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Are agarose-sucrose gels useful for studying the probing and feeding behavior of aphids?

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Abstract

The effects of novel chemicals on aphid probing and feeding can be more easily studied in gels than in plants; therefore, we investigated whether aphid probing behavior is similar on agarose-sucrose gels vs. plants. Electrical penetration graphs (DC EPG) were used to monitor the probing/feeding behavior of the bird cherry-oat aphid, *Rhopalosiphum padi* L. on wheat plants and agarose-sucrose gels. The EPG patterns generated by aphids feeding on plants were used to interpret the patterns generated on the gels. Based on terms previously used to describe EPG waveforms generated by aphids probing/feeding on plants we propose using a "g-" prefix to indicate agarose-sucrose gel EPG waveforms. The five main waveforms generated by aphids feeding on gels in this study are interpreted to be analogous to or representative of the patterns previously described for the feeding of aphids on plants. In waveform g-np, the aphids stylet is outside the gel (analogous to the stylet being outside the plant). Waveform g-C, indicates stylet activity in the gel (analogous to the stylet penetrating the epidermis and mesophyll). Waveform g-E1 indicates salivation into the gel (analogous to secreting saliva). Waveforms g-E2 and g-G represent ingesting the gel (analogous to ingestion of phloem and xylem sap). We report that EPG waveform shape, frequency, amplitude, electrical origin, and graphic display of peaks and waves are very similar for *R. padi* probing on agarose-sucrose gels and wheat plants. These results suggest that agarose-sucrose gels could be used for study aphid probing/ feeding behavior under controlled conditions.

Keywords: feeding behavior; *in vitro*; *Rhopalosiphum padi.*
Abbreviations: C (g-C)_stylet activity in the plant tissues (in the gel), cv._culticar, EPG_electrical penetration graph, E1 (g-E1)_ salivation into the plant (into the gel), E2 (g-E2)_ingestion from the phloem (from the gel), G (g-G)_ingestion from the xylem (from the gel), np (g-np)_waveform when the aphid's stylet is outside the plant (outside the gel).

Introduction

Aphids reduce crop yields by 8–15% worldwide (Oerke, 2006) and are among the most serious pests of various crops and ornamental plants (Sadeghi et al., 2009; Goggin, 2007). Aphids have devastating effects on plants. Their feeding on phloem sap causes plant stunting, discoloration, and deformation, and aphids are major vectors of plant viruses (Goggin, 2007; Sadeghi et al., 2009). Injury caused by aphids is both direct (through injection of chemicals within saliva, mechanical damage, and removal of sap) and indirect (through the production of honeydew) (Brault et al., 2007).

Aphids have traditionally been controlled by application of chemical insecticides. Although generally effective, chemical control of aphids has three major disadvantages: pollution of the environment by insecticide residues, development of aphid resistance, and potential toxicity to non-target organisms (Despres et al., 2007; Rharrabe et al., 2007). Because of these harmful effects, researchers have been investigating alternatives to conventional chemical control of aphids (Horowitz and Ishaaya, 2004; Rharrabe et al., 2007), including the use of resistant cultivars, transgenic plants containing novel genes that confer resistance to phloem-feeding insects, and chemicals that are compatible with integrated pest management. Understanding whether and how these alternative methods affect aphid feeding requires a method to monitor aphid feeding, and this is difficult because aphids feed on phloem sap. Although aphid stylet activity, saliva excretion, and food ingestion cannot be observed directly because the probing and feeding site is internal to the host, these activities can be monitored with the electrical penetration graph (EPG) method. This method has been widely used to study the probing and feeding behavior of aphids (Powell, 1993; Prado and Tjallingii, 1999; Pelletier and Giguere, 2009). A large number of bio-molecules with insecticidal activity against aphids have been attracting the attention of researchers. More studies on chemical factor effects have been done on plants (Denholm et al., 1998; Morita et al., 2007) and on artificial diets (Auclair, 1965; Febvay et al., 1988; Sadeghi et al., 2009). We have been using the EPG technique to monitor the effects of individual compounds on probing and feeding behavior *in vitro*, i.e., on agarose-sucrose gels (Goławska, 2007; Sprawka and Goławska, 2010; Sprawka et al., 2011, Goławska and Łukasik, 2012). Agarose or agar gels have also been used for salivary protein detection and staining (Urbańska et al., 1998, Cooper et al., 2010). Nevertheless there has been no research in which an electrophysiological approach, the DC EPG system, was utilized to compare the insects probing and feeding behavior on agarose-sucrose gels and on plants. Such studies using the agarose-sucrose gel substrates should assist in the assessment of potential insecticides/ aphicides on the insects probing and feeding behavior. EPG waveforms that represent aphid feeding activities and stylet locations in host

tissue have been well characterized (Tjallingii, 1978, 1988; Tjallingii and Hogen Esch, 1993) but information is lacking concerning how EPG data can be used to characterize and interpret waveforms produced by aphids feeding on agarose-sucrose gels. The objectives of the present research were to characterize the EPG waveform patterns produced by *Rhopalosiphum padi* feeding on gel membrane systems, to compare the EPG signals produced in plants and in agarose-sucrose gels, and to interpret the waveforms observed on gels.

Results

Waveform description

The EPGs were generally similar for *R. padi* probing and feeding on plants (Fig. 1) and on gels (Fig. 2). Data for EPG electrical origin, frequency, amplitude, and shape were also similar (Table 1). As noted previously, the behaviors ascribed to the waveforms in Table 1 are based on Tjallingii (1988, 1990, 1994). For waveforms observed on agarose-sucrose gels, we propose here the addition of the prefix g- to the terms commonly used for waveforms observed with plants.
The membrane potential in gels is positive outside the mesh and negative inside it (Fig. 3). No differences in waveform characteristics were observed when substrate voltage was adjusted to positive or negative. We reported similar associations with emf origin for waveforms of aphid on plants and on gels (Table 1).

Probing behavior

The first observed waveform was attributed to a non-probing phase (np, g-np). It appears as a "horizontal line" with plants (Fig. 1) and gels (Fig. 2). The non-probing phase was followed by stylet penetration (C, g-C). On plants, this waveform consisted of irregular, sharp positive peaks with a large number of spikes with a gradual decline in voltage level (Fig. 1). Amplitude, voltage and shape were variable over the experimental period but the waveform remained positive (above 0 V) for its entire duration (Table 1). On gels, this waveform was composed of a cyclic sequence of signals that strongly resembled the waveform on plants (Fig. 4; C vs. g-C). One characteristic of waveform C on plants but not on gels was the occurrence of short potential drops (pds), which has been associated with intracellular punctures across the cell membrane in all types of cells along the stylet pathway and also of phloem cells (Tjallingii and Hogen Esch 1993). Waveforms A and g-A (aphid is in contact with plant or gel) and B or g-B (excretion of gelling sheath saliva) (Table 1) are closely associated with waveforms C and g-C.

Feeding behavior

Waveform E1 (g-E1) was next. It consisted of a very regular repetition of small amplitude upward and downward peaks separated by short horizontal lines (Fig. 4; E1 vs. g-E1). The E1 (g-E1) waveform occurred following rapid sharp negative voltage (< 0 V) and generally remained negative (Table 1). Waveform E2 (g-E2) was characterized by sharp, mostly regular peaks that varied in amplitude (Fig. 4; E2 vs. g-E2). The wave frequency was almost identical on plants and gels. The voltage level of waveform E2 (g-E2) was always negative and relatively constant (Table 1).Waveform G (g-G) was monophasic with regular periodic signals, rounded peaks, and sharp basal troughs (Fig. 4; G vs. g-G). The waveform G (g-G) remained positive (Table 1).

Comparison of probing and feeding behavior on gels and plants. The proportion of the aphid activities on plant did not differ significantly from the proportion of the same activities on gels (G-test, G = 1.09, df = 4, p = 0.895)(Fig. 5).

Resume

Generally, the use of agarose-sucrose gels in combination with the EPG method is useful. The use of agarose-sucrose gels with EPG offers us a good background to investigate the mechanism of action of potential insecticides towards insects/ aphids. Moreover, the use of these methods together provide information helpful for (1) determining a differentiation between various chemicals' effects on sucking-piercing insects and (2) a better understanding of aphids' probing and feeding behavior. Analysis of penetration activities could therefore guide plant breeders when trying to incorporate different chemicals in plant breeders' programmers.

Discussion

This is the first study to compare EPG waveforms generated by the probing and feeding behavior of a sucking-piercing insect on agarose-sucrose gels and on plants. Previous studies using the DC EPG system have documented the feeding behavior of aphids on plants (Tjallingii, 1988; Tjallingii, 1995a, b; Tjallingii, 2001), and a standard EPG nomenclature describing aphid feeding in plants has been developed (Tjallingii, 1978). The EPG method and EPG nomenclature have also been used to study aphid probing and feeding on agarose-sucrose gels (Goławska, 2007; Sprawka and Goławska, 2010; Sprawka et al., 2011, Goławska and Łukasik, 2012) and on artificial diets (Sauvion et al., 2004). Although EPG nomenclature was used, these non-plant studies did not provide detailed descriptions of aphids probing and feeding behavior on gels. In the current study, waveforms generated by aphids on plants were very similar to those generated on gels, and we therefore propose that the same designations developed by Tjallingii (1978) for plants be used for gels (with addition of the prefix g-). Because the graphical representation of the waveforms and their frequency, amplitude, and electrical origin were similar on gels and plants, we infer that the probing and feeding behavior was also similar. Whereas five main waveforms were observed with gels (g-np, g-C, g-E1, g-E2, and g-G), only three (np, C and G) were previously reported with other artificial diets (Sauvion et al., 2004). Waveform g-np on gels is clearly analogous to np on plants, i.e., this waveform indicates that the aphid's stylet is outside the plant or outside the gel. Waveform g-C may be considered to indicate stylet activity in the gel (analogous to the stylet penetrating the plant tissues), while waveform g-E1 seems to suggest salivation into the gel (analogous to the excretion of saliva into the phloem), waveform g-E2 seems to suggest passive ingestion of fluids from the gel (analogous to the ingestion from the phloem), and waveform g-G seems related to active ingestion of fluids from the gel (analogous to the ingestion from the xylem). We believe that these inferences are valid not only because of the similarity of waveforms but also because of similarities in structure. Agarose gel is a cross-linked matrix that resembles a three-dimensional mesh or screen (Fig. 6). In addition, the gel is soft, transparent, and lacks ionic groups (so that the gel is neutral). These properties of agarose gels resemble those of plant tissues. The agarose-sucrose gels presumably mimics the tissues surrounding the sieve elements, whereas a Parafilm M® membrane containing sucrose syrup corresponds to sieve

Table 1. Characteristics of the EPG waveforms recorded during the feeding of *R. padi* on wheat plants and on agarose-sucrose gels (p-peak, w-weave).

Waveform		Waveform characteristics								Correlations			Proposed correlations*
Plant	Gel	Amplitude (plant)	Frequency (plant)	Cellular level[1]	Electrical origin[2]	Amplitude (gel)	Frequency (gel)	Pore level[1]	Electrical origin[2]	Position (plant)	Phase (plant)	Aphid activity (plant)	Aphid activity (gel)
A	g-A	100	5 – 10	e	R	100	6-10	e	R	epidermis	path	contact with plant	contact with gel
B	g-B	75	0,2 – 0,3	e	R	60	0.3-0.5	e	R	epidermis/ mesophyll	path	excretion of gelling sheath saliva	excretion of gelling sheath saliva
C	g-C	30	variable	e	R	40-80	variable	e	R	any tissue	path	path activity	path activity
E1	g-E1		2 – 7	i	emf		2-4	i	nd	phloem	phloem	saliva excretion	possibly saliva excretion
E2p	g-E2p	5	0,5 – 4	i	R	4	0.5-5	i	nd	phloem	phloem	water saliva excretion	possibly water saliva excretion
E2w	g-E2w		4 – 8	i	emf		5-10	i	nd	phloem	phloem	ingestion	possibly ingestion
G	g-G	0-60	4 – 8	e	R/emf	0-55	5-10	e	R/emf	xylem	xylem	ingestion	ingestion

[1] e, between cells or outside of gel pore (positive); i, within cells or within gel pore (negative). [2] R, resistance fluctuation; emf, electromotive force; nd, not determined. * activities assigned for similar waveforms in aphids on plant.

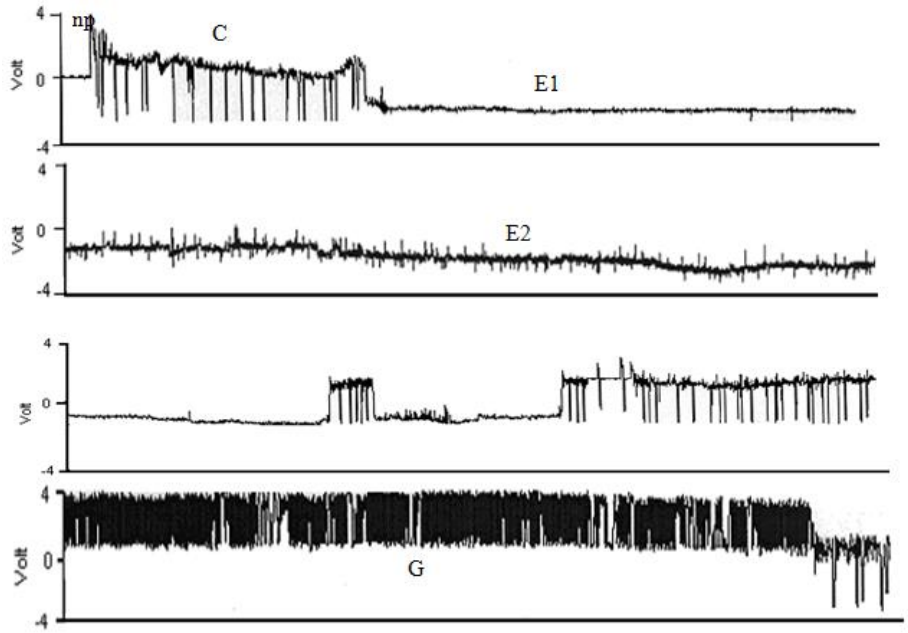


Fig 1. Representative electrical penetration graph for an *R. padi* aphid feeding on a wheat plant. Each panel represents 1 h, and all four panels together (from top to bottom) represent 4 h of continuous recording. Waveform details are described in Table 1 and Figure 4.

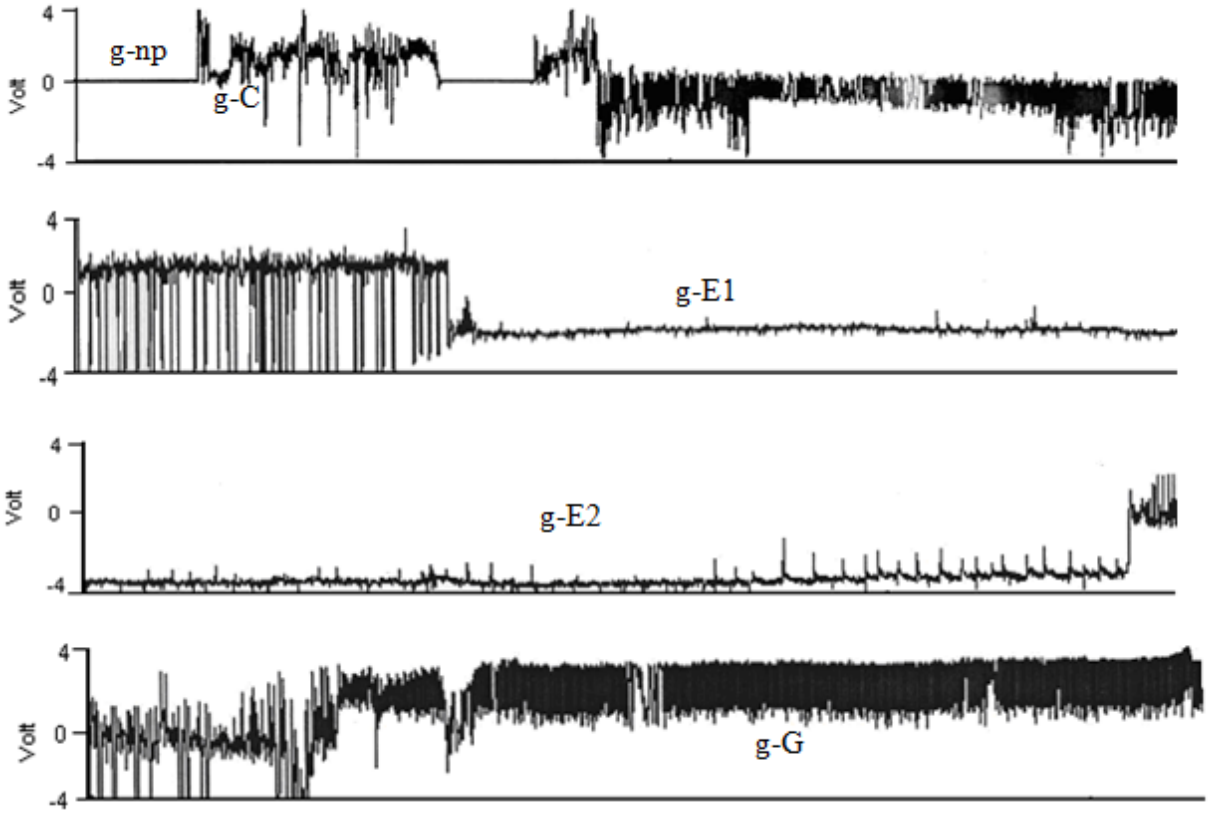


Fig 2. Representative electrical penetration graph for an *R. padi* aphid feeding on a agarose-sucrose gel. Each panel represents 1 h, and all four panels together (from top to bottom) represent 4 h of continuous recording. Waveform details are described in Table 1 and Figure 4.

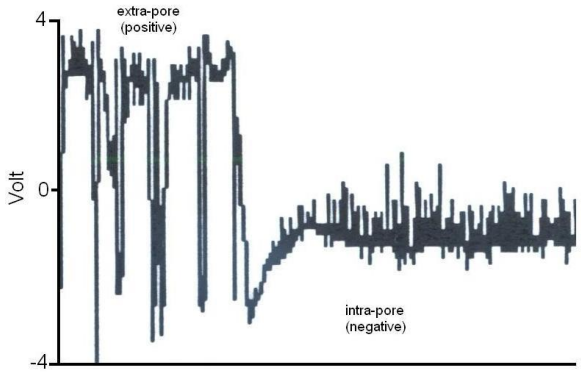


Fig 3. Scheme of changes in voltage level observed for R. padi feeding on agarose-sucrose gel. Two membrane potentials in gels were observed: positive outside (extra-pore) the mesh and negative inside (intra-pore) the mesh.

elements containing phloem sap (Urbańska et al., 1998). Waveform g-C always occurred after a non-probing phase, and similar waveforms have been reported by us on plants and by others with aphids and other homopterans on plants and on artificial diets (Calatayud et al., 2001; Lett et al., 2001; Sauvion et al., 2004; Goławska et al., 2006, 2008, 2010; Kordan et al., 2008; Goławska and Łukasik, 2009; Stafford and Walker, 2009; Cid and Fereres, 2010). This waveform is considered to represent the "pathway" phase, during which the aphid excretes gelling saliva that creates the salivary sheath. Urbańska et al. (1998) observed that the aphid *S. avenae*, when probing through a Parafilm M® membrane into agarose-sucrose gels, produces branched and/or simple stylet sheaths of variable length. This supports the notion that waveform g-C most likely corresponds to the pathway phase. Waveform g-E1 on gels was very similar to waveform E1 reported for aphids on plants, and this waveform on plants has been correlated with stylet activity in the phloem and the secretion watery saliva (Tjallingii, 1988; Tjallingii, 1995a; Tjallingii, 2001). Because watery saliva was detected when the aphid stylet punctured a Parafilm M® membrane into sucrose syrup (Urbańska et al., 1998), the generation of this waveform on gels indicates that the stylet is located in the gel and is excreting saliva. On gels, this waveform may be associated with the salivary pump action required for saliva injection. Waveform g-E2 on gels resemb-

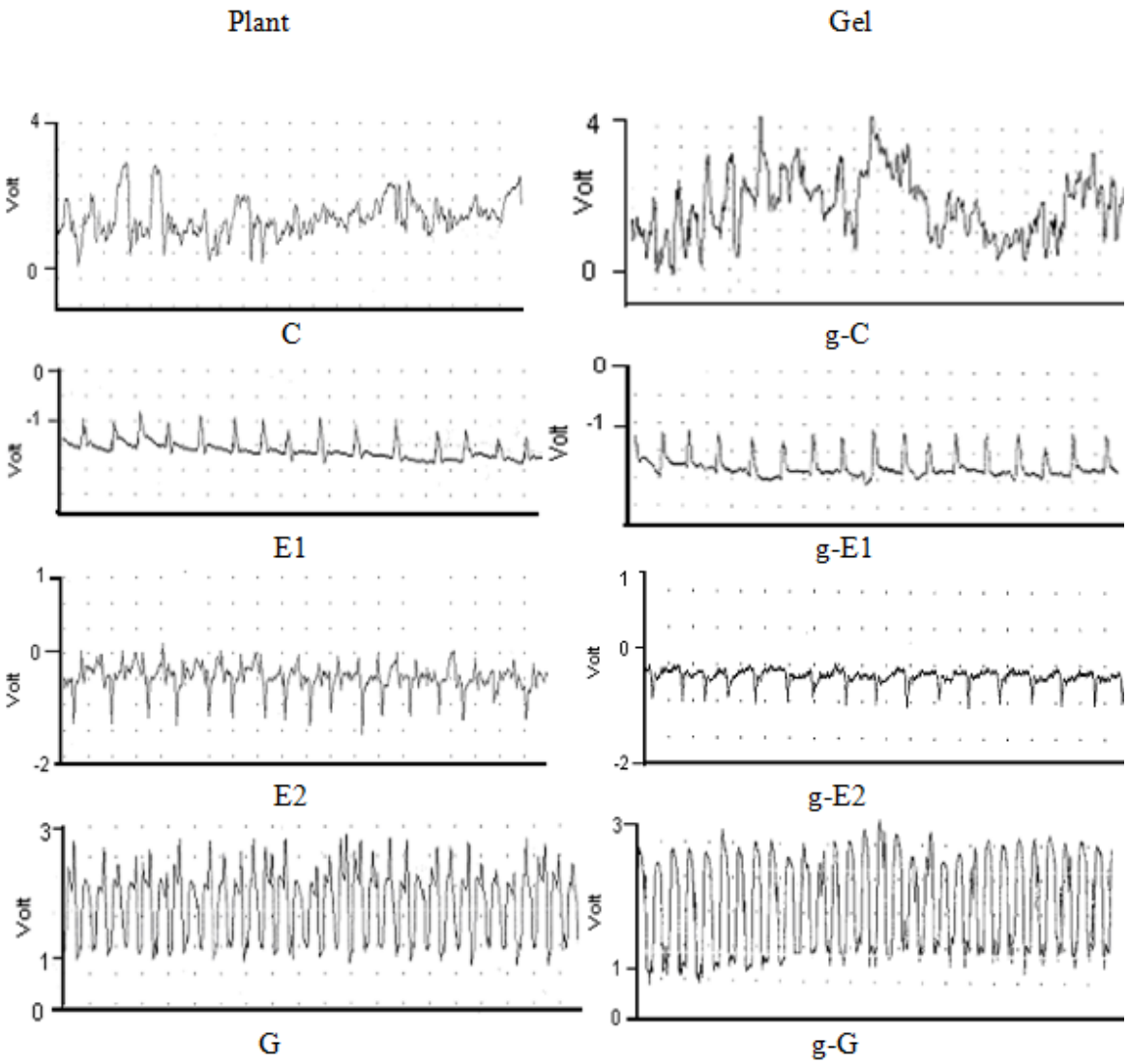


Fig 4. Details of EPG waveforms generated by *R. padi* feeding on plants and on agarose-sucrose gels. Additional information is provided in Table 1.

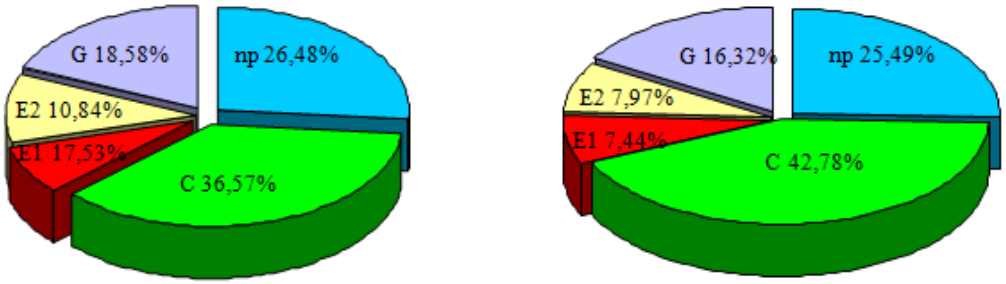


Fig 5. Cumulative percent of bird cherry-oat aphids probing and feeding activity on plants and gels during 4 hs of the EPG recordings.

les waveform E2 on plants (Tjallingii, 1988; Lett et al., 2001) and represents an ingestion phase. According to Tjallingii (1995a), phloem feeding by aphids does not involve active withdrawal of sap (i.e., the aphids do not suck during this phase) but instead depends on sap pressure, i.e., phloem feeding is mainly a passive form of ingestion. Aphids passively ingest the sap from the phloem because of the high hydrostatic pressure in that tissue (Prado and Tjallingii, 1994; Lett et al., 2001). To achieve this, aphids use their cybarial valve. During E2, the valve is open, and sap is forced into the stylet canal with the saliva (Tjallingii, 2001). Because the g-E2 waveform was also recorded on gels, we suspect that the stylet punctured the mesh wall of the gel matrix (Fig. 6) and that the hydrostatic pressure in the pore of the mesh was sufficient to support passive ingestion. This waveform also might be the result of "an intrinsically programmed motor pattern" in the aphids' brains. Waveform g-G was observed when aphids fed on agarose-sucrose gels. The waveform of g-G reported on gels is very similar to waveform G observed on plants for various aphids (Tjallingii, 1988; Prado and

Tjallingii, 1994). In all cases, this waveform has been associated with active feeding from xylem and/or mesophyll. The presence of waveform g-G for *R. padi* feeding on gel is caused by the fluid movements (evoking streaming potentials in the stylet food canal). In our study the proportion of the aphid activities on plants and gels were similar. These results together with our data as described above support the thesis that the sucrose- agarose gels are useful for investigating the influence of single chemical factor on aphids.

Materials and Methods

Aphids

Bird cherry-oat aphids, *Rhopalosiphum padi L.*, were obtained from a stock culture kept at the Siedlce University of Natural Sciences and Humanities, Poland. The stock culture was maintained on winter wheat (*Triticum aestivum* L. cv. Liwilla) in plastic pots in an environmental chamber at 21±1°C, L16:D8 photoperiod, and 70% RH. Adult apterous females were used for all experiments.

Experimental design

The experiments were arranged for winter wheat cultivars (cv. Liwilla) and agarose-sucrose gels with 10 replications. Plants of the wheat cultivar were grown in plastic pots (13 cm diameter; one plant per pot) containing sterile loamy soil. Gels were prepared by incorporating 1.25% agarose (Sigma A-0169) into a 30% sucrose solution. After the mixtures were stirred, they were heated in a water bath (75°C for 30 min) and then poured into plastic rings (10 mm high, 15 mm diameter) and covered with a stretched Parafilm M® membrane. Transparent gels formed after 1–2 min and were offered to aphids for probing.

Electrical monitoring of R. padi probing and feeding behavior on plants

The feeding behavior of adult aphids on winter wheat cultivar (cv. Liwilla) was recorded using DC EPGs (Tjallingii, 1988) (Fig. 7A). The EPG makes it possible to record different waveforms related to aphid activities and stylet locations during penetration of plant tissue or other substrates (Sauvion and Rahbe, 1999). EPG waveforms were recorded in a Faraday cage in the laboratory (21±1°C, L16:D8 photoperiod, and 70% RH). Apterous adults collected between 6 and 7 a.m. were dorsally tethered by the abdomen with a 20-µm-diameter gold wire and water-based, conductive, silver paint (Demetron, L2027, Darmstadt, Germany). After the insects were starved for 2 h to recover from tethering, EPGs were started (between 9 and 10 a.m.) by carefully transferring the aphids to plants (one aphid per plant); the plants were 7 days old. A second electrode (a copper wire 9 cm long and 1 mm in diameter) was introduced into the soil. Aphids were connected to the Giga-4 EPG amplifier (Wageningen, Agricultural University, Entomology Department, The Netherlands) coupled to an IBM-compatible computer through a DAS 8 SCSI acquisition card (Keithley, USA). The EPGs were recorded under continuous laboratory lighting per day, and all EPG recordings were made for 10 aphids on 10 different plants. Aphid probing and feeding behavior was monitored for 4 h. EPGs were acquired and analyzed with STYLET 2.2 software provided by W.F. Tjallingii. Five waveforms were identified according to Tjallingii (1988, 1990, 1994): np (non-penetration), the aphid's stylet is outside the plant tissues; C (stylet pathway phase), the stylet

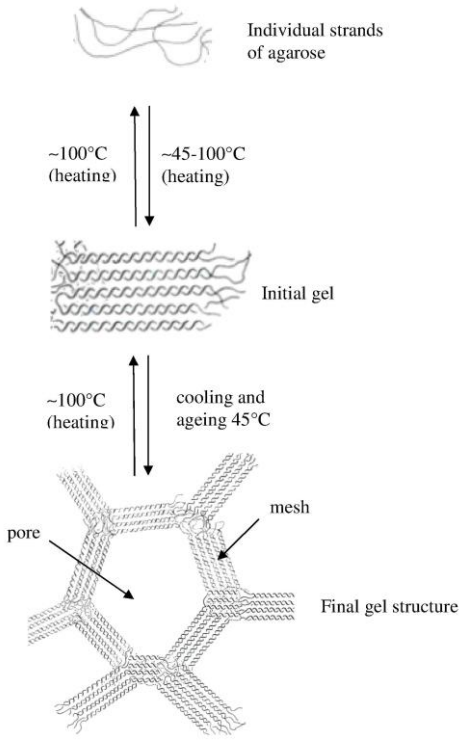
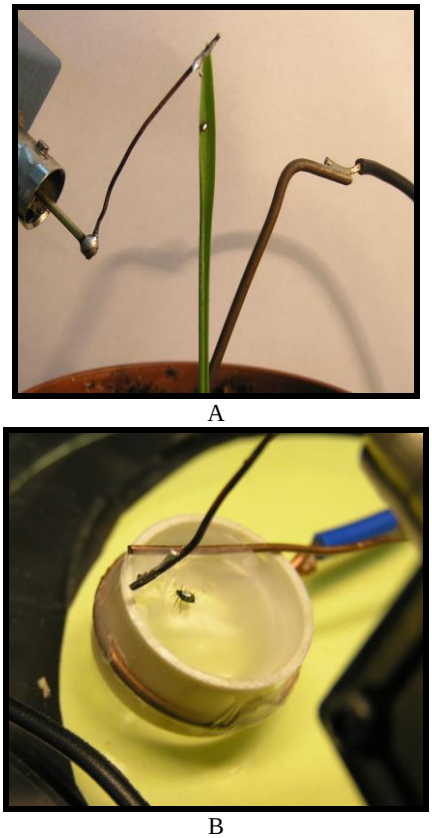


Fig 6. Gel structure of agarose. The final gel structure resembles a three- dimensional network of plant tissue. The gel strands of agarose (mesh) separated by sucrose solution (pore).



A B

Fig 7. The experimental EPG systems used to monitoring feeding behavior of bird cherry-oat aphid on plants (A) and agarose-sucrose gels (B).

is moving intercellularly through mesophyll or vascular tissues, and the salivary sheath is formed; E1 (sieve element phase), saliva is being excreted into the phloem sieve tubes; E2 (phloem phase), phloem sap is being passively ingested; and G (xylem phase), xylem sap is being actively ingested. The time spent on each EPG waveform was measured and expressed per one insect.

Electrical monitoring of R. padi probing and feeding behavior on gels

R. padi probing and feeding behavior was investigated *in vitro* using agarose-sucrose gels (Fig. 7B). Aphids were dorsally tethered as described in the previous section and individually placed in the center of the membrane on the gel (one aphid per gel). A second electrode (a copper wire 4 cm long and 0.5 mm in diameter) was introduced into the gel. The probing and feeding behavior of adult aphids on gels was then recorded and EPG data were acquired and analyzed as described in the previous section. Changes in voltage level were used to determine whether the stylet was located outside of or within the three-dimensional mesh formed by the gel.

The main electrical origin

The following characteristics were determined for the EPG waveforms: amplitude spectrum (maximum and minimum), frequency (Hz), voltage level (extra- or intracellular/ pore), and electrical origin (R, resistance or emf, electromotive force). To determine the electrical origin, voltage adjustments to positive and negative levels were done in different periods for each waveform. The waveform amplitude and frequency were estimated based on the average of 30 observations for each waveform (three observations per recorded insect).

Statistical analysis

The cumulative percent of bird cherry-oat aphids probing and feeding activity on plants and gels were compared using G tests (Sokal and Rohlf 2001).

Conclusion

In conclusion, the results presented here are the first to associate the probing and feeding behavior of aphids on agarose-sucrose gels and on plants as indicated by EPGs. The bird cherry-oat aphid, *R. padi*, readily fed on the agarose-sucrose gels. The agarose-sucrose gel was superior to a liquid sucrose diet because the gel elicited nearly all of the typical EPG waveforms elicited by plants. The use of agarose gels in combination with the EPG method will facilitate the study of how chemoreception, phagostimulation, and deterrence of sucking-piercing insects are affected by various chemicals and should therefore be useful for biopesticide research. To increase our understanding of aphid probing and feeding behavior on agarose-sucrose gels, we suggest that researchers should combine various techniques including light and electron microscopy, stylet amputation, radioactive tracers, and electromyography.

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