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Infection of potato plants with potato leafroll virus changes attraction and feeding behaviour of *Myzus persicae*

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Abstract

Potato leafroll virus (PLRV; genus *Polerovirus*, family Luteoviridae) is a persistently transmitted circulative virus that depends on aphids for spreading. The primary vector of PLRV is the aphid *Myzus persicae* (Sulzer) (Homoptera: Aphididae). *Solanum tuberosum* L. potato cv. Kardal (Solanaceae) has a certain degree of resistance to *M. persicae*: young leaves seem to be resistant, whereas senescent leaves are susceptible. In this study, we investigated whether PLRV-infection of potato plants affected aphid behaviour. We found that *M. persicae*'s ability to differentiate headspace volatiles emitted from PLRV-infected and non-infected potato plants depends on the age of the leaf. In young apical leaves, no difference in aphid attraction was found between PLRV-infected and non-infected leaves. In fact, hardly any aphids were attracted. On the contrary, in mature leaves, headspace volatiles from virus infected leaves attracted the aphids. We also studied the effect of PLRV-infection on probing and feeding behaviour (plant penetration) of *M. persicae* using the electrical penetration graph technique (DC system). Several differences were observed between plant penetration in PLRV-infected and non-infected plants, but only after infected plants showed visual symptoms of PLRV infection. The effects of PLRV-infection in plants on the behaviour of *M. persicae*, the vector of the virus, and the implications of these effects on the transmission of the virus are thoroughly discussed.

Introduction

Potato leafroll virus (PLRV) (genus *Polerovirus*, family Luteoviridae) is a persistently transmitted circulative virus that depends on aphids for dispersal and transmission to host plants. Potato leafroll virus is restricted to the phloem. Aphids acquire PLRV during ingestion of phloem sap from infected plants and they inoculate it into new plants during salivation into the phloem sieve elements. The green peach aphid, *Myzus persicae* (Sulzer) (Homoptera: Aphididae), is the most efficient vector of PLRV. In this triangular relationship, direct interactions occur between virus, host, and vector. Moreover, virus infections can change hosts in such a way that interactions between host and vector are influenced. Vector activity and behaviour are important determinants of the rate and extent of epidemic virus development (Jeger et al., 1998, 2004). Changes in the attraction between the aphid vector and the infected plant and changes in the benefits obtained by the aphid from this relationship will certainly influence the probability of virus dispersal.

In some plant–virus–aphid interactions, the presence of viruses negatively affects the performance of the vector. On wheat, the presence of barley yellow dwarf virus (BYDV) reduces the concentration of total amino acids in the phloem. BYDV-infection also leads to a less efficient use of phloem sap by the aphid *Sitobion avenae* (F.) (Fiebig et al., 2004).

Benefits for the vector that favour the transmission of viruses have been described for various plant–pathogen–vector combinations. Belliure et al. (2005) showed that

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Frankliniella occidentalis (Pergande) benefits indirectly from the tomato spotted wilt virus, which it transmits, through effects of the virus on host-plant characteristics. They hypothesized that infection with the virus has a negative effect on induced defences against the thrips vector. *Aphis gossypii* Glover transmits zucchini yellow mosaic virus (ZYMV) to *Cucurbita pepo* L. and it lives longer and produces more offspring than on non-infected plants (Blua et al., 1994). In contrast to the negative effects mentioned above (Fiebig et al., 2004), BYDV has been reported to benefit its vector *S. avenae* (F.) by disrupting the development of a braconid parasitoid within the aphid vector (Christiansen-Weniger et al., 1998). BYDV-infection of wheat plants increases the attractiveness to the aphid *Rhopalosiphum padi* (L.) as a result of producing more volatiles than non-infected plants (Jiménez-Martínez et al., 2004).

Castle & Berger (1993) found that *M. persicae* performance improves in terms of growth rate, reproduction, and longevity on cultivated *Solanum tuberosum* L. (Solanaceae) plants when infected by PLRV (vector-borne virus) as compared to virus-free potato plants, plants infected by potato virus Y (PVY, stylet-borne virus that can be briefly associated to aphids but also mechanically transmitted), or potato virus X (PVX, vector-independent mechanically transmitted virus). It was also found that more *M. persicae* individuals settled on PLRV-infected leaves of *S. tuberosum* L. than on leaves of virus-free, PVY-infected, or PVX-infected leaves (Castle et al., 1998). Eigenbrode et al. (2002) reported that *M. persicae* preferred PLRV-infected to either non-infected potato plants, PVY-infected plants, or PVX-infected plants. They found an increased emission of several volatiles by PLRV-infected plants when compared to non-infected plants. These volatiles may act as attractants and arrestants of *M. persicae*. Srinivasan et al. (2006) found that the preference of *M. persicae* for PLRV-infected *Solanum* spp. plants over non-infected plants relies primarily on olfactory cues rather than on visual cues. The role of plant volatiles in host recognition and settling behaviour by aphids has been reviewed by Pickett et al. (1992). Vargas et al. (2005) have recently shown that alate virginoparae of the tobacco-adapted subspecies *Myzus persicae nicotianae* recognized and chose their host plant more efficiently than the generalist *Myzus persicae* s.s., on the basis of olfactory and visual cues and factors present at cuticular and subcuticular levels.

In addition to olfactory cues, aphids must insert their mouthparts into plant tissues (probing) in order to select a suitable host and find the phloem. Therefore, knowledge of mechanical or biochemical cues in host plants during probing by aphids is crucial. The electrical penetration graph (EPG) technique (Tjallingii, 1978, 1985, 1988) is a robust tool to study plant penetration by aphid stylets. EPG waveforms have been correlated with aphid activities as well as with tissue locations of the stylet tips (Tjallingii, 1978; Kimmins & Tjallingii, 1986; Tjallingii & Hogen Esch, 1993). Strategies for controlling plant viruses depend highly on the understanding of the virus–plant–vector interactions. The partial plant resistance present in potato cultivar Kardal slows down aphid population growth on young plants and, consequently, limits acquisition and dissemination of PLRV. The resistance to aphids in Kardal is phloem located and occurs in young apical leaves, it declines in mature and senescent leaves (Alvarez et al., 2006). Here, we investigated whether infection with PLRV changed the host plant to the advantage of the aphid through improving probing and feeding behaviour in young apical leaves of cv. Kardal. We were especially interested in changes regarding vector attraction.

Our specific aim was to study (i) the effects of PLRV-infection of potato plants on vector attraction by using an olfactometer assay for apical and mature leaves, and (ii) the impact of PLRV-infection on plant resistance, as expressed by feeding behaviour, by using the EPG technique. We hypothesized that the better performance of *M. persicae* previously found on PLRV-infected potatoes plants was due to structural or chemical changes in plant tissues that enhanced probing and feeding behaviour.

Materials and methods

Plants and aphids

Virus-free potato plants (*S. tuberosum* L. cv. Kardal) were propagated in vitro (Alvarez et al., 2006). After 2 weeks, the rooted plantlets were transferred to soil in a glasshouse at 22 ± 2 °C, about 70% r.h., and an L16:D8 photoperiod. A new colony of *M. persicae* was established on radish *Raphanus sativus* L. starting with a single virginoparous apterous aphid from a colony maintained at the Laboratory of Entomology, Wageningen University, Wageningen, The Netherlands. The colony was reared in a climate chamber at 22 ± 2 °C, 30–40% r.h., and an L16:D8 photoperiod.

Virus infection

One week after transfer to soil, nine potato plants were exposed to 15 viruliferous *M. persicae* nymphs during 96 h (treatment) and nine potato plants were exposed to 15 virus-free nymphs during the same time (control). After 96 h, aphids and nymphs were gently removed with a brush. Three *Physalis floridana* Rybd. plants, very susceptible to *M. persicae* and PLRV, were also exposed to 15 viruliferous nymphs during 96 h as a positive control. Viruliferous aphids had been obtained by placing apterous adult aphids on PLRV-infected (isolate PLRV-Wageningen) *P. floridana* plants for 24 h. After adults were removed, newborn

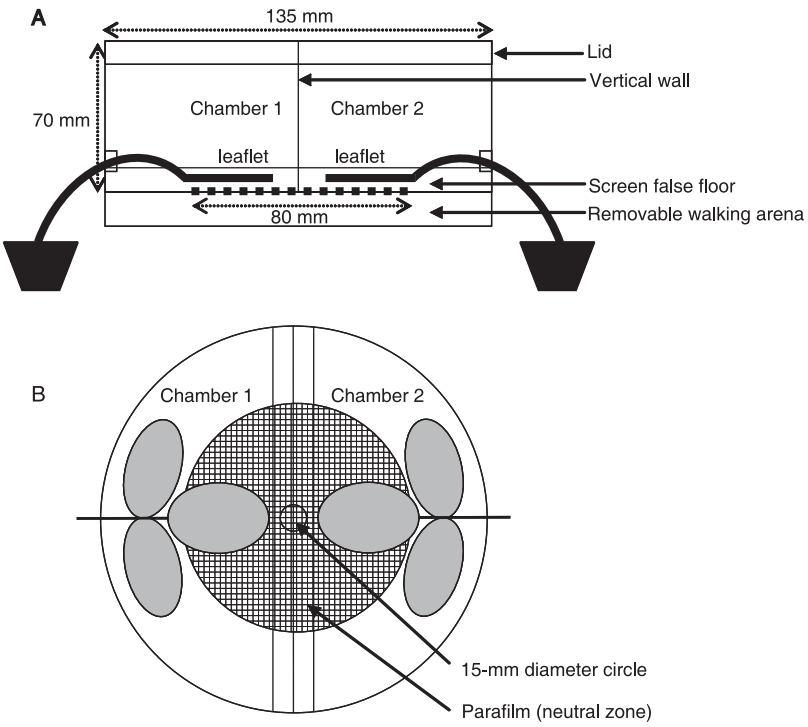

Figure 1 Static air two-chamber olfactometer. (A) Side view; (B) top view. Forty aphids were released within the 15-mm diameter circle drawn on the centre of the removable walking arena (Petri dish beneath the screen false floor). The arena was used to close the olfactometer from below. The leaflets always remained attached to live plants (see Materials and methods for details).

nymphs were allowed to feed for 24 h on the source plants before being used for the inoculation of test plants.

Twenty-seven days after inoculation with PLRV, infection was determined by enzyme-linked immunosorbent assay (ELISA) (van den Heuvel & Peters, 1989) using antisera from Prime Diagnostics, Wageningen, The Netherlands. Enzyme-linked immunosorbent assay results were considered positive for PLRV-infection when absorbance at 405 nm exceeded the mean absorbance of the non-infected control plants by four times the standard deviation or more.

Potato leafroll virus-infection symptoms were not visible 27 days after exposure of the plants to viruliferous nymphs. Seven out of nine potato plants were successfully inoculated and only these plants, positive by ELISA, were used for the EPG recordings 27 days after virus-inoculation.

Static air two-chamber olfactometer

The preference of non-viruliferous *M. persicae* to headspace volatiles from PLRV-infected or non-infected potato plants was tested on Day 65 after infection, when symptoms of PLRV-infection were evident. Preference was observed in a two-chamber olfactometer without airflow (Figure 1). This olfactometer was a modified version of the one-chamber olfactometer described by Eigenbrode et al. (2002). A plastic cylinder (135 mm in diameter) was divided into two chambers by a vertical plastic plate. The cylinder was closed at the bottom by a removable polystyrene Petri dish lid with a false floor. The false floor was made by opening a 80-mm diameter circle in the middle of the Petri dish lid and covering it completely with polyethylene screen (200 μm mesh) (Figure 1); a line was drawn over the mesh, indicating the position of the vertical wall in between the two chambers. To create a neutral zone between the chambers, a 20-mm strip of parafilm was put on the mesh over the line, just below the vertical wall between the two chambers. Two leaflets that were still attached to plants were placed above the false-floor Petri dish, one in each chamber (Figure 1). A strip of parafilm was used to support the leaves in the correct position at a distance of approximately 3 mm above the screen, to prevent the leaflets from touching the screen, and then the top of the cylinder was closed with a Petri dish lid. The aphid walking arena was a second Petri dish, in which a circle of 15 mm in diameter was drawn in the centre and where 40 apterous adult aphids were released at the start of each replicate. After releasing the aphids, the Petri dish was immediately used to close the olfactometer from the bottom. To avoid visual cues, the trials were performed in a dark room. Aphids on the walking arena could move freely but could not touch the leaves so they were not able to acquire any gustatory or contact cues.

The two-chamber olfactometer was used in three tests: (1) non-infected mature leaflet vs. empty chamber, where one mature leaflet (leaf 5–7 from the apex) of a non-infected control potato plant was enclosed in one chamber while the other chamber remained empty; (2) virus-infected

apical leaflet vs. non-infected apical leaflet, leaflets of the third fully expanded leaves (from apex) were each enclosed in one of the separate olfactometer chambers; (3) virus-infected mature leaflet vs. non-infected mature leaflet, leaflets of leaves 5–7 (from apex) were each enclosed in an olfactometer chamber. Each test was repeated six times. The entire olfactometer was rotated 180 degrees after every recording and the plants were changed after two opposite recordings.

In Experiment 1, aphid positions were recorded only once, after 60 min. In Experiments 2 and 3, aphid positions were recorded at 10, 20, 30, 40, 50, and 60 min. During observation, the choice arena was illuminated from below by a red light for 30 s. Aphids were counted as having chosen for one of the options only if they were present on the mesh directly below any leaflet part. In the experiment where a non-infected mature leaflet was compared with an empty chamber, any aphid present on the mesh of the empty chamber was counted as having chosen for the empty chamber. The total number of aphids showing a choice was counted and the mean number of aphids was calculated over time for each of the experiments.

EPG monitoring

The EPG (DC system) technique was used to monitor plant penetration by young apterous adult aphids for 8 h (Alvarez et al., 2006) on PLRV-infected and non-infected plants at three different times: (i) on Day 0, the same day of the inoculation with PLRV (8-day-old plants); (ii) 27 days after PLRV-inoculation (35-day-old plants), when PLRV symptoms were not yet visibly expressed (called PLRV-27 and control-27 in Table 1); and (iii) 65 days after PLRV-inoculation (73-day-old plants), when PLRV symptoms were evident on infected plants (called PLRV-65 and control-65 in Table 1).

Four plants of each treatment were placed in a Faraday cage and signals of two individual aphids per plant (eight aphids in total) were monitored simultaneously. Fifteen to 20 replicates (individual aphids) per treatment were obtained (n, Table 1). Aphids were placed on the abaxial side of the third leaf from the apex, which was nearly fully expanded. Electrical penetration graphs were recorded at about 20 °C and constant light, immediately after wiring the aphids.

EPG waveforms, waveform patterns, and parameters

The recorded EPGs were analysed by distinguishing the following waveforms or waveform patterns: (i) waveform C, stylet pathway phase; in fact, waveform C includes four pooled pathway waveforms/activities that partly overlap and/or cannot be separated as such, that is, waveform A, first stylet contact and epidermis penetration; waveform B, intercellular sheath salivation; waveform C, stylet movements (mainly); and waveform pd (potential drop), an intracellular stylet puncture. Waveform E, phloem phase, is separated into (ii) waveform E1, sieve element salivation and (iii) waveform E2, phloem sap ingestion with concurrent salivation; (iv) waveform E1e, assumed to reflect extracellular watery salivation; (v) waveform F, derailed stylet mechanics (stylet penetration difficulties); and (vi) waveform G, active intake of water from xylem elements (Tjallingii, 1990). Waveforms were characterized in a number of EPG features or parameters, divided here into four categories: (i) number of times waveforms occurred; (ii) total (summed) duration of each waveform; (iii) time to the first occurrence of waveforms; and (iv) numbers of aphids with sustained phloem ingestion (sE2) within each plant treatment (Table 1).

Statistical analysis

The response of aphids to either of two odour sources at each time-point (olfactometer assay) was scored by counting the number of aphids present directly underneath a leaflet or, in the case of the empty chamber, any aphid present on the mesh below the empty chamber. The binomial test was used to analyse the data after 60 min, assuming the null hypothesis of no preference between the treatments (aphids not found below any leaflet were not taken into account in the statistical analysis).

The proportions of aphids preferring PLRV-infected leaves or control leaves (relative to the total number of aphids per treatment) over time were studied by regression analysis. Student's t analysis was used to test for linear relationship between percentages of aphids and time (regression coefficient). For statistical analysis, we used the software package SPSS 12.0.1 for Windows (SPSS Inc., Chicago, IL, USA).

The EPG parameter values were established for each individual aphid, and then the mean and standard error of the mean (SEM) of the total number of aphids per treatment were calculated. Times to first E2 and first sE2 since the start of the first probe in the experiment were calculated only for those aphids that showed any E2 or sE2, respectively. The Mann–Whitney U-test was used to test for plant penetration differences between infected and control plants (SPSS 12.0.1). Differences in numbers of aphids showing sustained phloem ingestion (sE2) in the 8-h EPG recordings were tested by Fisher's exact test (Preacher & Briggs, 2001). In order to assess the probing and feeding behaviour of *M. persicae* on Kardal leaves of the same developmental stage (third leaf from apex) but increasing plant age, we analysed the EPG data of the three non-infected controls (control-0, -27, and -65) by Kruskall–Wallis followed by multiple comparisons with Bonferroni correction, hence the α-value was lowered to account

Table 1 Electrical penetration graph (EPG) parameters (mean ± SEM) for 8-h monitoring of *Myzus persicae* on potato cv. Kardal plants, 27 and 65 days after potato leafroll virus (PLRV)-infection and on non-infected control plants of the same ages. The parameters are divided in four categories of data processing: number of waveform periods, total time, time to a certain event, and number of aphids with sustained phloem sap ingestion

Treatment[1]	Symptoms PLRV	Category 1: number of waveform periods[3] n[2]	Probes	Probes <3 min	C	E1e	Single E1[4]	E1 fraction[4]	E2	sE2	F	G
Control-27	Non-infected	20	59 ± 4	14 ± 2	70 ± 4	3.6 ± 0.5	9.6 ± 1.0	0.7 ± 0.2	1.1 ± 0.3	0.2 ± 0.1	1.0 ± 0.3	0.5 ± 0.1
PLRV-27	No symptoms	20	56 ± 3	13 ± 3	62 ± 5	5.1 ± 0.6	11.3 ± 1.3	0.3 ± 0.1	0.3 ± 0.1	0	1.4 ± 0.5	1.1 ± 0.2
Control-65	Non-infected	15	42 ± 6	12 ± 2	52 ± 6	0.9 ± 0.3	6.7 ± 1.5	0.8 ± 0.2	1.3 ± 0.4	0.3 ± 0.2	2.7 ± 1.0	0.8 ± 0.2
PLRV-65	Symptoms	17	41 ± 5	6 ± 1*	51 ± 5	2.8 ± 0.6*	9.4 ± 1.0*	1.5 ± 0.4	2.0 ± 0.6	0.6 ± 0.2	0.5 ± 0.2*	0.7 ± 0.2
		Category 2: total time (min) n	Probing		C	E1e	Single E1[4]	E1 fraction[4]	E2		F	G
Control-27	Non-infected	20	297 ± 12		183 ± 12	2.5 ± 0.3	37 ± 5	12.9 ± 7.4	10 ± 8		35 ± 12	16 ± 5
PLRV-27	No symptoms	20	332 ± 11		203 ± 14	10.7 ± 4.8	39 ± 4	2.1 ± 1.3	1 ± 1		33 ± 11	26 ± 5
Control-65	Non-infected	15	322 ± 20		174 ± 17	1.3 ± 0.7	25 ± 8	7.2 ± 2.7	28 ± 15		44 ± 13	45 ± 15
PLRV-65	Symptoms	17	312 ± 21		186 ± 15	0.7 ± 0.2	26 ± 6	10.5 ± 4.2	62 ± 26		11 ± 5*	17 ± 7
		Category 3: time (min) to event n	First probe from start of recording	First E1 in the experiment from first probe	First E1 from start of the probe	First E2 in experiment from first probe[5]	First sE2 in experiment from first probe[5]		Category 4[6] sE2 n (%)			
Control-27	Non-infected	20	2.9 ± 0.8	95 ± 19	9.0 ± 0.9	253 ± 51 (n = 9)	358 ± 23 (n = 3)		3 (15)			
PLRV-27	No symptoms	20	0.9 ± 0.2*	104 ± 27	12.8 ± 1.2	397 ± 31 (n = 6)	– (n = 0)		0 (0)			
Control-65	Non-infected	15	5.0 ± 1.7	189 ± 35	13.1 ± 2.4	262 ± 56 (n = 8)	151 ± 109 (n = 3)		3 (20)			
PLRV-65	Symptoms	17	4.4 ± 1.7	61 ± 19*	10.0 ± 1.7	190 ± 32 (n = 11)	176 ± 57 (n = 6)		6 (35)			

[1]Treatment: control-27 and control-65, non-infected plants 27 and 65 days, respectively, after the exposure to non-viruliferous aphids; PLRV-27 and PLRV-65, infected plants 27 and 65 days, respectively, after the exposure to viruliferous aphids.

[2]n, number of replicates (aphids).

[3]Category 1: probes <3 min, probes shorter than 3 min before first E1; C, pathway phase; E1e, salivation at extracellular voltage level; E2, ingestion at intracellular voltage level; sE2, sustained ingestion at phloem level (E2 lasting >10 min); F, derailed stylet mechanics; G, xylem ingestion.

[4]Salivation E1 periods split into single E1 (i.e., without subsequent E2), and E1 fractions (i.e., embedded in periods with one or more subsequent E2 periods); E2 periods only occur in phloem phase together with E1.

[5]First E2 in experiment from first probe and first sE2 in experiment from first probe were calculated only with the aphids (n) showing E2 and sE2, respectively (n is indicated next to each value between brackets).

[6]Category 4: number and percentage of aphids with sustained E2. No statistical differences between control and PLRV-infected plants of the same age, $P<0.05$, Fisher's exact test.

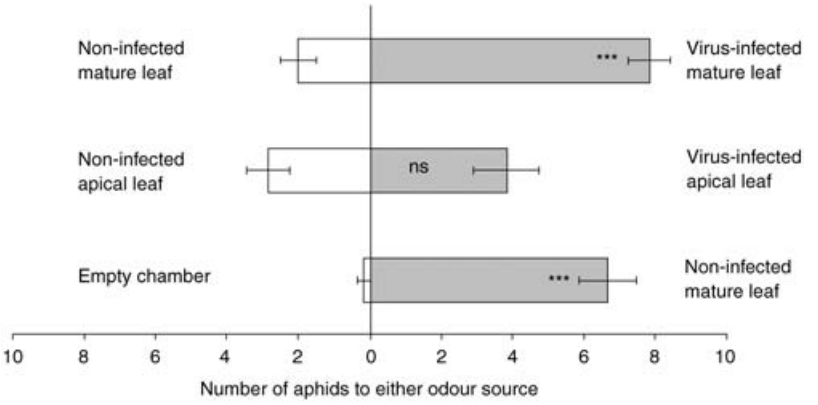

Figure 2 Choice test results of *Myzus persicae* to headspace volatiles from potato leaves in olfactometer assay: (1) non-infected mature leaflet vs. empty chamber (bottom bars), (2) virus-infected apical leaflet vs. non-infected apical leaflet (middle bars), and (3) virus-infected mature leaflet vs. non-infected mature leaflet (upper bars) are compared. Only the number of aphids located directly below potato leaves was scored after 60 min. For each replicate test, 40 aphids have been used. Error bars (SEM) are indicated next to each bar. ***Significant preferences within test ($P<0.001$), binomial test. ns, no significant preferences within test.

for the number of comparisons performed (Weisstein, 1999).

Results

Two-chamber olfactometer test

The results of the preference tests (Figure 2) showed: (i) preference of aphids for a non-infected mature leaflet over an empty chamber ($P<0.001$); (ii) no difference in attraction between apical infected and non-infected leaves after 1 h ($P = 0.22$); and (iii) a preference of aphids for mature PLRV-infected leaves over non-infected mature leaves ($P<0.001$). There was a clear increase in the proportion of aphids preferring virus-infected mature leaves over time ($R = 0.968$, $t = 7.713$, $P = 0.002$) to the non-infected mature leaves ($R = 0.140$, $t = 0.808$, $P = 0.464$). On the contrary, no such increase in proportion was found with virus-infected apical leaves ($R = 0.751$, $t = 2.276$, $P = 0.085$) or non-infected apical leaves ($R = 0.567$, $t = 2.287$, $P = 0.084$) (Figure 3).

EPG monitoring of plant penetration behaviour

Electrical penetration graph parameters were divided into four categories (Table 1). Twenty-seven days after infection of the plants with PLRV, the time to the first probe was the only parameter that differed significantly between aphids on infected plants not yet showing PLRV symptoms (PLRV-27) and aphids on non-infected control plants (control-27) (Mann–Whitney U-test: $U = 83$, $P<0.05$). In contrast, we found several EPG parameters to differ at 65 days after infection between aphids on PLRV-infected plants with symptoms (PLRV-65) and non-infected control plants (control-65) (Table 1).

Electrical penetration graphs showed fewer probes shorter than 3 min before the first phloem salivation activity E1 (Mann–Whitney U-test: $U = 78.5$, $P<0.05$), and fewer F periods (waveform reflecting derailed stylet mechanics) in aphids on PLRV-65 plants than on control-65 plants (Mann–Whitney U-test: $U = 60$, $P<0.05$); also aphids on PLRV-65 plants had shorter summed duration of F periods than on control-65 plants (Mann–Whitney U-test: $U = 66.5$, $P<0.05$) (Table 1). But higher number of extracellular salivation (E1e) periods (Mann–Whitney U-test: $U = 65.5$, $P<0.05$) and sieve element salivation (E1) were shown on PLRV-65 plants than on control-65 plants (Mann–Whitney U-test: $U = 71.5$, $P<0.05$) (Table 1). Most of these E1e periods started as E1 (i.e., the transmembrane potentials of punctured sieve elements). Furthermore, aphids on control plants had decreased the total duration on E1e and the number of E1e with plant age (i.e., from control-27 to control-65, total time on E1e: 2.5 ± 0.3 and 1.3 ± 0.7 min, respectively; Mann–Whitney U-test: $U = 90$, $P<0.05$; and number of E1e: 3.6 ± 0.5 to 0.9 ± 0.3, respectively; Mann–Whitney U-test: $U = 37.5$, $P<0.05$).

The start of F events within probes could give us information on the tissue depth at which the possibly causal factors/constraints were located. We ordered all the F periods into two classes according to the starting times from the start of probes (irrespective of aphid individuals) for each treatment (i.e., F starting between 0 and 8 min, and later than 8 min from start of the probe) (Figure 4). The time to the first phloem activity (sieve element salivation E1) from the first probe was shorter on PLRV-65 than on control-65 plants (Mann–Whitney U-test: $U = 38.5$, $P<0.05$) (category 3, Table 1).

The numbers of aphids showing sustained phloem ingestion during the 8 h of recording period (number of aphids showing sE2) on the PLRV-65 plants compared to non-infected control plants were not significantly different (number of aphids showing sE2: 6 out of 17 and 3 out of 15 aphids, respectively; Fisher's exact test $P = 0.287$). We found no differences between EPG parameters on control-0

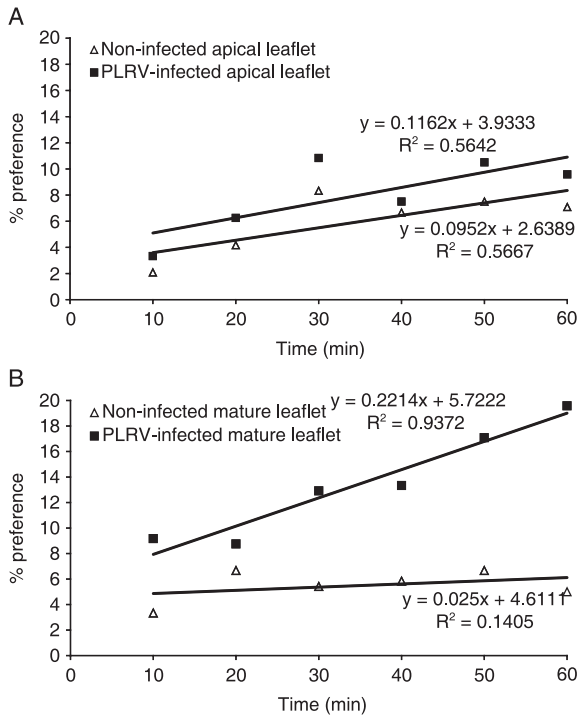

Figure 3 Choice test results of *Myzus persicae* to headspace volatiles from potato leaves of different age. Regression analysis was used to study the relationship between observation time and treatment of two separate experiments contrasting (A) potato leafroll virus (PLRV)-infected apical leaflets vs. non-infected apical leaflets, and (B) PLRV-infected mature vs. non-infected mature leaflets. Numbers of aphids located directly below potato leaves were scored every 10 min for 1 h. For each replicate, 40 aphids had been used. The percentages of aphids located either below PLRV-infected leaflets or control leaflets for each treatment were calculated over the total number of aphids. Dots are means of six replicates. Regression lines are shown for each treatment. PLRV-apical leaflet, $t = 2.276$, $P = 0.085$; non-infected apical leaflet, $t = 2.287$, $P = 0.084$; PLRV-mature leaflet, $t = 7.713$, $P = 0.002$; non-infected mature leaflet, $t = 0.808$, $P = 0.464$.

(8-day-old plants) and control-27 (35-day-old plants), but control-65 (73-day-old plants) showed more waveform F (number of periods and total time), more total G (xylem drinking), and a longer time to the first phloem activity in experiment than control-0 leaves (Table 2).

Discussion

Attraction of aphids by headspace volatiles

The resistance to aphids in potato cv. Kardal strongly depends on the developmental stage of the leaves (Alvarez et al., 2006). This cultivar has a high level of resistance to aphids in young apical leaves but older leaves become

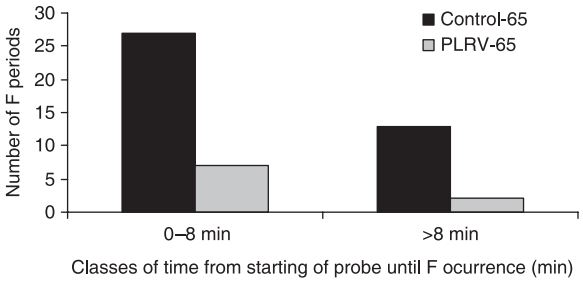

Figure 4 Number of derailed stylet mechanics (F waveform) per class of time from the starting of the probe. The time until the first F on each probe across all aphids per treatment (control-65 and PLRV-65) was counted, then the F periods were sorted into two classes of time: 0–8 min and >8 min.

susceptible with maturity and senescence. Production of different headspace volatiles could be one of the factors contributing to the increased attraction of *M. persicae* to mature leaves of potato cv. Kardal. The response of *M. persicae* to headspace volatiles from PLRV-infected and non-infected potato plants also seemed to depend on the age of the leaf. Mature leaves of virus-infected plants

Table 2 Electrical penetration graph (EPG) parameters (mean ± SEM) changing with plant age. *Myzus persicae* on potato cv. Kardal plants (potato leafroll virus non-infected controls) (8-h monitoring)

		Number of waveform periods	
Treatment[1]	n	E1e	F
Control-0	35	2.2 ± 0.4ab	0.3 ± 0.2a
Control-27	20	3.6 ± 0.5a	1.0 ± 0.3ab
Control-65	15	0.9 ± 0.3b	2.7 ± 1.0b
		Total time (min)	
	n	F	G
Control-0	35	6 ± 4a	9 ± 3a
Control-27	20	35 ± 12ab	16 ± 5ab
Control-65	15	44 ± 13b	45 ± 15b
		Time (min) to event	
	n	First E1 in the experiment from first probe	
Control-0	35	69 ± 14a	
Control-27	20	95 ± 19ab	
Control-65	15	189 ± 35b	

[1]Treatment: control-0, -27, -65, non-infected plants, 0, 27, and 65 days, respectively, after exposure to non-viruliferous aphids; the age of the plants on EPG recording were 8, 35, and 73 days old, respectively; parameter descriptions are the same as in Table 1. Numbers in a column followed by different letters are significantly different at $P<0.002$ according to Kruskal–Wallis test followed by multiple comparisons with Bonferroni correction.

attracted more aphids than mature non-infected leaves. In contrast, virus-infection of apical leaves did not result in an increase in aphid attraction, suggesting that volatile induction is leaf age dependent.

Plant penetration behaviour

In this study, we used EPG parameters to evaluate whether the resistance to aphids in young leaves of potato cv. Kardal can be suppressed by the presence of the virus. PLRV-infection appeared to change probing behaviour of aphids only after visual symptoms of PLRV disease had developed, except for one case. Electrical penetration graph data were processed into 23 quantified behavioural features (Table 1). The only one parameter that differered between PLRV and non-infected plants before symptoms were show (–27 plants) was the time to (before) the first probe. This is in fact the only parameter that is likely to reflect odour effects. But this is not supported by the olfactometer results, which would suggest an effect in opposit direction and with plants after showing symptoms, in which no difference was found. We cannot explain this discrepancy. Six of other parameters that differed significantly were between plants after showing symptoms (–65 plants). Three of them parameters suggested enhanced plant penetration by the aphid stylets on PLRV-infected plants at the epidermal/mesophyll level, that is, (i) the lower number of probes shorter than 3 min before first phloem activity (E1), (ii) the lower number of derailed stylet mechanic (F), and (iii) shorter total duration of (F) events.

Probes shorter than 3 min before the first E1 waveform suggested the presence of some resistance factors in these tissues that are already active before the stylets reach the phloem. The lower number of early withdrawals in PLRV-infected plants suggested a breakdown of resistance components. On virus-infected plants (PLRV-65), waveform F occurred less often, and the total time was shorter than in control-65 (non-infected plants), mainly within the first 8 min of a probe (Figure 4). Stylets are assumed to penetrate about one cell layer per min. Thus, the mechanical derailment of stylet movements (waveform F) seems to be caused mainly by some factors in epidermal/mesophyll tissue. In general, waveform F has been reported to occur in the mesophyll (Tjallingii, 1987). Waveform F was also found more often and with longer duration in other *Solanum* spp. plants resistant to *M. persicae* (Alvarez et al., 2006). The constraints to stylet movement diminished with the visual developments of PLRV-infection symptoms. Leaf roll symptoms induced by PLRV have been visually observed by an upwards curling of the pale, yellowish leaf edges (Beemster & de Bokx, 1987), thickening of cell walls in primary phloem cells of stems and petioles, accumulated callose in sieve elements (Thomas, 1996), and by phloem necrosis and excessive callose formation in the phloem (de Bokx, 1987). From these PLRV symptoms, especially those related to the thickening or reinforcement of the cell wall, an opposite effect on F occurrence was expected. It seems that other, yet unknown, factors were responsible for diminished mechanical constraint of the stylet movements in PRLV-65 plants. On the other hand, aphids on non-infected plants showed an increase of F with plant age (Day 0–65; Table 2). Plant age seemed to play a role regarding F waveform on cv. Kardal. These phenomena need further study.

Aphids probing on plants with PLRV symptoms (PLRV-65) had a higher number of periods with extracellular watery salivation (E1e) than on non-infected control plants of the same age. Waveform E1e is very similar to waveform E1, which is related to salivation at phloem sieve elements (Tjallingii & Hogen Esch, 1993). However, E1e occurs without initial potential drop, which indicates the transmembrane potential when the stylet tips puncture a cell. Thus, during E1e the stylet tip remains extracellular whereas the other waveform features suggest watery salivation activity as in a sieve element. Waveform E1e is embedded in stylet pathway (waveform A, B, and C) and mostly occurs in the mesophyll or non-phloem vascular tissue. In our study, however, E1e mainly occurred after normal E1. During normal sieve element salivation, the voltage level changed from intra- to extracellular, which suggests that the membrane potential of the punctured sieve element collapsed, which is very likely a symptom of cell death. E1 salivation always preceded phloem ingestion (E2) and seemed necessary to suppress primary wound reaction in sieve elements (Tjallingii, 2006; Will & Van Bel, 2006). Long periods of extended sieve element salivation (E1) were normally observed in young Kardal leaves (Tjallingii, 2006). Now, it appeared that these extended E1 periods on Kardal lead to the death of a sieve element after which the aphids continued with watery salivation, at least for some time. Hence, extended sieve element salivation and sieve element death can be considered as resistance factors at the phloem level, which decrease with plant age (control-27 to control-65). On PLRV-65 leaves, in contrast, the number of E1e (and so this phloem resistance) remained at the initial high level of control-27 leaves (Table 1). Our data did not provide enough information to understand the E1e phenomena and further studies are needed on this topic.

The period before the first phloem activity in the experiment (time to first E1 in experiment from first probe; Table 1) is determined by epidermal, mesophyll, general vascular, and early phloem factors. The significantly shorter time to first E1 on PLRV-infected than on non-infected mature leaves suggests reduced resistance in these tissues. Time to first E1 parameter increased with plant age from control-0 to control-65, and the total time in G periods

(xylem intake) increased with plant age (G increased when no phloem feeding occurred, suggesting that longer time in G indicates starvation). However, on PLRV-65 leaves, the time to first salivation at sieve element and the time in G remained at the initial control-0 and control-27 level, respectively. As phloem salivation is essential for inoculation of persistently transmitted viruses like PLRV (Prado & Tjallingii, 1994), early E1 might imply early virus inoculation after landing, although this behaviour has no advantage for the virus in already-infected plants (such as our PRLV-65).

Early phloem sap ingestion (E2) on PLRV-infected plants, on the other hand, will enhance virus transmission as aphids will acquire the virus sooner. However, we found that PLRV-infected plants did not significantly affect the time to first E2. Other EPG parameters related to phloem sap ingestion were not different either, for example, the number of E2, total time in E2, and total number of aphids with sE2. Thus, the phloem sap uptake by aphids on PLRV-infected plants was not different from non-infected plants. Our EPG recording period of 8 h did not provide data with respect to long-term sap ingestion, but Castle & Berger (1993) showed a better performance of *M. persicae* feeding on PLRV-infected potato plants. Whether this is due to a better phloem sap quality or to improved feeding behaviour remains unclear.

The presence of PLRV leads to important structural and metabolic changes in the host plant. Herbers et al. (1997) found that distorted plasmodesmata occur within the phloem tissue of infected plants, and that there was an altered carbohydrate allocation pattern causing impaired phloem sucrose loading, an accumulation of soluble sugars and starch, and a reduced photosynthetic capacity of the leaves. Phloem exudates of *C. pepo* infected by ZYMV showed a changed amino acid composition (although total concentration of amino acids remained the same), which may have changed the performance of aphids (Blua et al., 1994). Changes in phloem sap composition may affect the dispersion of the vector as well and, thus, the epidemiology of the virus. Fiebig et al. (2004) found that BYDV-infection affected plant suitability for its aphid vector and promoted the production of aphid alatae, and this might be the driving force for increased virus spreading in the field. According to McElhany et al. (1995), the implications of vector preferences on patterns of disease dispersion are complex. The dynamics of vector-borne diseases depend on ecological factors. They are the complex result of the changing frequency of sick plants in the population, local spatial structure of the host, and pathogen and vector populations. Results of McElhany et al. (1995) for BYDV showed that the vector remained infective for a long period after visiting a diseased host. Therefore, a vector preferring healthy hosts would spread the disease more than a vector preferring diseased hosts. Predictions of the way in which a vector preference towards PLRV-infected plants would affect the spreading of the disease should be investigated under field conditions.

Conclusions

In our plant–virus–vector system, PLRV-infection directly or indirectly influenced the plant–aphid interaction as (i) odours of infected plants attracted significantly more vectors than non-infected plants although no such odour effect was supported by EPG data and (ii) virus-infected plants enhanced stylet penetration into the plant tissue (as reflected by the reduced number of short probes, reduced constraints in terms of mechanical derailment, and reduced time to the first phloem salivation); on the other hand, (iii) phloem factors showed an opposite effect as indicated by increased E1e and E1 numbers and by the fact that phloem feeding did not improve significantly.

During the 8-h monitoring, the phloem sap uptake was not affected by PLRV-infection of the plants. Therefore, better aphid performance on PLRV-infected potato plants, as reported by Castle & Berger (1993), might have been related to enhanced phloem quality. It would be interesting to investigate whether PLRV-infection results in increased nutritional quality of the phloem sap on potato cv. Kardal. The transmission efficiency of the persistent circulative PLRV is also expected to be affected on PLRV-infected plants because these plants attract more aphids than non-infected plants.

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