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Tissue location of resistance in apple to the rosy apple aphid established by electrical penetration graphs

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Abstract

A study of the constitutive resistance of the apple cultivar Florina, *Malus domestica* Borkh. (Rosaceae), to the rosy apple aphid, *Dysaphis plantaginea* (Passerini) (Homoptera Aphididae), was performed for the first time by the electrical penetration graph (DC-EPG) system, using the susceptible apple cultivar Smoothe as control. All experiments were conducted with apterous adult virginoparae. The results showed a constitutive resistance in Florina due to a much longer period before the first probe reflecting surface factors. Some weak indications were found for pre-phloem resistance and initiating phloem access was not affected as inferred from equal time to show phloem salivation. However, the complete absence of phloem ingestion indicates a major resistance factor in the phloem sieve elements, most likely in the sieve element sap. Surface factors could have affected tissue related variables and this should be studied further. Anyhow, the strong constitutive resistance in Florina, either on the surface alone or in the phloem as well, effectively prevented reliable experiments on induced resistance, previously detected by molecular methods.

Key words: *Dysaphis plantaginea*, *Malus domestica*, EPG, Florina, plant resistance, plant-aphid relationships.

Introduction

Rosy apple aphid, *Dysaphis plantaginea* (Passerini) (Homoptera Aphididae), is one of the most common aphid pests on apple (*Malus domestica* Borkh., Rosaceae) trees in Europe (Rat-Morris, 1993), causing economical losses of up to 80% when not controlled. Geographically, the rosy apple aphid is spread all over Asia, North Africa, North America and Europe including the whole Italian territory (Barbagallo *et al.*, 1996). The aphid is a dioecious species whose primary host is apple (sometimes quince, *Cydonia oblonga* Mill.) and its secondary hosts are herbaceous plants of the genus *Plantago* (*P. lanceolata* L., *P. media* L., *P. major* L., Plantaginaceae): *P. lanceolata* is the preferred secondary host (Blommers *et al*., 2004).

Damages due to *D. plantaginea* probing, i.e. stylet penetration and feeding, include floral abortion, high loss of buds of immature flowers and fruits, and leaf-roll in shoots. Generally, apterous virginoparae settle on the abaxial side of leaves, causing leaf roll with chlorosis and early phylloptosis. The most relevant damage is fruit deformation caused by stylet penetration in fruits, likely due to injected salivary components during probing. Either the saliva or transduced plant signals can spread systemically for some distance from the probing site. The aphids produce abundant honeydew on leaves and fruits, on which sooty moulds develop (Faccioli *et al*., 1985; Pasqualini *et al*., 1996).

Natural control of *D. plantaginea* is often difficult at the beginning of attacks, generally from the end of March to about mid April, because of very low densities of entomophagous insects and unfavourable weather conditions for activities of natural enemies. Since natural factors are insufficient to control this aphid, active control treatments has always been used so far. Control strategies have changed considerably, from traditional treatments with specific aphicides (Memmi *et al*., 1979) to currently used systemic insecticides, such as Vamidothion and Oxydemeton-methyl; or carbammates, such as Pirimicarb and Ethiofencarb. These yielded good results, especially in late treatments or reinfestations (Bylemans, 1999). However, in apple orchards some cases of efficiency failure were detected since early 1990's with respect to the above insecticides and for mixed treatments with other compounds, such as humic acids and/or leaf nutrients, which initially seemed to solve the problem. Consequently, pest control became partially useless (Bylemans, 1999). The introduction of neonicotinoids, such as Imidacloprid and Acetamiprid, and Triazamate (belonging to the group of carbamil-triazoles) contributed to improve *D. plantaginea* control but they could become ineffective due to rapid development of resistance in aphid populations.

The apple cultivar Florina has been developed at the INRA research station in Angers (France) by crossing *Malus floribunda* 821 x Rome Beauty to obtain resistance to apple scab *Venturia inaequalis* (Cooke) Winter (Ascomycota Venturiaceae). Florina was found to be tolerant also to fireblight *Erwinia amylovora* (Burrill) Winslow *et al*. (Enterobacteriaceae) and to red mite *Panonychus ulmi* Kock (Acarina Tetranychidae) (Lespinasse *et al*., 1985) as well as resistant to the rosy apple aphid. According to Rat-Morris (1994) Florina expressed also a resistance against the aphid *D. plantaginea*, characterized by tolerance and antibiosis. Therefore, this cultivar was considered a solution to the problems of this aphid, although some cases of resistance-breaking were reported, probably due to the spread of other aphid biotypes (Rat-Morris *et al*., 1999). Florina is grown for fruit juices production or as a field pollinator, within plant breeding and biological produc-

tion programs. The quality of fresh fruits is unsuitable for marketing (Rat-Morris *et al*., 1999).

Qubbaj *et al*. (2005) demonstrated that the preinfestation of Florina by *D. plantaginea* resulted in induced transcription changes of several putative resistance genes after 72h. They suggest that these changes are related to plant stress defence and might also play an important role in resistance mechanism against aphids. Others (Sauge *et al*., 2002) showed that in the peach cultivar "Rubira" the induced resistance to *Myzus persicae* Sulzer (Homoptera Aphididae) increased after 48h of preinfestation as compared to the same cultivar without aphid preinfestation. Also, aphid attack was found to affect aphid fitness (Wood and Hales, 1996), aphid feeding behaviour (Hays *et al*., 1999), and plant phytochemistry (Van der Westhuizen *et al*., 1998). The interest in the special role of aphids and aphid saliva in activating defensive responses in plants is increasing (Miles, 1999; De Vos *et al*., 2005; Prado and Tjallingii, 2006).

Although induction certainly plays a role in some plant aphid interactions, constitutive resistance in plants seems to dominate the generally occurring high host specificity in most aphid species. Constitutive resistance factors may occur at every tissue level as demonstrated in many plant resistance studies using the electrical penetration graph (DC-EPG) technique (Tjallingii, 1978; 1985; 1988), for example by Alvarez *et al*. (2006). This technique, now widely used, allows monitoring the aphid activities as well as the stylet tip positions in the plant tissues.

The purpose of this research is to study the constitutive resistance of Florina cultivar to *D. plantaginea* by using the EPG technique. We analyzed EPG variables including intracellular punctures in non-phloem tissue (pd waveforms), which reflect successive activities - as derived from virus transmission experiments (Martin *et al*., 1997; Powell, 2005) - presumably playing an important role in host plant discrimination. Our initial aim was to study induced resistance as well but the colonization of Florina by free moving aphids was too low to get a controlled degree of induction. So far, EPGs have not been used with apple pests, except for the woolly apple aphid, *Eriosoma lanigerum* (Hausmann) (Sandanayaka and Hale, 2003). This paper deals with *D. plantaginea* behavioural responses on resistant and susceptible apple cultivars.

Materials and methods

Plants and aphids

Two years old apple cultivars, 6 plants of resistant Florina and 6 of susceptible Smoothe, both grafted on M26, were grown in pots containing mixed soil and mould substrate, watered daily and fertilized every week. Plants were chemically treated with "Stroby" fungicide (140 mg/l, Basf, Milan Italy) against powdery mildew, *Podosphaera leucotricha* (Ellis et Everh.) E.S. Salmon (Ascomycota Erysiphaceae) 15 days before the beginning of EPG recording. All apple trees were kept in glasshouse at 25 ± 1 °C, about 50% RH, and a 16:8 L:D photoperiod for 2 month before EPG recording, which was done in spring after the blossom stage.

The aphid colony was established from one virginoparous apterous female of *D. plantaginea*, field collected from an apple orchard in Ferrara (Italy), April 2006. The colony was reared on Smoothe in a growth chamber at 23 ± 1 °C, 50% RH, and a 16:8 L:D photoperiod. All experiments were conducted with apterous adult aphids.

Preinfestation

Two-years old apple trees of the resistant Florina (5 plants) and susceptible Smoothe cultivar (6 plants), about 1 m high and with about 40 leaves, were each preinfested with 10 apterous adults, free to move on each of ten different leaves, i.e. 100 aphids per tree. Aphids and their progeny were removed after 96 h and about 1 h before EPG recording.

EPG recording and signal analysis

Aphids were individually placed on the lower surface of terminal leaves of apple trees, in the after blossom stage. Before each experiment, test aphids were carefully collected by using a fine brush, then fixed by a vacuum device to apply a small drop of electrically conductive glue (water based silver glue) on their dorsum for attachment to a thin (20 μm) gold wire electrode of about 2 cm long. All the operations were performed under a stereo-microscope. EPGs of aphids on preinfested and non-preinfested plants were performed in spring in the laboratory at 21 ± 1 °C and artificial fluorescent HF light (4000 Lux) with a 16:8 L:D photoperiod. Aphids were recorded for 8 hours resulting in 17 and 16 replicates for Smoothe and Florina, respectively. We used a DC EPG device (Giga-4 model, Wageningen University, The Netherlands) with an input resistance of 1 GΩ (Tjallingii, 1985). After A/D conversion at 100 Hz (KPCI-3102, Keithley Instruments, Cleveland, OH, USA), the EPG signals were stored on a computer hard disk, data acquisition mediated by PROBE 3 software (for Windows; Wageningen University, The Netherlands), used also for signal analysis. The variables (parameters) measured were comparable to those used by Alvarez *et al.* (2006) and accordingly indicated. Concerning potential drop waveform periods (pd), the data of the three subphases, recorded for 8 hours, were analyzed only for the first 3 hours, since these were the most significant to detect possible differences, according to preliminary observations.

Statistical analyses

EPG variables were split in non-sequential variables - mean frequencies (occurrences) and total durations of waveform periods, sequential variables - mean numbers or durations of waveform periods before or after certain events per treatment (cultivar), and percentages of aphids showing a certain waveform matching a certain criterion. From figures per individual aphid, means and standard errors of variables were calculated per treatment and differences were analysed by non-parametric Mann-Whitney *U*-test (software STATISTICA 6). Whenever percentages were involved, the χ^2 test was applied (software STATISTICA 6).

Results and discussion

On preinfested apple cultivars, i.e. 96 h after aphids had been put on plants to allow induced effects, almost all aphids were disappeared from Florina, whereas on susceptible Smoothe parental aphids and progeny were found, uniformly distributed on all leaves. Moreover, on the susceptible cultivar some leaf curling was already visible after 72 h, but no leaf curling was detected on preinfested Florina apple trees. We analysed the EPGs recorded after Florina preinfestation but the results were unclear. Apparently the constitutive resistance prevented settling, thus interfering with induction activities, especially in Florina: actually, unlike in the previous molecular study by Qubbaj *et al.* (2005), the aphids in our study were not in clip cages but free to move. Although interesting, the data we obtained on preinfested Florina appeared unreliable because the induction dose was very different from susceptible Smoothe. Moreover, aphids will normally never meet induced Florina trees in the conditions used in the molecular study. Thus we presume that it would not make any sense to evaluate induced resistance plants with a clear constitutive resistance.

Our EPG results from the two apple cultivars (non-preinfested) showed that aphids spent a much longer period without stylet penetration (non-probing) before the first probe on Florina (59 min) than on susceptible Smoothe (8 min) (table 1, variable 6). This indicates a rather strong surface repellence (leaf volatiles, wax, trichomes, leaf colour, etc.): presumably the aphid would have walked off, if not tethered by the gold wire electrode, similarly to what happened during the Florina preinfestation by non tethered aphids.

Nevertheless, once probing had started, the total time (table 1, variable 2) as well as the total number of non-probing periods (table 1, variable 4) and the average duration of np period (table 1, variable 5) scarcely differed between Florina and Smoothe. The number of probes (or the number of non-probing periods separating them) and total duration of stylet pathway (waveform ABC), without or with short intracellular punctures (potential drop periods; pd) was not different between susceptible and resistant plants (table 2, variables 12 and 13). With respect to intracellular punctures, some differences between susceptible Smoothe and resistant Florina apples were shown by the aphids. During these punctures, saliva is injected into, and sap samples are taken from most cells bordering the stylet track, normally leaving the cells intact. In comparison to the duration of stylet pathway (ABC), the total pd duration was relatively short and did not significantly influence the total duration of probes (variables 12 and 13). However, pd values as such showed several differences between Florina and Smoothe. The total pd duration was shorter in the resistant cultivar (table 2, variable 24), and fewer number of pd and number of pd/ min ABC occurred than on the susceptible one (table 2, variable 25 and 26), in spite of the fact that the average pd lasted longer (variable 27). This longer pd was mainly caused by much longer subphases II-1 and II-2 on Florina than on Smoothe (table 2, variables 28 and 29). The longer (II-1) suggests that aphids salivated longer into non-phloem cells of Florina than into those of Smoothe, but sap sampling (II-3 subphase; variable 30) was comparable. So far, sub-phase II-2 has not been related to any aphid activity and therefore its function and possible effects remain unclear. It also is unclear why aphids showed less intracellular punctures and salivated longer intracellularly on Florina, and on the basis of what possible cues. As shown by virus transmission studies, sap sampling (subphase II-3) occurs after the salivation (subphase II-1), not before. Thus cues may have come from earlier experiences, on the leaf surface, intercellularly, or non-gustatory cell properties. Anyhow, there could be consequences for the plant, since the intracellular salivation could induce transcriptional changes. More research will be needed to study these aspects.

The first probe duration appeared shorter in Florina (5 min) than in Smoothe (59 min) but the differences were not statistically significant because of the very large variation in Smoothe (table 2, variable 16). Both cultivars showed a similar number of probes shorter than 3 min, reflecting the fact that stylet tips did not penetrate

Table 1. Total duration, frequency and average duration (mean ± SE) of non-probing variables in 8 h of recording of *D. plantaginea* on susceptible (Smoothe) and resistant (Florina) apple cultivar. Time in minutes.

Variable n.	EPG variable	Smoothe n = 17	Florina n = 16	P value
		Non Probing phase		
1	total duration of np	104.45 ± 16.83	197.28 ± 22.56	0.004**
2	total duration np between probes	96.55 ± 17.71	138.28 ± 21.25	0.13
3	var. 2 as % of susceptible	100.00%	143.20%	0.18 [a]
4	total number of np	14.12 ± 2.20	15.50 ± 3.05	0.84
5	duration of np between probes	9.20 ± 1.93	11.85 ± 1.96	0.18
6	duration of 1st np period	7.88 ± 2.61	58.98 ± 17.76	0.001**
7	% 1st np on total np	13.57%	28.65%	0.054
8	duration of 2nd np period	9.18 ± 3.78	11.21 ± 3.15	0.49
9	duration of np after 1st E	7.58 ± 2.11	13.43 ± 6.06	0.38
10	duration of np after 1st E2	19.36 ± 9.78	-	-

[a] χ^2 test.

Table 2. Total duration, frequency and average duration (mean ± SE) of probing variables in 8 h of recording of *D. plantaginea* on susceptible (Smoothe) and resistant (Florina) apple cultivar. Time in minutes.

Variable n.	EPG variable	Smoothe n = 17	Florina n = 16	P value
		Probing phase		
11	total duration of probe (ABC + pd + E)	338.16 ± 25.33	220.85 ± 24.95	0.002**
12	total duration of path (ABC + pd)	250.63 ± 20.9	217.21 ± 24.7	0.29
13	total duration of path (ABC)	228.18 ± 19.36	204.23 ± 23.71	0.54
14	duration of probe	66.06 ± 26.01	33.86 ± 7.60	0.25
15	number of probes	13.88 ± 2.19	15.00 ± 3.02	0.98
16	duration of 1st probe	58.96 ± 30.33	4.97 ± 1.26	0.13
17	total number of probes < 3 min before 1st E1	2.93 ± 0.72	3.89 ± 1.31	0.67
		F phase		
18	total duration of F	11.23 ± 11.23	61.55 ± 23.81	0.003**
19	total number of F	0.06 ± 0.06	1.94 ± 0.91	0.001**
20	duration of F period	11.23 ± 11.23	30.75 ± 9.55	0.004**
		G phase		
21	total duration of G	26.08 ± 9.73	3.00 ± 3.00	0.045*
22	total number of G	0.47 ± 0.12	0.06 ± 0.06	0.045*
23	duration of G period	26.08 ± 9.13	3.00 ± 3.00	0.045*
		Potential drop (pd)		
24	total duration of pd	25.30 ± 1.98	12.98 ± 1.86	0.0012**
25	number of pd	3.91 ± 0.35	1.78 ± 0.26	<0.001***
26	number of pd/min ABC	1.45 ± 0.03	0.55 ± 0.06	<0.001***
27	duration of pd [c]	6.13 ± 0.26	7.44 ± 0.36	0.001**
28	duration of pd subphase II-1 [ab] (salivation)	2.21 ± 0.04	2.60 ± 0.09	0.002**
29	duration of pd subphase II-2 [ab] (unknown)	1.39 ± 0.07	2.49 ± 0.23	<0.001***
30	duration of pd subphase II-3 [ab] (ingestion)	1.94 ± 0.11	2.21 ± 0.10	0.11

[a] first 3 hour of EPG registration.
[b] time in seconds.

Table 3. Total duration, frequency and average duration (mean ± SE) of phloem variable in 8 h of recording of *D. plantaginea* on susceptible (Smoothe) and resistant (Florina) apple cultivar. Time in minutes.

Variable n.	EPG variable	Smoothe n = 17	Florina n = 16	P value
		Phloem phase		
31	total duration of E1	17.41 ± 2.61	3.63 ± 1.16	<0.001***
32	total duration of E2	70.10 ± 23.26	-	-
33	total number of E1	14.29 ± 1.89	1.94 ± 0.62	<0.001***
34	total number of E2	9.71 ± 2.01	-	-
35	duration of E1	1.21 ± 0.13	1.63 ± 0.53	0.34
36	duration of E2	6.15 ± 2.08	-	-
37	time to 1st E from start penetration	117.40 ± 20.15	231.10 ± 38.66	0.76
38	No. penetrations before 1stE	6.06 ± 1.30	9.91 ± 1.98	0.028*
39	duration of 1st E	4.96 ± 1.40	1.45 ± 0.53	0.02*
40	duration of 1stE1	1.70 ± 0.35	2.10 ± 0.56	0.25
41	% 1stE1 (duration 1stE1/ duration 1stE)	52.02%	100%	0.02* [a]
42	% single E1 of 1stE (number)	50% (8/16)	100% (16/16)	0.001** [a]
43	duration of np after 1st E	7.58 ± 2.11	13.43 ± 6.06	0.38
44	time to 1st E2 from start penetration	41.10 ± 4.26	-	-
45	No. of penetration preceding 1st E2	7.87 ± 2.13	-	-
46	duration of 1st E2	3.80 ± 1.26	-	-
47	duration of np after 1st E2	19.36 ± 9.78	-	-
48	% aphids with E2 > 10 minutes (number)	41.17% (7/17)	-	-

[a] χ^2 test.

deeper than about 3 cells (1 cell/min; i.e. epidermal/ mesophyll tissue) before the first phloem activity (E1). Together with a similar second non-probing period (table 1, variable 8), this suggests that epidermal or mesophyll factors do not play a role in the Florina resistance. Also, deeper mesophyll and vascular factors do not appear to be relevant, as the time required by *D. plantaginea* to show the first phloem activity within a probe was similar for both cultivars, due to high variability (table 3, variable 37). However, the somewhat higher number of probes before first E in Florina (table 3, variable 38) may suggest some weak resistance in the mesophyll, vascular tissue or sieve elements. No sieve element factor affected the average salivation (E1) period. However, phloem ingestion (waveform E2) was totally absent in Florina; thus all E2 variables (table 3, variables 32, 34, 36, and 44-48) indicate a major resistance factor in the phloem sieve elements. On susceptible Smoothe the percentage of aphids with prolonged ingestion (> 10 min) from phloem is about 41% (table 3, variable 48).

With respect to xylem ingestion (G), Florina showed a somewhat shorter duration than Smoothe (table 2, variable 21). On the other hand, derailed stylet mechanics (penetration difficulties, F) was higher in Florina (table 2, variable 18) but whether or not this plays any role in the Florina resistance is uncertain, as what triggers this derailed activity is generally unknown.

Overall, a clear phloem-based plant resistance is apparently detected by the EPG results. However, the rather strong surface resistance might have affected the later and deeper tissue variables as shown by trichome effects on the leaves of potato (Alvarez *et al.*, 2006). Further studying of deeper tissue factors, with elimination or reduction measures of the surface factors, is recommended. On the other hand, if the phloem ingestion (E2) would have been strongly affected by surface factors, why would phloem salivation (E1) is much less affected? We think it is rather safe to conclude that, in addition to surface resistance, Florina likely shows phloem sap resistance, too. Also, regardless of additional factors, the Florina resistance is rather effective, as shown by our failed preinfestation attempts. Surface resistance as such may certainly be useful for plant breeding, thus it is worthwhile to study Florina resistance mechanisms in more detail.

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