloem sap was recorded in aphids on S-limonene-treated leaves. On average, aphids spent 11% of probing time on the ingestion of phloem sap on S-limonene- treated leaves, while on control and lactone-treated leaves – 42% and 25%, respectively. The duration of the first

phloem sap ingestion period was the shortest on S-limonene-treated leaves (Tab. 2). All aphids reached phloem vessels during the 8-hour experiment, although aphids on S-limonene and lactone-treated leaves much later than those on control leaves (Fig. 3). During the first four hours of the experiment, aphid probing was not restrained on S-limonene and lactone-treated leaves in comparison to the control, but almost 100% of it were pathway activities (Fig. 4). The phloem sap ingestion was visibly suppressed on S-limonene and lactone-treated leaves although 24% and 55% of aphids reached phloem vessels with their stylets during that time, respectively. On control leaves, phloem sap ingestion engaged 30–40% of stylet penetration activities and 80% of aphids found sieve elements during that time (Figs. 3, 4). During the 5th–8th hours of the experiment, more aphids on S-limonene and lactone-treated leaves started feeding (Fig. 3). At the end of experiment, time spent on phloem sap ingestion was similar for aphids on lactone-treated and control leaves. Phloem sap ingestion on S-limonene-treated leaves was strongly suppressed until the end of the recording time (Fig. 4).

DISCUSSION

The compounds that interfere with food selection and consumption by insects may function as preingestive inhibitors affecting activity of gustatory receptors,

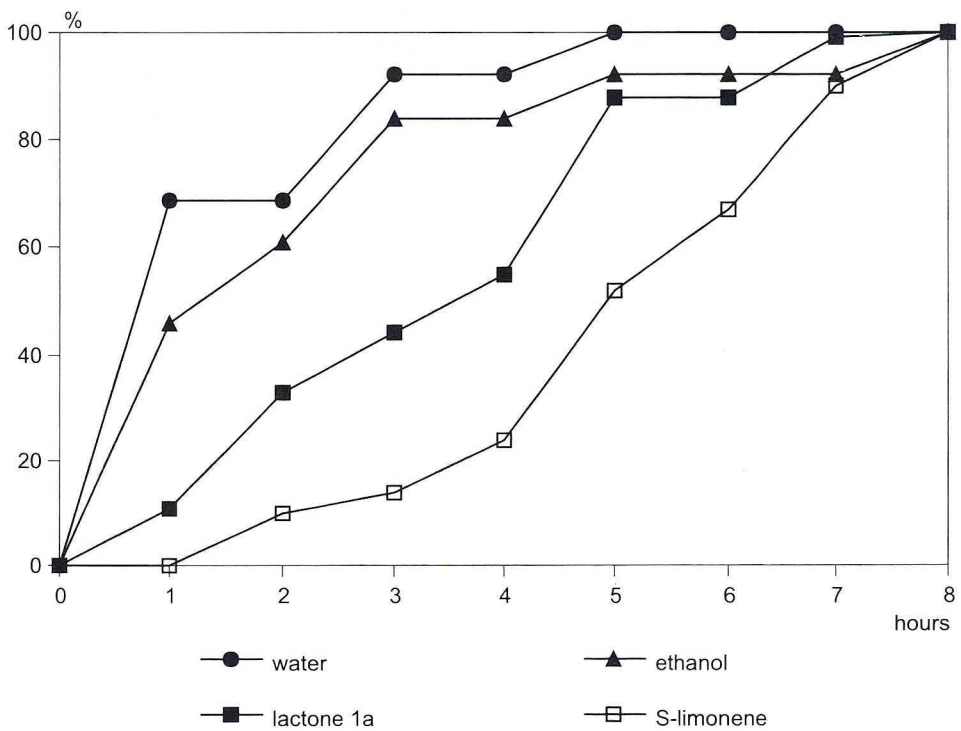

Fig. 4. Cumulative percent of aphids showing phloem sap ingestion activities during EPG recorded 8-hour experiment

ingestive inhibitors suppressing salivary enzymes or transport of food, and postingestive inhibitors affecting physiological processes after food had been ingested (Frazier and Chyb 1995).

In this study we found all levels of the deterrent activity of S-limonene and (+)-1S,4S,6S-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one: the immediate, preingestive and ingestive and the delayed, postingestive suppression of probing, feeding, and settling.

The preingestive inhibition of aphid feeding was demonstrated by (+)-1S,4S,6S-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one. The free moving aphids avoided the lactone-treated leaves: the entire time spent on these leaves was nearly twice shorter than on control and S-limonene-treated ones, and the probes were very short. Considering the average duration of a probe on lactone-treated leaves, which did not exceed 2 min., the suppression of probing must have occurred when aphid stylets were positioned within epidermis or mesophyll. Aphid stylets penetrate one cell layer in approximately 2 minutes (van Hoof 1958). The continuation of probing demonstrated in the EPG experiment might have been an effect of tethering: aphids were not free to move away from the unsuitable substrate.

The ingestive inhibition of aphid feeding was caused by S-limonene as shown in the significant reduction of time spent on phloem sap ingestion.

The postingestive suppression of settling was demonstrated by (+)-1S,4S,6S-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan–8-one in the previous study when we found that aphid settling was suppressed by the lactone during the 24-hour experiment (Wawrzeńczyk et al. 1998). EPG experiments in the present study showed that the phloem sap ingestion was not restrained from the 5th hour of experiment onwards. It is possible that the concentration of the compound in the plant decreased due to phloem translocation, or metabolising it by the plant to a value below the level evoking changes in aphid feeding behaviour. However, it is likely that the delayed effect of (+)-1S,4S,6S-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-one resulted from the ingestion of the deterrent compound in a quantity that affected the physiology of the digestive system.

CONCLUSIONS

1. Only S enentiomers of limonene and limonene-derived bicyclic lactone showed strong and long term feeding deterrent properties.
2. S-limonene affected aphid feeding during ingestive stage. It inhibited phloem sap ingestion and reduced the number of phloem sap ingestion phases during aphid stylet penetration of plant tissues.
3. Limonene-derived lactone (+)-1S,4S,6S-4-(1-methylethenyl)-9-oxabicyclo [4.3.0] nonan-8-one affected aphid feeding during the preingestive and postingestive stages. It restrained aphid probing on the level of mesophyll and phloem.

Acknowledgement

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POLISH SUMMARY

WPŁYW KONFIGURACJI PRZESTRZENNEJ LIMONENU I BICYKLICZNYCH LAKTONÓW POCHODNYCH LIMONENU NA ŻEROWANIE I ZASIEDLANIE ROŚLIN PRZEZ MSZYCĘ BRZOSKWINIOWĄ *MYZUS PERSICAE* (SULZ.)

Badano deterentny wpływ enancjomerów limonenu i jego pochodnej: (+)-(1S,4S,6S)-4-(1-methylethenyl)-9-oxabicyclo[4.3.0]nonan-8-on-u, na *Myzus persicae*. Jedynie enancjomery „S" badanych związków wykazywały właściwości deterentne. S-limonen ograniczał pobieranie soku floemowego i obniżał liczbę pojedynczych okresów żerowania we floemie podczas penetracji tkanek roślinnych przez mszyce. Pod wpływem badanego laktonu mszyce przebywały dwa razy krócej na liściach w stosunku do mszyc na liściach kontrolnych i pokrytych S-limonenem. Na liściach pokrytych laktonem, nakłucia były krótsze niż 2 minuty. Niniejsze badania potwierdziły opinie, że konfiguracja centrum chiralnego laktonów jest istotna dla uzyskania efektu deterencji pokarmowej.