

Location of the Mechanism of Resistance to *Amphorophora agathonica* (Hemiptera: Aphididae) in Red Raspberry

D. M. LIGHTLE,¹ M. DOSSETT,² E. A. BACKUS,³ AND J. C. LEE⁴

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ABSTRACT The aphid *Amphorophora agathonica* Hottes (Hemiptera: Aphididae) is an important virus vector in red (*Rubus idaeus* L.) and black (*Rubus occidentalis* L.) raspberries in North America. Raspberry resistance to *A. agathonica* in the form of a single dominant gene named Ag_1 has been relied upon to help control aphid-transmitted plant viruses; however, the mechanism of resistance to the insect is poorly understood. Aphid feeding was monitored using an electrical penetration graph on the resistant red raspberry ‘Tulameen’ and compared with a susceptible control, ‘Vintage’. There were no differences in pathway feeding behaviors of aphids as they moved toward the phloem. Once in the phloem, however, aphids feeding on resistant plants spent significantly more time salivating than on susceptible plants, and ingested significantly less phloem sap. This suggests that a mechanism for resistance to *A. agathonica* is located in the phloem. Reduced ingestion of phloem may result in inefficient acquisition of viruses and is a likely explanation for the lack of aphid-transmitted viruses in plantings of resistant cultivars.

KEY WORDS electrical penetration graph, plant resistance, feeding behavior, antixenosis

Aphid-transmitted viruses are an important problem for red (*Rubus idaeus* L.) and black (*Rubus occidentalis* L.) raspberry production in North America. Viruses such as Raspberry leaf mottle virus (family *Closteroviridae*, genus *Closterovirus*, RLMV), Raspberry latent virus (family *Reoviridae*, genus *Reovirus*, RpLV), and Rubus yellow net (family *Caulimoviridae*, genus *Badnavirus*, RYNV) in red raspberry and Black raspberry necrosis virus (family *Secoviridae*, genus unassigned *Secoviridae* species, BRNV) in black raspberry, cause a decline in cane health and fruit quality, resulting in a shortened life of the infected plantings (Halgren et al. 2007, Tzanetakis et al. 2007, Quito-Avila et al. 2011). In North America, the most important vector of these viruses is the large raspberry aphid, *Amphorophora agathonica* Hottes (Hemiptera: Aphididae). *A. agathonica* is distributed throughout the United States and Canada and colonizes only *Rubus* species (Blackman and Eastop 2000).

Host plant resistance has long been recognized as an effective method for reducing virus spread (van Emden 2007). Indeed, this practice has played a major role in reducing the spread of viruses in resistant red raspberry by *A. agathonica* in North America and by the closely related *Amphorophora idaei* Börner

(Hemiptera: Aphididae) in Europe (Isaacs and Trefor Woodford 2007). Resistance to *A. agathonica* is conferred by the Ag_1 gene, which was originally found in ‘Lloyd George’ and has since been used widely in breeding (Daubeny 1966). Ag_1 , a single dominant gene, has been effective for >50 yr, and resistance has not been overcome by the most common biotype of *A. agathonica*, although an Ag_1 -breaking biotype of *A. agathonica* has been reported in British Columbia (Daubeny and Anderson 1993). Despite reliance upon this gene, the mechanism of resistance is not entirely understood.

Kennedy and Schaefer (1974b) presented evidence that Ag_1 plants were resistant through antixenosis, or aphid nonpreference for the host, which led to host rejection and eventual aphid death. In choice trials, aphid colonies became established only on susceptible cultivars, whereas in no-choice trials, aphids experienced decreased survival and high desertion rates on resistant cultivars compared with susceptible cultivars (Kennedy and Schaefer 1974b). However, phloem contact was not entirely avoided because histological studies of stylet sheath pathways showed that aphids on resistant plants reached the phloem sieve elements (Kennedy 1974). Furthermore, Kennedy and Schaefer (1975) showed that the ingested from resistant plants was more dilute than on susceptible plants based on honeydew and whole-body homogenate analysis. They hypothesized that in addition to antixenotic mechanism, there was a nutritional role to the resistance against *A. agathonica*. Yet, questions about the resistance mechanism of Ag_1 remain. It is unlikely that a substantial nutritional deficit exists in

¹ Corresponding author: Oregon State University, 4017 Ag & Life Sciences Bldg., Corvallis, OR 97330 (e-mail: lightled@onid.orst.edu).

² Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada, 6947 #7 Hgwy., Agassiz, BC, Canada V0M 1A0.

³ USDA-ARS, Crop Diseases, Pests and Genetics Research Unit, 9611 Riverbend Ave., Parlier, CA 93619.

⁴ USDA-ARS, Horticultural Crops Research Unit, 3450 SW Campus Way, Corvallis, OR 97331.

Ag₇ raspberries because several other aphid species, including *Aphis rubicola* Oestlund (Hemiptera: Aphididae) and the closely related *A. idaei*, will readily feed on *Ag₇* plants without an impact on survival or ability to colonize these plants (Kennedy et al. 1973, Kennedy and Schaefer 1975).

Insight on potential mechanisms of resistance can be obtained by studying the feeding behaviors of aphids on resistant and susceptible plants. The electrical penetration graph (EPG) technique has been invaluable in measuring the feeding behavior of aphids and other hemipterans (Walker 2000). In EPG, the insect is wired into an electrical circuit with a host plant. The insect's stylets then act as a switch, completing the circuit when the stylets are inserted into the plant. Changes in output voltage over time, known as waveforms, represent different activities, such as phloem salivation or ingestion, within the plant. The objective of this study was to compare the feeding behavior of *A. agathonica* on resistant and susceptible hosts using EPG to determine which plant tissues were most important for resistance.

Materials and Methods

Plants and Insects. The raspberry cultivars selected for this study were the resistant 'Tulameen' and susceptible 'Vintage,' a new release from the USDA Horticultural Crops Research Unit and Oregon State University cooperative breeding program. Tulameen was selected for aphid resistance conferred by gene *Ag₇* (Daubeny and Anderson 1991, Daubeny and Kempler 2003). Vintage and Tulameen plants were obtained as hardened-off tissue culture plugs from Sakuma Brothers (Burlington, WA). Randomly sampled individuals of each cultivar tested negative for the presence of all known aphid-transmitted raspberry viruses. Plugs were grown individually in 10-cm pots (Dura-Pot, Lake Oswego, OR) of Sunshine Professional Growing Mix (Sun Gro Horticulture, Bellevue, WA) amended with 8 g/gal of 21-2-11 N-P-K fertilizer (Apex, Boise, ID). The plants were grown in a greenhouse at a photoperiod of 16:8 (L:D) h and 21°C daytime and 15.5°C nighttime temperatures, and used when they were ≈30 cm tall.

Six apterous parthenogenic female *A. agathonica* were collected from commercial red raspberry fields in Whatcom Co., WA, in July 2010 and offspring from these females were combined into a single colony. This aphid colony was maintained in a Percival growth chamber at 18 ± 2°C under fluorescent growth lights at a photoperiod of 16:8 (L:D) h. The aphids were provided virus-free 'Meeker' red raspberry grown from root stock (Sakuma Brothers, Burlington, WA) in 12.5-cm pots (Dura-Pot) of Sunshine Professional Growing Mix (Sun Gro Horticulture) amended with 8 g/gal of 21-2-11 N-P-K fertilizer (Apex, Boise, ID). Aphids in the study were used within 3 d of molting into the adult stage unless noted otherwise.

Aphid Performance on Tulameen. Because *Ag₇*-breaking clones of *A. agathonica* have been reported (Daubeny and Anderson 1993), Tulameen plants were

tested to verify that they were resistant to the aphid clones used in this study. Two Tulameen plants were placed in one mesh aluminum cage (35.5 by 35.5 by 35.5 cm) and two Vintage plants were placed in a second cage. Twenty adults and 20 nymphs were evenly distributed in each cage. The plants and aphids were maintained in this insect cage in a greenhouse at a photoperiod of 16:8 (L:D) h and 21°C daytime and 15.5°C nighttime temperatures. After 2 wk, the aphid populations in each cage were counted. This procedure was replicated three times.

Aphid Settling Behavior. To test whether resistant plants may affect aphid settling behaviors or tendency of an aphid to immediately leave the plant (Pelletier and Giguere 2009), aphids were observed for differences in behavior on Tulameen or Vintage leaves. Adult aphids were starved in a petri dish for 1 h to be consistent with the 1-h handling time aphids underwent in the electronic monitoring protocol (see below), then placed individually on a trifoliate leaf cutting from a resistant or susceptible plant. Leaf cuttings were obtained by excising the leaf with a razor blade and immediately submerging the petiole in water, which reduces the likelihood that the resistance properties are lost (Kennedy and Schaefer 1974a). The aphids' position (on top of or under leaf, on stem, desertion of plant) and activity (walking, settled) was recorded every 5 min for 1 h. Observations on aphids in each treatment were replicated 15 times.

Electronic Monitoring. The EPG system used for this study was the AC-DC EPG developed by Backus and Bennett (2009). Before the beginning of monitoring, adult aphids were starved for 1 h, during which time the aphids were immobilized and attached to the insect electrode via a 1-2-cm long, 25.4- μ m-diameter gold wire using silver conductive glue (1 part school glue:1 part water:1 part silver flake by weight). A second copper electrode was inserted into the soil at the base of the plant. Direct current (DC) signal (40 mV) was applied to the plant and data were collected using a giga-Ohm (10^9) input resistor. The data sample rate was 100 Hz. EPG recordings were acquired using a DI-710 and Windaq Acquisition Software (Dataq Instruments Inc., Akron, OH).

Recordings began every evening at 1800 hours and lasted for 12 h. Recordings were conducted overnight to reduce interference resulting from laboratory activities during the daytime. The plants and aphids were set up in the laboratory in a metal Faraday cage to reduce extraneous electrical noise. The temperature ranged from 20 to 24°C, and ambient light was provided. Each aphid and plant was used in only one recording. Electronic monitoring for each insect-plant combination was replicated 20 times for each treatment.

Waveform data were imported into The Observer XT version 10.0 (Noldus Information Technology, Wageningen, The Netherlands), and each waveform event (an uninterrupted performance of a behavior during a probe) was coded as one of the common DC system aphid waveform names (Tjallingii 1988). The waveforms scored were as follows: nonprobing, C,

potential drops, G, F, E1, and E2. Waveform C encompasses waveforms A, B, and C that represent initial stylet penetration, sheath salivation, and intercellular movement through the epidermis and mesophyll, respectively. These behaviors are commonly referred to as pathway phase and were scored together for simplicity. Potential drops (pd) represent intracellular punctures made by the stylets as they travel between parenchyma and mesophyll cells. Waveform G is correlated with ingestion from xylem. Together E1 and E2 comprise phloem phase; E1 represents salivation into the phloem sieve elements and E2 represents ingestion from the phloem sieve elements. The waveform F, signifying penetration or stylet difficulties by the aphid, was rarely observed ($n = 3$ on Vintage, $n = 2$ on Tulameen; $P > 0.1$; data not shown) and was pooled with pathway behaviors because it was typically bordered on both sides by the C waveform.

Statistics. Statistical variables for EPG, whose names were adapted from Backus et al. (2007), were calculated using an automated Excel workbook (Microsoft, Redmond, WA; Sarria et al. 2009). All variables concern the durations or numbers of behaviors, either as a mean per insect or a mean of a mean, (i.e., mean per event per insect) because each insect was a statistical unit. Individual aphids that did not perform a certain behavior were excluded. Differences in EPG variables between Vintage and Tulameen were tested using analysis of variance (ANOVA) mixed models (PROC GLIMMIX), with treatment as a fixed factor and the night tested (i.e., block) as a random factor. Degrees of freedom were calculated with the Kenward–Rogers adjustment as recommended for use in mixed models by Littell et al. (2006). The GLIMMIX models were iteratively optimized using the protocol of Littell et al. (2006). Differences between treatments were calculated using Wald's F-test. For the aphid settling observations, differences in aphid position (top or underside of leaf, or off leaf) at each 5-min time interval were tested with a chi-square analysis, and the number of times an aphid changed location on the leaf was tested with an ANOVA (PROC GLIMMIX). All statistics were conducted using SAS 9.2.3 (SAS Institute 2008) with $\alpha = 0.05$.

Results

Aphid Performance on Tulameen. No aphid colonies formed on Tulameen plants in the greenhouse in any of the three replicates, whereas aphids on the corresponding Vintage plants formed colonies of >50 aphids on each plant. Tulameen was thus considered to be resistant to the aphid clones used for the EPG study. Additional observation of *A. agathonica* derived from the same clones on Tulameen in a no-choice situation also failed to maintain a colony (M.D., unpublished data).

Aphid Settling Behavior. There were no significant differences in aphid position or movement on Tulameen leaf cuttings versus Vintage leaf cuttings. At each 5-min time interval, there was no significant difference in the number of aphids that moved to the

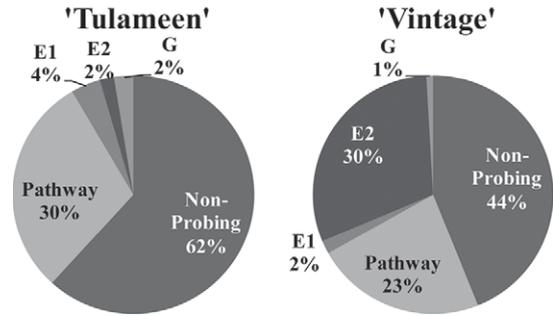


Fig. 1. Proportion of time (percentage) spent performing each feeding behavior by aphids on resistant Tulameen ($n = 18$) and susceptible Vintage ($n = 17$) during 12 h of EPG monitoring. Waveform definition: nonprobing, stylets withdrawn from plant; pathway, stylet activities in epidermis and mesophyll including cell punctures (potential drops); E1, salivation into phloem sieve elements; E2, ingestion from phloem sieve elements; and G, ingestion from xylem.

top or bottom of the leaf ($P > 0.1$ for each interval). At 1 h, 80% of aphids on resistant leaves and 72% of aphids on susceptible leaves had moved to the underside of the leaf and settled with their rostrum against the leaf and held their antennae back, an indication of settling. There was no difference in the number of times aphids changed location on the plant (susceptible = 2.0 ± 0.58 , resistant = 1.8 ± 0.39 ; $F = 0.08$; $df = 1, 27$; $P > 0.5$). Only one aphid on a susceptible plant deserted the leaf altogether.

Electronic Monitoring. Three aphids on Vintage and two aphids on Tulameen did not probe during the entire recording period; data from these aphids were discarded from analyses. The proportion of recording time that aphids spent performing different feeding behaviors is shown in Fig. 1; when combined, all probing behaviors accounted for only 38% of the time spent on Tulameen compared with 56% on Vintage. Accordingly, the mean duration of probing per insect was significantly lower on Tulameen (Table 1). Despite this, the number of probes per insect was greater on Tulameen, including a greater number of short probes (<3 min in duration; Table 1). There were no differences in the number of xylem ingestion events or the duration of xylem ingestion between the two cultivars (Table 1; waveform G).

There were no differences in the mean durations (per event or per insect) of pathway behaviors; nor were there differences in the mean potential drops per insect (Table 1; waveform C, pd). In contrast, there was a significantly higher number of pathway waveform events per insect on Tulameen (Table 1). Aphids on Tulameen were able to reach the phloem more quickly than aphids on Vintage (Table 1; variable shortest duration C before E1).

There were significant differences in the variables relating to phloem phase behaviors. For example, there was no difference in the number of aphids that reached the phloem, with 10 of 18 aphids on resistant plants and 12 of 17 on susceptible plants reaching the sieve elements ($\chi^2 = 0.85$, $df = 1$, $P > 0.1$). However,

Table 1. Mean and standard error of pathway and xylem behaviors performed by *A. gathonica* on resistant Tulameen and susceptible Vintage red raspberry

Parameter	Tulameen	Vintage	df	P
Duration of probing per insect	286.37 ± 47.53a	416.80 ± 48.10b	1, 21.06	0.0112
No. probes	7.77 ± 1.27a	5.17 ± 0.90b	1, 33	0.0045
No. short probes ^a	2.87 ± 0.78a	1.81 ± 0.53b	1, 33	0.0361
No. G events ^b	0.32 ± 0.14	0.12 ± 0.08	1, 33	0.2310
Duration of G per event ^c	55.08 ± 24.71	84.82 ± 38.05	1, 3.98	0.6226
Duration of G per insect ^d	68.90 ± 34.39	90.53 ± 45.19	1, 4.16	0.7743
No. C events ^b	10.23 ± 1.38a	6.14 ± 0.92b	1, 14.38	0.0042
Duration of C per event ^c	26.77 ± 5.02	29.49 ± 5.53	1, 33	0.7513
Duration of C per insect ^d	196.27 ± 33.70	146.24 ± 25.57	1, 22.32	0.1709
No. of pd events ^b	96.95 ± 28.94	71.84 ± 21.63	1, 11.73	0.4448
Shortest duration of C event before E1	39.67 ± 0.71a	61.33 ± 1.04b	1, 20	0.0335
No. E1 events ^b	1.86 ± 0.60	3.00 ± 0.92	1, 20.04	0.2953
No. single E1 probes ^e	1.15 ± 0.33a	0.27 ± 0.13b	1, 33	0.0071
Duration of E1 per event ^c	14.23 ± 3.13a	4.16 ± 0.91b	1, 20	0.0030
Duration of E1 per insect ^d	52.53 ± 10.61a	19.49 ± 3.94b	1, 20	0.0232
No. E2 events ^b	0.48 ± 0.24a	2.23 ± 0.85b	1, 25.92	0.0210
No. sustained E2 events	0.32 ± 0.16a	1.89 ± 0.60b	1, 15.79	0.0078
Duration of E2 per event ^c	18.91 ± 7.12a	104.58 ± 39.42b	1, 14	0.0114
Duration of E2 per insect ^d	23.75 ± 45.29a	312.08 ± 41.35b	1, 20	0.0001

Durations are reported in minutes. Means followed by different letters are significantly different.

^a Defined as probes <3 min in duration.

^b Waveform events; defined in text.

^c Waveform duration per event per insect (Backus et al. 2007).

^d Waveform duration per insect (Backus et al. 2007).

^e Defined as a single E1 event in a probe, without entering E2 or another phloem sieve element.

aphids spent 77% of phloem phase salivating when they were on Tulameen, whereas aphids on Vintage spent only 17% of the time salivating (Fig. 2). Aphids on Tulameen were significantly more likely to perform a single salivation event, withdraw from the sieve elements without ingesting phloem sap, and not re-enter a sieve element in that probe (Table 1; single E1). In addition, the mean durations of phloem salivation, both per event and per insect, were significantly higher on Tulameen (Table 1).

On Vintage, 12 of 17 aphids ingested from the phloem, whereas significantly fewer aphids (4 of 18) feeding on Tulameen were able to successfully do so ($\chi^2 = 6.61$, $df = 1$, $P = 0.011$). An average aphid was

significantly less likely to engage in sustained phloem ingestion >10 min long on Tulameen (Table 1; sustained E2). The mean durations of phloem ingestion, both per event and per insect, were also significantly greater on Vintage than on Tulameen (Table 1; waveform E2).

Discussion

The differences in feeding behaviors recorded between aphids on Tulameen and Vintage largely seem to be confined to factors within the phloem sieve elements. Aphids on Tulameen salivated for much longer into phloem sieve elements and often did not initiate phloem sap ingestion. One role of aphid salivation into the phloem is to prevent sieve tube occlusion through the release of Ca^{2+} binding proteins (Will et al. 2009). An induced defense by the raspberry plant may play a role in preventing the proteins contained in aphid saliva from interfering with callous deposition and wound repair (Tjallingii 2006). In the aphid-resistant melon line 'TGR-1551,' *Aphis gossypii* Glover displayed extremely long salivation durations, often without entering into passive phloem ingestion (i.e., >10 min) (Garzo et al. 2002). TGR-1551 resistance is controlled by the *Agr* gene, which belongs to a group of NBS-LRR resistance genes important in plant defenses against diseases and wounding (Garzo et al. 2002). A similar phenomenon may be occurring in raspberry; thus, future studies of *Ag*₁ should include identifying the class of protein that the gene encodes.

This study did not find evidence of a resistance mechanism located outside of the phloem (Sarria et al. 2009), based on the EPG waveforms for 12 h, as well as the observed behavior of aphids on excised leaves

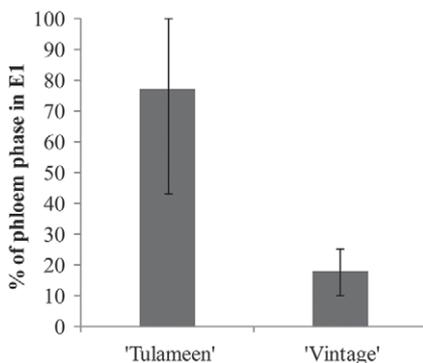


Fig. 2. Percentage of the phloem phase (E1 + E2) spent salivating (E1) by aphids on susceptible Vintage and resistant Tulameen plants. Aphids on Tulameen spent a significantly greater proportion of time salivating ($P = 0.024$). Waveform definition: E1, salivation into phloem sieve elements; and E2, ingestion from phloem sieve elements.

for 1 h. In the settling study, aphids were just as likely to move to the underside of the Tulameen leaves and begin probing as were aphids on Vintage leaves, suggesting that a prepenetration factor is not a significant deterrent to probing. In addition, there was no difference in the amount of time spent in pathway activities or the duration of potential drops. In fact, aphids feeding on resistant plants reached the phloem in a significantly shorter time than those on susceptible plants (Table 1).

Past research has suggested that one mechanism for resistance may be that the phloem sap is nutritionally deficient for *A. agathonica* survival (Kennedy and Schaefer 1975). Their evidence was that although aphid stylets reached the phloem sieve elements on resistant plants, the honeydew collected and analyzed was much more dilute than from aphids feeding on a susceptible control. The data from our study also suggest that aphids were able to easily locate the phloem; however, instead of ingesting, more time was spent salivating and aphids were unable to engage in sustained phloem sap ingestion. A possible explanation for the dilute honeydew measured by Kennedy and Schaefer (1975) is that their aphids may have engaged in xylem ingestion because the aphids were caged on the resistant plant for four days before the honeydew was analyzed. Xylem sap is a very dilute source of nutrition, yet xylem ingestion is common in aphids that are starved and may serve as an easy-to-access water source (Powell and Hardie 2002). Although the aphids in our study rarely engaged in xylem ingestion (1–2% of the 12-h study time), they might have engaged in this behavior more if they had been EPG monitored for longer.

Vector resistance is one of the best methods for controlling plant viruses, but the location of the resistance mechanism is important in determining how the spread of different types of viruses will be affected. Nonpersistent viruses could be acquired and inoculated by *A. agathonica* on Ag_1 resistant plants because aphids will readily probe. In addition, spread may increase because the aphids may become restless and desert the unsuitable host in search of another. The main viruses currently of concern (RLMV, RpLV, RYNV, and BRNV) are phloem limited and transmitted both semipersistently and persistently, whereas nonpersistent viruses are not a research focus.

Because aphids feeding on Ag_1 -resistant plants are able to access and salivate into phloem sieve elements, it is probable that they could inoculate plants with both semipersistent and persistent viruses, should they be viruliferous. However, because there is very little phloem ingestion occurring on Ag_1 resistant plants, common strains of *A. agathonica* are inefficient at acquiring persistent or semipersistent viruses from an infected aphid-resistant plant. This hypothesis is supported by Stace-Smith (1960) who found that aphids feeding on resistant raspberry plants infected with BRNV, a semipersistent virus, were unable to acquire and inoculate the virus to aphid-susceptible indicator plants efficiently. Also, because aphids are

unable to build colonies on resistant plants, there would be little secondary virus spread within a field.

The interactions between plant defenses and aphid saliva are poorly understood in many systems, including raspberry. Further research into these interactions would help explain why *A. agathonica* salivation apparently was unsuccessful in overcoming phloem sieve element defenses (Will et al. 2009), preventing initiation of phloem sap ingestion. Future research also should focus on the mechanism of resistance in raspberry containing resistance genes Ag_2 and Ag_3 , as well as resistance genes recently reported in black raspberry (Daubeny and Stary 1982, Dossett and Finn 2010). Thorough knowledge of the mechanism of each gene will facilitate breeding of cultivars that pyramid multiple defensive mechanisms to slow the advance of new *A. agathonica* biotypes and maintain the effectiveness of resistant red raspberries for virus control.

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