The resistance of lettuce to the aphid *Nasonovia ribisnigri*

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The resistance of lettuce to the aphid *Nasonovia ribisnigri*

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STELLINGEN

1. Een AIO heeft geen luizenbaantje.
   Dit proefschrift

2. The resistance of lettuce to the aphid *Nasonovia ribisnigri* is based on a chemical factor in the phloem sap.
   Dit proefschrift

3. The assumption that phloem sap is poor in secondary plant compounds is caused by a general lack of knowledge on phloem sap composition.

4. The ingestion rate of aphids during phloem feeding is much lower than the sap exudation rate from amputated stylets. Therefore, the relation between aphid resistance and exudation from amputated stylets in *Medicago sativa* as suggested by Girousse & Bournoville is premature.
   Girousse, C. & R. Bournoville, 1994. Role of phloem sap quality and exudation characteristics on performance of pea aphid grown on lucerne genotypes. Entomol. Exp. Appl. 70: 227-235

5. There are no scientific arguments to prefer the AC feeding monitor to a DC-EPG amplifier for the recording of homopteran feeding behaviour.
   Reese, J.C., Tjallingii, W.F., Helden, M. van & E. Prado, 1994. Waveform comparison among AC and DC systems for electronic monitoring of aphid feeding behaviour. In: E.A. Backus & G. Walker, Homopteran feeding behaviour: Recent research advances and experimental techniques. Special issues Ent. Soc. Am.: *in press*)

6. The results of experiments with aphids on artificial diet should not be extrapolated directly to the situation on the plant, especially in view of the compartmentalisation of chemicals in plant tissues.

7. Naturally occurring host-plant resistance is the most widespread plant protection method and therefore, deserves more research and plant breeding effort.

8. Deeltijdwerken vergroot de efficiëntie per gewerkte tijdseenheid.

9. De beslissing om op een project een AIO dan wel een Post-doc aan te stellen wordt te vaak genomen op financiële gronden.

10. Een opgeruimd humeur is het halve werk.

11. Veel wetenschappers zijn te eigenwijs om dat zelf toe te geven.

12. Het houden van exotische huisdieren dient rigoureus te worden beperkt door de invoering van zogenaamde positieflijsten die zowel voor handelaren als houders gelden.

13. De toepassing van fonteinen en filters in tuinvijvers is uit ecologisch oogpunt ongewenst.

14. Het belang van een flinke lichamelijke inspanning voor de geestelijke ontspanning wordt sterk onderschat.

Stellingen behorende bij het proefschrift "The resistance of lettuce to the aphid *Nasonovia ribisnigri* " door Maarten van Helden.

Wageningen, woensdag 22 februari, 1995

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1. INTRODUCTION

APHID-PLANT INTERACTIONS

Aphids are highly specialised phloem sap feeders. This specialisation on the phloem sap has made it very difficult to study the feeding behaviour in detail. What happens during the penetration of the stylets through the different plant tissues on their way to the sieve elements was unknown. The only way to observe ingestion was by the recording of honeydew excretion (see Pollard, 1973 for a review). Knowledge of the events which occur during the period between the insertion of the stylets in the plant and ingestion from a sieve element is essential to understand aphid-plant interactions.

The introduction of the "feeding monitor" by McLean and Kinsey (1964, 1965) was a major breakthrough. By connecting the aphid and the plant in an electrical circuit it was possible to monitor electrical resistance fluctuations during probing. Freddy Tjallingii greatly improved this method with the introduction of the Direct Current (DC) Electrical Penetration Graph (EPG) amplifier (Tjallingii, 1978, 1985). The DC-EPG amplifier made it possible to identify many more different waveforms, including so-called Electro Motive Force (EMF) components. Through combination with various other techniques (TEM, radioactive tracer studies, virus transmission, stylectomy etc.) EPG waveforms could be correlated with aphid activities, stylet location and plant histology (Kimmins & Tjallingii, 1985; Spiller *et al.*, 1990; Tjallingii, 1985, 1988, 1990, 1994; Tjallingii & Hogen Esch, 1993; Tjallingii & Mayoral,

1992; Prado & Tjallingii, 1994). This work has enriched our knowledge of aphid feeding behaviour enormously.

We now know that aphid stylets penetrate extracellular, via the cell walls. It takes a considerable amount of time, and often several different penetrations before a sieve element is reached for the first time. The stylets frequently puncture cells during the extracellular probing to the phloem, but the aphid retracts its stylets after a few seconds to continue their extracellular route until a sieve element is reached. Salivation into the sieve element precedes the actual ingestion of phloem sap. Often several sieve elements are punctured before sustained ingestion occurs.

Increased knowledge of the events during feeding behaviour multiplied the number of questions. Understanding of the aphid-plant interactions during stylet penetration and their role in host plant acceptance or rejection is still very poor. Does the aphid taste extracellular fluid during probing or does it ingest fluid during the cell punctures? How do aphids locate and recognise the phloem sieve elements, is this strictly by trial and error or are there plant cues to guide the aphid? Why is there a stylet sheath excreted during stylet penetration and what is the role of salivary enzymes? Why does probing sometimes induce (hypersensitive) plant reactions and how could these influence the aphid? How do aphids avoid defence reactions like P-protein gelation and callose formation during tapping of the phloem sap?

Because of this shortage of fundamental knowledge, it is difficult to interpret the behaviour during probing. The behavioral sequence that leads to plant acceptance appears complex and highly variable. The early theory of aphids making only a short test probe to decide on host-plant suitability was certainly an oversimplification. Especially on resistant varieties of normally susceptible plant species aphids usually make numerous penetrations and often reach the phloem before differences in behaviour appear (Caillaud *et al.*, 1992; Cole, 1993). Rejection is not always straightforward and, even on absolutely resistant plants, aphids can continue probing while their motivational state gradually shifts and finally reaches a point where the aphid decides to leave the plant. On partially resistant plants changes in behaviour

can be small and difficult to trace in the large natural variation that occurs. Aphid-plant interactions during host-plant selection form a dynamic process, the result of plant suitability, aphid motivation and stochastic variability, which may vary in time.

RESISTANCE MECHANISMS

Several possible resistance mechanisms have been proposed for various aphid-plant combinations, ranging from volatiles which deter alighting to toxic compounds in the phloem. In most cases aphids seem to land at random on green/yellow surfaces (Moericke, 1962; Müller, 1964) and penetration is necessary to evaluate plant resistance (Klingauf, 1972, 1987; Müller, 1962). The mechanisms that play a role during stylet penetration to the phloem might be either chemical or mechanical. A chemical resistance mechanism acting in this stadium would imply that aphids do ingest fluid during penetration to be tested by the contact chemoreceptors in the epipharyngeal cavity (Wensler & Filshie, 1969; Tjallingii, 1985). Though ingestion during cell punctures has never been shown unequivocally, it probably occurs, aphids can acquire viruses during these punctures (Powell, 1991). Mechanical resistance mechanisms could lay in the toughness of the tissue, especially of the secondary cell wall through which the stylets penetrate. Dreyer and Campbell (1984) suggested that a higher degree of polymerisation of the pectin in wheat caused resistance to the greenbug *Schizaphis graminum* by making penetration more difficult.

Once an aphid reaches a phloem sieve element and attempts to establish ingestion, blocking of the sieve element or the aphids stylets by plant defence reactions like P-protein gelation or callose formation can occur. Aphid salivation into the sieve element (Prado & Tjallingii, 1994) might normally avoid this.

Chemical composition of the phloem sap is very important. This is the only food of the aphid and therefore it should contain all the dietary

requirements of the aphid. Phloem sap is rich in sugars and contains many different amino acids, organic acids and vitamins. So phloem sap seems to contain everything needed for the aphid's metabolism and some additional essential compounds are synthesized by symbionts (Douglas, 1990; Houk & Griffiths, 1980).

Apart from the nutritional aspects the chemicals in the phloem sap influence the acceptance of the plant by their gustatory properties. Sucrose and some amino acids are feeding stimulants while the strong feeding stimulancy of other amino acids can be shown only in combination with sugars (Mittler & Dadd, 1964).

Many secondary plant metabolites can also play an important role in host plant acceptability. Phenolic acids, hydroxamic acids, alkaloids and many other compounds show feeding deterrent action in artificial diet experiments (Nault & Styer, 1972; Schoonhoven & Derksen-Koppers, 1976; Argondoña *et al.*, 1980; Herrbach, 1985; Mittler, 1988; Harrewijn, 1990; Niemeyer, 1990 and many others). The artificial diet assays used for these experiments are very different from the natural situation, especially in the physical aspects (pressure, compartmentation). Therefore, the effect of a compound in an artificial diet cannot be extrapolated directly to the natural situation. Some plant specific secondary compounds stimulate feeding of monophagous species while deterring others (Klingauf *et al.*, 1972; Nault & Styer, 1972), but the preference of an aphid for a certain plant species is usually unexplained. Host plant recognition of mono- and oligophagous species is expected to be based on secondary compounds rather than on differences in primary nutrients.

Phloem sap is thought to be poor in secondary plant chemicals. Knowledge about the role of secondary compounds in aphid-plant relations is very limited (Herrbach, 1985; Wink & Witte, 1991; Cole, 1993). Whether the compounds which show allomonal effects *in vitro* are present in the phloem sap or in other plant compartments sampled by the aphid during penetration, and in concentrations high enough to affect the aphid, is usually unknown. Often correlations between the concentration of a certain compound in whole

plant extracts and plant resistance were thought to represent causal relationships (Todd *et al.*, 1971; Argandoña, 1980; Dreyer & Jones, 1981; Herrbach, 1985; Leszczynski *et al.*, 1985; Niemeyer 1990; Kanehisha *et al.*, 1990; Luczak & Gaweda, 1993), even while it is in some cases unlikely that these compounds are present in the phloem sap.

Research in this field is hampered by problems of phloem sap collection, which is necessary to allow conclusions about the biological activity of its compounds. Differences in phloem sap composition will not show in whole plant analysis since phloem sap is only a very small fraction of the total plant.

THE MODEL SYSTEM

The aphid-plant combination chosen for this work consisted of the aphid *Nasonovia ribisnigri* (Mosley) and lettuce (*Lactuca sativa* L.) on which this aphid is a major pest in large parts of the world (Forbes & Mackenzie, 1982; MacKenzie & Vernon, 1988; Reinink & Dieleman, 1992). Chemical control is difficult because aphids settle inside the head of the lettuce where they cannot be reached by insecticides. Frequent spraying (often twice weekly) is necessary for effective control. Aphid feeding deforms plants, aphids can transmit viruses, and - most important - the presence of aphids in lettuce makes it unmarketable. Plant resistance could be a good alternative for chemical control provided that the level of resistance is very high or even absolute, the plant contains resistance against all important aphid species, and the resistance is durable.

During the last 25 years a breeding program has been performed at the CPRO-DLO to increase aphid resistance in lettuce (Eenink *et al.*, 1982a,b; Eenink & Dieleman, 1982; Reinink & Dieleman 1990, 1992). Complete resistance to *N. ribisnigri* was found in *Lactuca virosa* L., a wild lettuce species. This resistance was transferred to *L. sativa* via interspecific crosses using *Lactuca serriola* L. as a bridge. The resistance is based on a single

dominant gene (Nr gene, Eenink *et al.*, 1982 a, b). The resistance was not effective against other aphid species like *Myzus persicae* (Sulzer) or *Macrosiphum euphorbiae* (Thomas). Breeding is continuing to improve resistance to other species (Reinink & Dieleman, 1990). So far, field trials have not shown any sign of breaking of the resistance by *N. ribisnigri* (Reinink & Dieleman, 1992). Lettuce lines with the Nr-gene are used by seed-breeding companies trying to develop commercial lines with aphid resistance. Segregation, occurring after several generations of inbreeding, produced sets of near isogenic resistant (genotype NrNr) and susceptible lines (nrnr) as a by-product. These sets are used for comparison of the aphid feeding behaviour and chemistry.
This model system has several important characteristics. The absolute resistance is a guarantee for clear differences in behaviour of the aphids on these lines, and therefore probably also between lettuce lines. The availability of (near) isogenic lines with and without monogenic resistance is an important advantage, since the risk of differences between resistant and susceptible plants which are not related to resistance is reduced to a minimum. Simultaneously the genetic differences are as small as possible, reducing the risk that differences related to resistance are so large that identification of a single factor is impossible. Still, it is important to realise that statements about the isogenicity are only based on breeding experiments and not on a more elaborate genetic analysis, so genetic difference will be somewhere between one set of chromosomes (out of 9) and one gene, depending on the amount of crossing-over.

OBJECTIVES

The main goals of the research project described in this thesis was the identification of the resistance mechanism to *Nasonovia ribisnigri* in lettuce. This included the localisation of the resistance in the plant, the effect of the resistance on the aphid's feeding behaviour, and -if possible- the

identification of the responsible chemical compounds (allomones). When successful, the results could be used to explain why this resistance is not effective against other species like *Myzus persicae* or *Macrosiphum euphorbiae*.

Identification of the mechanism could also enable predictions about the durability of the resistance, important for possible commercial use of the resistance gene.

EXPERIMENTS

The work described in this thesis started in 1988. The project proposal on which it was originally based was entitled "The role of secondary plant substances in the resistance of lettuce to aphids". The plant lines were newly developed in the breeding program of a seed-breeding company. Therefore, the study started with a basic characterisation of the resistance using aphid developmental parameters (Chapter 2). After this Electrical Penetration Graphs were used to localise the resistance in the plant (Chapter 3). Since aphids are connected to a wire during EPG recording, they are very restricted in their movements. In chapter 4 we compared EPG recordings with visual observations of the behaviour of freely moving (not wired) aphids. Chapter 5 describes our efforts to collect phloem sap from lettuce plants using different methods, with some unexpected results. The phloem sap samples were chemically compared for sugars, amino acids, proteins and secondary compounds in chapter 6. Chapter 7 finally describes the development of a bioassay based on phloem sap.

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2. BIONOMICS OF *N. RIBISNIGRI*

ABSTRACT

The intrinsic rate of increase (r_m-values), mean relative growth rates and mortality of *Nasonovia ribisnigri* (Mosley) (Homoptera, Aphididae) on different lines of lettuce (*Lactuca sativa* L.) were determined. Near isogenic resistant and susceptible lines, plus their ancestors, were used. Bionomics of *N. ribisnigri* on the resistant lines (NrNr) with the dominant *Nasonovia* resistance gene (Nr-gene) differed clearly from the susceptible lines (nrnr). Mortality was high, no larvae reached adulthood, and no reproduction nor honeydew production was seen on the resistant lines. Transfer of aphids to susceptible plants after a period of 2 days on the resistant lines showed no signs of intoxication of aphids. Apparently there is no feeding on the resistant lines. It is not clear whether the aphids can not reach the phloem or do not accept it on the resistant line.

INTRODUCTION

Aphids are a major problem in lettuce growing. Aphids transmit viruses and aphid-infested heads are unmarketable. The aphid *N. ribisnigri* occurs frequently and causes severe problems in Western Europe and Canada (Eenink *et al*, 1982a, 1982b, Forbes & Mackenzie, 1982).

During a breeding program at the Institute for Horticultural Plant Breeding (IVT) absolute resistance to *N. ribisnigri* was transferred from the wild *L. virosa* to the cultivated *L. sativa* (Eenink *et al.*, 1982a, Eenink & Dieleman, 1982a). This resistance is based on a single dominant gene (Nr gene, Eenink & Dieleman, 1989; Eenink *et al.*, 1982b) and is extremely effective against *N. ribisnigri* but it gives little or no protection against other important aphid species such as *Myzus persicae* and *Macrosiphum euphorbiae* (Reinink & Dieleman, 1989). Breeding companies are currently developing commercial lines with this resistance gene. In this study the effect of the resistance gene on developmental parameters of *N. ribisnigri* was determined. Future experiments on the aphid resistance will focus on its localization in the plant and identification of the mechanism (chemical or mechanical).

MATERIALS AND METHODS

All experiments and rearing of aphids were conducted in a greenhouse at 20 ± 2°C, r.h. 70% and L16:D8 photoperiod (extra light provided by SONT high pressure sodium lamps). Seeds were sown in a standardized soil mixture in trays and transferred to 15 cm plastic pots (1.35 l) in the 2-3 leaf stage. Plants for experiments were about five weeks old and had about 6 full-grown leaves. For the transfer experiment 4 week old plants (3-4 full-grown leaves) were used from a soilless culture system as described by Harrewijn & Dieleman (1984) in a greenhouse at 18 ± 1°C, r.h. 70%, L16:D8 photoperiod.

Plant genotypes: Three susceptible lines (A,B,C, genotypes nrnr) and one resistant line 411 (genotype NrNr) were used in a backcross breeding program to produce a commercial line. Pedigree: [F6((((F3((C*F1(B*411)))*A))))]. Segregation in the F6 generation resulted in two near isogenic lines: RES (NrNr) and SUS (nrnr). The susceptible cultivar "Taiwan" (nrnr) was used as a control. These seven lines were used in our experiments.

Aphids: Mass rearing of an orange biotype of *N. ribisnigri* (WNr1) was performed on lettuce plants of cv. Taiwan. Neonate (<24 h) larvae were selected by placing adult virginoparae (alates) in clip-on cages on lettuce plants or in plastic containers on excised leaves for 24 hours after which adults were removed and larvae were used for experiments.

Mean Relative Growth Rate (MRGR): Five young larvae (<24 h, total weight 250±19 µg) were placed in a 2.5 cm diameter clip-on cage. After 8 days the aphids were counted and weighed. MRGR was calculated as {Ln(mean weight per aphid at day 8) - Ln(mean weight per aphid at day 1)}/8 (Kogan, 1986). Four replicates of a randomized block design with the seven lettuce lines and five plants (= blocks) per line were performed. One clip-on cage was used per plant resulting in 25 aphids per line per replicate and 4 x 25 = 100 aphids per line in total.

Intrinsic rate of increase and larval mortality:

Experimental design: Six replicates were performed with the seven lines in a randomized block design with one plant as a block. There were five (repl. 1-4) or seven (repl. 5 and 6) plants per line in each replicate.

Larval development time (D) and mortality (L_m): Five young larvae (age < 24 h) were placed in a 2.5 cm diameter clip-on cage. Two clip-on cages were used per plant. Each day the number of surviving larvae and adults was determined and adults were removed. Mortality per plant was calculated as the fraction of initial number of larvae (10) that moulted to adults: (10-total nr. of adults)/10. Results were based on a total of 34 plants and 340 aphids per line.

Reproduction (R_d): Two adults from the larval development time and mortality experiment were used. They were placed individually in clip-on cages on new plants of the same genotype. For tests on the resistant lines, where all larvae died, adult virginoparae from cv. Taiwan were used to provide aphids in replicates 1 to 4. Every second day the offspring was counted and removed to avoid crowding effects. R_d was the total number of larvae produced in a period equal to the larval development time D (D is depending of lettuce line as determined in larval development time and mortality experiment). Estimates of the number produced during the time

between two observations were made by linear interpolation. Reproduction was based on 68 virginoparae per line on a total of 34 plants.

Calculation of intrinsic rate of increase (r_m): The formula developed by Wyatt & White (1977) was used to calculate the intrinsic rate of increase (Birch, 1948) on the susceptible lines: $r_m = 0.738 * Ln (R_d)/D$.

Honeydew production: 30 Larvae (age 96±12 h) were placed individually in honeydew production cages as described by Eenink *et al.* (1984) on plants of the lines RES and SUS. The number of honeydew droplets was counted every 24 hours during 3 days.

Transfer experiment: On day 0, larvae reared on Taiwan (age 96±12 h, mean weight 163±32 µg) were weighed and placed individually in 1 cm clip-on cages on small plants (4 weeks old, 3-4 full grown leaves) of the lines SUS and RES (50 larvae per line). After 2 days they were weighed and transferred to plants of the other genotype. At day 4 they were weighed again. The larvae from RES were transferred to SUS and at day 6 they were all weighed for the last time (resulting treatments RES->SUS->SUS and SUS->RES->SUS). As a control 10 aphids per line were not transferred to the other genotype and weighed only at day 4 of the experiment (continuous treatments RES_{cont} and SUS_{cont}) or weighed at day 2 and 4 and placed back on the same plant but on a new leaf (not transferred treatments RES_{nt} and SUS_{nt}). Weights on day 6 were either not detectable because all adults died (on the resistant line) or not determined because they had moulted to adults (on the susceptible line).

Statistics: In all experiments one plant represented one block. All results were first calculated per plant. Means and standard deviations were then calculated over all plants. Kruskal-Wallis tests with multiple comparison ($\alpha < 0.05$) (Conover, 1980) were used to compare the lines for each parameter. For the MRGR experiment Tukey tests with multiple range analysis were used to show differences between lines.

RESULTS

The results of all developmental parameters are listed in Table 1.

MRGR: The weight growth (MRGR) of the aphids on the resistant lines was only half of the weight growth on the susceptible lines. There were no significant differences among the susceptible lines. The results for the resistant lines were based on only very few surviving aphids (3 on 411 and 8 on RES).

Larval development time (D) and mortality L_m: The larval development time on the susceptible (nrnr) lines ranged from 8.20 (A) to 8.44 (SUS) days (Table 1). These minor differences were not statistically significant. On the resistant lines (NrNr) only 7 (line 411) or 3 (Line RES) out of 340 larvae reached adulthood, therefore D could not be determined. The mortality ranged from 15.15% (Line A) to 30.47% (Taiwan) on the susceptible lines, and was always > 99% on the resistant lines.

Reproduction: R_d values on the susceptible lines did not differ significantly. On the resistant lines (RES and 411) very few larvae were produced during replicates 1 to 4 when virginoparae of Taiwan were used for the reproduction experiment to make up the shortage of aphids developed on these lines. In replicates 5 and 6 no larvae were produced. Usually the adults died within 4 days on the resistant lines.

Intrinsic rate of increase: The r_m showed small but significant differences among the susceptible lines. For the resistant lines it was not possible to calculate the r_m-values.

Honeydew production: Essentially no honeydew was produced on the resistant line RES; on the susceptible line SUS 7-8 droplets per aphid per day were produced (Table 2).

Transfer experiment. The results of this experiment showed a complete absence of weight gain and even a small decrease in weight, on the resistant

Table 1. Bionomics of *Nasonovia ribisnigri* on seven different lettuce lines

Line	Genotype	MRGR	D	R_d	r_m	L_m
A	nrnr	.365[a]	8.20[a]	36.06 [b]	0.310 [b]	15.15[a]
B	nrnr	.358[a]	8.42[a]	34.41[ab]	0.297[ab]	17.35[a]
C	nrnr	.380[a]	8.28[a]	37.14 [bc]	0.307[ab]	17.50[a]
411	NrNr	.181 [b]	N.D.	3.85 [d]	N.D.	99.36 [c]
RES	NrNr	.164 [b]	N.D.	0.97 [d]	N.D.	99.85 [c]
SUS	nrnr	.364[a]	8.44[a]	32.79[a]	0.296[a]	18.52[a]
Taiwan	nrnr	.389[a]	8.39[a]	39.35 [c]	0.310 [b]	30.47 [b]

MRGR = Mean Relative Growth Rate, D = Larval Development Time (days), R_d = Mean reproduction in time eq. D (nr), r_m = Intrinsic rate of increase (formula by Wyatt and White, 1977)(/day), L_m = Larval mortality in percentage. N.D. = Not determinable. Values that have no letter in common are significantly different (Kruskal-Wallis test with multiple comparison $\alpha < 0.05$, Conover, 1980).

lines (Fig. 1). After 2 days on the resistant line and transfer to the susceptible line the weight growth was resumed at the same rate (parallel lines) as found on the susceptible line (Fig. 1), resulting in equal mean weights of 285 µg on day four for the treatments RES->SUS->SUS and SUS->RES->SUS. This weight was comparable to the weight of the treatments SUS_{nt} and SUS->RES->SUS on day two.

DISCUSSION AND CONCLUSIONS

Differences between resistant and susceptible lines: For all parameters the performance on the resistant lines was much less than on the susceptible lines. In addition the MRGR values on the resistant lines (Table 1) were overestimated because they were based only on the surviving aphids whereas 94% of the aphids died . Over a shorter period when no mortality was observed the weight gain was zero (see transfer experiment, Fig 1.). However some aphids did grow and apparently reached adulthood on the resistant lines.

Table 2. Honeydew production of *N. ribisnigri* on RES and SUS during 24 hours (Mean standard error, N=30)

Line	# droplets on day:		
	1	2	3
SUS	7.41 ± 1.08	8.28 ± 1.27	7.11 ± 1.11
RES	0.04 ± 0.04	0.05 ± 0.05	not detected

On the susceptible lines there were significant differences for the R_d, r_m and L_d, but these differences were not very large. Only the mortality was clearly higher on Taiwan compared to the other susceptible lines. Nevertheless, the small differences among the susceptible lines appear negligible in comparison to the differences between susceptible and resistant lines.

The resistant lines showed no significant differences. The resistance appeared to be based exclusively on the Nr gene. No loss of the resistance occurred during its transfer from the 411 parent line to the resistant line RES. No evidence of any change in the resistance during this transfer was found.

The absence of honeydew on the resistant line RES indicated that there was a complete absence of uptake of phloem sap or any other plant fluid on this line.

In the transfer experiment a total absence of weight gain on the resistant lines was found, indicating that there was either no food uptake on these lines and/or very short term toxic effects. The immediate resumption of growth on the susceptible lines showed that there was no lasting intoxication on the resistant line.

The results show that the resistance is probably based on a total absence of feeding on the resistant line. The complete absence of weight increase and honeydew production indicates that there is no food uptake on the resistant lines rather than a poor food quality or a toxic effect of the ingested food in which case a reduced weight gain and honeydew would have been present. The course of the mortality of the aphids on the resistant lines is comparable to the mortality in a petri dish with some moist cotton wool in the total absence of food (unpublished results) and similar to the results of

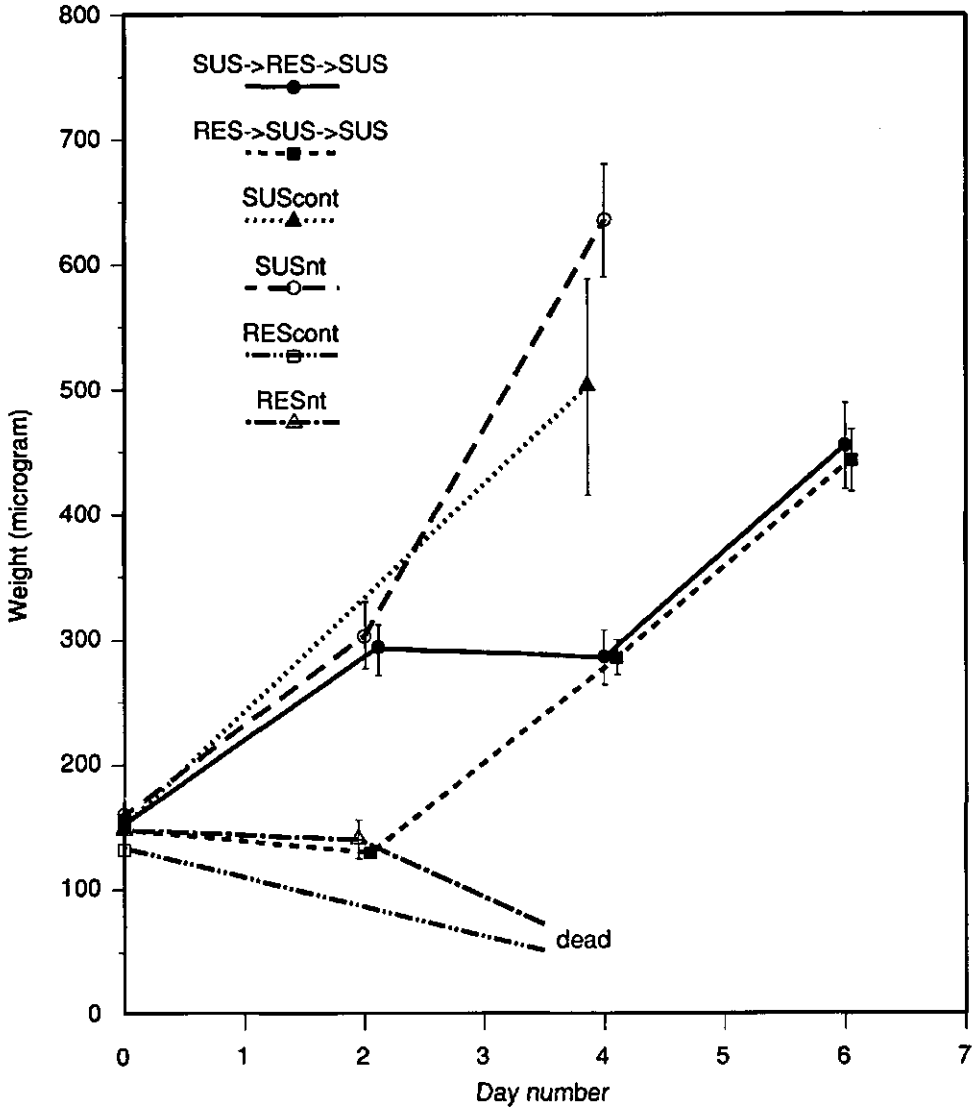

Figure 1. Weight of *N. ribisnigri* on resistant and susceptible lettuce lines with transfers from one genotype to the other at two-day intervals. For explanation of treatments see Materials and Methods. Aphids of RES_{nt} and RES_{cont} died after 3 to 4 days. Points represent mean ± standard errors.

Eenink & Dieleman (1982b) who worked with a different lettuce carrying the same resistance gene. A toxic effect would also have influenced the growth after transfer for a certain period. Although nearly all aphids died on the resistant lines, those that survived for a longer period showed some growth (MRGR experiment) suggesting that feeding is possible but strongly suppressed. *N. ribisnigri* is a specialist with only *Lactuca* species and some related compositeae as secondary hosts. Therefore the presence of a feeding deterrent or absence of a feeding stimulant are possible resistance mechanisms, interfering with host plant discrimination in this monophagous species and not giving resistance against other more polyphagous species like *M. persicae* or *M. euphorbiae* which are not influenced by the resistance (Reinink & Dieleman, 1989). Other mechanisms like food quality or mechanical resistance of the leaf during penetration probably would effect all species to a certain degree. However, it is not yet clear whether the absence of feeding is due to problems locating or reaching the phloem-vessels on the resistant line or due to problems feeding on or accepting the phloem. This is investigated in a study of the Electrical Penetration Graphs (EPG's) on both the resistant and susceptible line (van Helden, 1990; van Helden & Tjallingii, 1990). Behavioral studies and EPG recording will give more information of the possible nature and location of this resistance mechanism.

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3. TISSUE LOCALISATION OF THE RESISTANCE

ABSTRACT

Electrical penetration graphs (EPG's) of *Nasonovia ribisnigri* (Mosley) (Homoptera, Aphididae) on resistant and susceptible lettuce (*Lactuca sativa*, Compositae) showed a large reduction in the duration of the food uptake pattern (E2) on the resistant line. No differences in EPG's were observed before the phloem was reached. Therefore, resistance is believed to be located in the phloem vessel. Both mechanical blocking of the sieve element after puncturing and a difference in composition of the phloem sap are possible resistance factors. However, a chemical factor seems more likely because of the specificity of the resistance against *N. ribisnigri*.

INTRODUCTION

Absolute resistance to *N. ribisnigri* was transferred from *Lactuca virosa* to *L. sativa* (Eenink *et al.*, 1982b). The resistance is based on a single dominant gene (Nr gene) (Eenink & Dieleman, 1982; Eenink *et al.*, 1982a; Reinink & Dieleman, 1989). Here we studied the resistance using the direct current electrical penetration graph (DC-EPG) technique as developed by Tjallingii (1978, 1985, 1988). This technique is especially suitable for studying possible resistance mechanisms because it provides information about both

the behaviour of the aphid and the stylet tip positions in the plant during penetration (Montllor & Tjallingii, 1989; Niemeyer, 1990; Spiller *et al.*, 1990; Tjallingii, 1978, 1985, 1988, 1990). Table 1 provides a summary of the different waveform patterns which can be distinguished in the EPG (modified after Tjallingii, 1990). Electrical Penetration graphs of *Nasonovia ribisnigri* on a resistant and a susceptible lettuce (*L. sativa*) line were compared. These lines were isogenic except for the resistance gene (Van Helden & Tjallingii, 1990). The frequency of occurrence, mean and total duration of the waveform patterns, their sequence and other derived parameters were compared to get information about the possible tissue location of the resistance.

MATERIALS AND METHODS

Aphids and plants. Mass-rearing of *N. ribisnigri* on *L. sativa* cv "Taiwan" and culture of plants were performed as described by Van Helden *et al.* (1992a). The plants used in the experiments were two nearly isogenic lines (RES and SUS) which differed only in the *Nasonovia* resistance gene (Nr gene) (Van Helden *et al*, 1992a). Plants were approximately 6 weeks old (5-6 leaves) when used.

Production of alates. Alates were produced on *L. sativa* cv "Taiwan" by placing 5-7 females in a 2.5 cm clip cage. After two days the clip cages and adults were removed. More than 90% of the larvae developed as alates due to crowding in the first larval stage. Recently moulted (< 2 hours) young alates were used in the tests.

Pretreatment of the aphids. Since the extensive handling of the aphids before the EPG recording can strongly influence their behaviour, special care was taken to standardize it as much as possible (see discussion). Teneral alate virginoparae were collected with a brush from their food plant (lettuce cv "Taiwan") into a petri dish between 8.30 and 9.00 a.m. They were tethered on the thorax (not on the abdomen) to a 20 µm gold wire using a vacuum device by sucking them onto a pipette tip and attaching them to the wire

Table 1. Summary of EPG patterns and their correlations to aphid activities (modified after Tjallingii, 1990)

EPG PATTERN		CORRELATIONS PLANT TISSUE	APHID ACTIVITY	REMARKS
np		stylets not inserted	non penetration (walking etc.)	
A		epidermis	electrical on/off stylet contacts	first wave-form only.
B		epidermis /mesophyll	sheath salivation	| A, B, and C | overlap:
C		all tissues	activities during stylet pathway	| in analysis | often pooled
pd		all living cells	stylet tip puncture of cell membrane	
E1e		unknown	unknown	
E1		sieve elements	unknown	
E2	p[1]	sieve elements	(watery?) salivation	
	w	"	(passive) ingestion	
F		all tissues	mechanical stylet work	"penetration difficulties"
G	p[1]	xylem	unknown	"drinking"
	w	"	active ingestion	

[1] p= peaks, w= waves.

using water-based conductive silver paint (Tjallingii, 1988). After tethering, the aphids were placed back on a Taiwan lettuce plant for four hours. The EPG recordings started between 13.00 and 14.00 p.m. To transfer the aphids to the test plant they were carefully lifted off the Taiwan plant by pulling the gold wire. The electrode was connected to the amplifier placed in a micromanipulator and the aphid was lowered on the abaxial side of a reversed, fully expanded leaf of the test plant (RES or SUS) immediately.

EPG recording. The recordings were performed in the laboratory at 22 ± 1°C under continuous artificial illumination (HF fluorescent tubes *ca* 4000 Lux). The amplifier used was a 10^9 Ohm input impedance DC amplifier (Tjallingii, 1988). Each day three EPG's were recorded for 16 hours on susceptible and resistant plants simultaneously. Fifteen recordings were made on plants of the susceptible line (SUS) and 20 on the resistant line (RES).

EPG analyses. EPG's were analyzed with the computer program STYLET (Tjallingii & Hogen Esch, 1993). In the EPG's the patterns np (non penetration), A, B, C (with numerous potential drops), E1e (E1 at extracellular voltage level), E1 (intracellular or pd level), E2, F and G could be distinguished (see Table 1 and Tjallingii, 1990 for an overview). For analysis A, B and C were regarded as one pattern (ABC) because they always occurred together at the start of a penetration and were not always clearly separated. The term E pattern refers to any E pattern at the intracellular level, either E1 or E2. The sequence of E patterns on a suitable host plant is a short period of E1, followed by a short transient period and a long E2 (Tjallingii, 1990). Sometimes shifts from E2 back to E1 did occur, mostly on the resistant line. The transition from E1 to E2 and vice versa was not always clear, particularly on the resistant line and the two patterns sometimes occurred simultaneously. Ambiguous mixtures were classified as E1 and only clear E2 patterns as E2. The actual aphid activities during E1 are not yet known (Tjallingii, 1990).

The parameters extracted from the EPG's can be divided into two groups. The "Non Sequential" parameters (Table 2), like *total time* and *frequency* of each pattern, do not take into account the order of the events in time but use the pooled data of each EPG. The "Sequential" parameters (Table 3), like registration time to 1st E (time from start of experiment to 1st E), refer to the sequence of the patterns as they occur in the EPG. Several of these sequential parameters are explained in figure 1.

Calculations and statistical analysis. The different parameters were determined or calculated per aphid, and after that the mean and standard errors over all aphids of the treatment were calculated. Mann-Whitney rank sum test was used to test for significance. The patterns which were

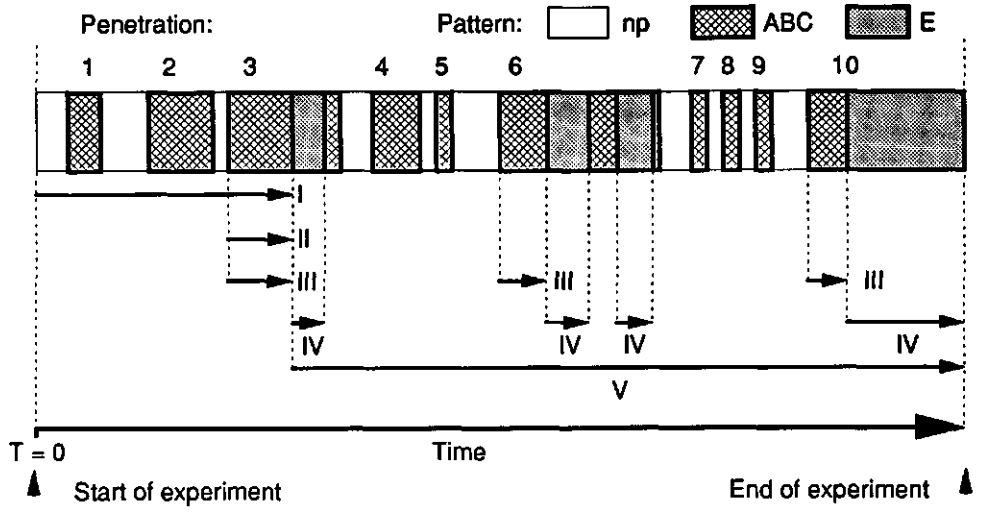

Figure 1. Simplified overview of an EPG and some parameters.
After placing the aphid on the leaf the recording starts (T=0). It takes some time before penetration starts (1st np period). Usually several penetrations without E pattern (pen. 1 and 2) precede the first "successful" penetration (3) showing E. Registration time to 1st E is the time between T=0 and the 1st E pattern (arrow I) and includes several penetrations. Penetration time to 1st E is the time between the start of the successful penetration (3) and the start of E (arrow II). During an EPG several penetrations showing E patterns can occur with a different penetration time to E (arrows III). To calculate the Potential E index (see text) the sum of arrows IV is calculated as a percentage of arrow V.

terminated by the end of the experimental period (after 16 hours) and not by the aphid itself, were not excluded from the calculations (see discussion).

RESULTS

Table 2 presents the *total time, frequency* and *mean duration* (= total time / frequency) of each pattern during 16 hour of EPG recording. Significant differences between the resistant and susceptible lines occurred

Table 2. *Total time*, *frequency* and *mean duration* of each EPG pattern in the 16 h EPG's of *N. ribisnigri* on resistant and susceptible lettuce

PARAMETER	RESISTANT (N=20)			SUSCEPTIBLE (N=15)			
TOTAL TIME(in seconds x 10^3)							
np	17.3	±	2.4	9.9	±	1.7	*
ABC	33.6	±	2.3	23.3	±	3.6	*
E1e	0.05	±	0.04		ND		
E1	2.5	±	1.3	1.4	±	0.4	
E2	2.1	±	0.8	22.1	±	5.0	**
F	1.0	±	0.4	0.2	±	0.2	
G	1.8	±	0.4	1.8	±	1.1	
FREQUENCY							
np	64.9	±	5.1	52.8	±	9.0	
ABC	70.2	±	5.3	58.4	±	9.7	
E1e	0.1	±	0.1		ND		
E1	4.6	±	0.7	5.7	±	1.0	
E2	2.2	±	0.4	3.9	±	0.8	
F	1.1	±	0.6	0.3	±	0.2	
G	1.3	±	0.3	1.1	±	0.5	
MEAN DURATION (in seconds)							
np	287	±	50	184	±	20	
ABC	510	±	41	467	±	77	
E1e	45	±	37		ND		
E1	623	±	448	207	±	45	
E2	621	±	213	9548	±	3743	***
F	716	±	407	113	±	77	
G	1350	±	399	574	±	264	

Time in seconds, Mean ± standard error. Mann-Whitney tests for comparison of 2 samples, * = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.001$. ND = not detected.

in *total time* of non penetration (np) ($p < 0.05$), ABC ($p < 0.05$) and E2 ($p < 0.005$), and in the *mean duration* of E2 ($p < 0.0001$). In Table 3 the parameters associated with the *start of the EPG* include: Duration of 1st np, duration of the first penetration period and of the second non penetration

period (Table 3 : Start of EPG). No significant differences were observed. The parameters associated with the *1st E* (Table 3) were: The penetration time, from the start of the "successful" penetration, to the 1st E pattern; the time from the start of the registration to 1st E; the number of penetrations preceding the 1st E; the duration of the 1st E and the duration of the np period after the 1st E . These parameters showed no significant differences between RES and SUS, except for the penetration time to first E, which was longer on the susceptible line (2.47×10^3 versus 1.34×10^3 s, $p < 0.05$). Similar parameters are provided for the *1st E2* (Table 3). The duration of the 1st E2 pattern was much longer on the susceptible line (7.7 versus 1.1×10^3, $p < 0.005$). The last part of Table 3 (*All E*) shows some other parameters related to all intracellular E patterns (both E1 intracellular and E2). No significant differences were observed in the time from the start of the penetration to E, the number of penetrations showing E, the number of penetrations showing E2 and the percentage of penetrations showing E.

On the resistant line the E periods were difficult to separate in E1 and E2 patterns. On the susceptible line we usually found the characteristic sequence of a short (< 1 min) E1 pattern and a short "transient period" (ca. 15 s) preceding a long E2 pattern (many minutes to hours) as also described by Tjallingii (1990). On the resistant line this sequence showed many irregularities. At the beginning of a sieve element puncture E1 was always found but quite often the shift to E2 did not occur or occurred very late. Irregular shifts from E1 to E2 and back occurred and sometimes the EPG signal showed characteristics of both patterns. Patterns E1e, F, and G, occurred in only a few cases and no relation with susceptibility was observed.

DISCUSSION

Pretreatment. The pretreatment (from rearing to final transfer to the test plant) is known to strongly influence the aphid's behaviour, in particular the duration of the first non penetration period as reported by Montllor and Tjallingii (1989). A number of different methods have been reported, especially with regards to the wiring and handling of the aphids. In order to

Table 3. Other parameters and events during 16 h EPG recording of *N. ribisnigri* on susceptible and resistant lettuce

EVENT	RESISTANT (N=20)			SUSCEPTIBLE (N=15)			
START OF EPG							
Duration of 1st np period	209.1	±	49.7	178.2	±	33.9	
Duration of 1st penetration period	59.6	±	15.0	115.6	±	56.7	
Duration of 2nd np period	237.6	±	39.0	210.7	±	68.7	
1ST E							
time to 1st E							
a. from start registration (x 10^3 s)	17.8	±	3.0	9.7	±	2.9	
b. from start penetration (x 10^3 s)	1.34	±	0.18	2.47	±	0.52	*
Nr of penetrations preceding 1st E	19.9	±	3.3	17.7	±	5.5	
Duration of 1st E (x 10^3 s)	1.0	±	0.5	5.2	±	3.8	
Duration of np after 1st E	322.9	±	90.2	313.5	±	134.1	
1ST E2							
Time to 1st E2							
a. from start registration (x 10^3 s)	20.0	±	3.8	11.8	±	3.2	
b. from start penetration (x 10^3 s)	1.49	±	0.21	1.80	±	0.18	
Nr. of penetrations preceding 1st E2	20.1	±	3.8	19.3	±	5.1	
Duration of 1st E2 (x 10^3 s)	1.1	±	0.6	7.7	±	4.0	**
Duration of np after 1st E2	191.8	±	70.3	122.4	±	19.2	
All E							
Time to E from start penetr.(x 10^3 s)	1.56	±	0.20	1.57	±	0.15	
Nr. of penetrations showing E	3.2	±	0.5	3.7	±	0.7	
Nr. of penetrations showing E2	1.8	±	0.3	2.6	±	0.6	
% of penetrations showing E	5.42	±	0.85	8.84	±	1.70	

Time in seconds unless otherwise stated, Mean ± standard error. Mann-Whitney tests for comparison of 2 samples, * = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.001$. Notice the difference between 1stE which refers to only one event per aphid (the first time it reaches E) and ALL E which may be the result of several penetrations showing E patterns for one aphid.

attach the gold wire to the aphid three methods have been used: inactivation of the aphid by cooling or anaesthetization (Harrewijn, 1990, pers. comm.), immobilizing the aphid by applying a vacuum (in this case) or attaching the wire on the walking aphid (C.B. Montllor & W.F. Tjallingii pers. comm). After wiring the aphid was either used directly (Tjallingii, 1988), after a period of

recovery on a plant (Montllor & Tjallingii, 1989) or starved for a certain period (McLean & Kinsey, 1967). Durations of the first non penetration period of up to 15 minutes have been found when aphids were cooled before tethering (P. Harrewijn, 1990). We developed a pretreatment with a four hour period on the Taiwan plant between the wiring and the EPG recording to recover from the stress experienced during tethering. This reduced the first non penetration period to about three minutes.

Morphs. Alates were chosen because they are the migrating morph and therefore most relevant, and they might be more sensitive to host-plant differences. Alates of *N. ribisnigri* have more receptors on their antennae than apterae (Bromley *et al.*, 1979). The wire had to be connected to the thorax because the wings over the abdomen of the alates interfered with the "normal" wire connection on the abdomen. Attaching the wire on the small thorax is slightly more difficult than connection on the abdomen, but this seemed to reduce the burden of the wire on the aphids movements.

Total time, frequency and *mean duration* (Table 2). These "non sequential" parameters showed that the only clear difference observed in the EPG's was a strong reduction of the duration of the E2 pattern on the resistant line ($p < 0.001$). This resulted in a strongly reduced total time in E2 on the resistant line of 0.5 hour versus 6 hours on the susceptible line ($p < 0.005$). Pattern E2 reflects passive food uptake from a sieve element (Tjallingii, 1985, 1988), so feeding was almost completely absent on the resistant line. These results are comparable to the results of Montllor and Tjallingii (1989) who used different lettuce lines but the same resistance gene. They did not distinguish between E1 and E2 pattern but used the old classification E(pd) which consists of both E1 and E2 (Tjallingii, 1990).

The calculation of the mean durations was somewhat biased by the termination of the EPG recording after 16 hours. However, we decided not to exclude the pattern that was in progress at the end of the EPG from the calculations of the mean duration. This would have influenced the results even more, in particular for the mean duration of the E2 pattern. Six long (> 2 h) E2 patterns were ended on the susceptible line by the end of the experimental time, against one on the resistant line. Therefore, the actual

Table 4. Potential indices of np, ABC, E and E2

A. Potential E indices as percentage of time used as E (both E1 and E2) or only E2 after subtraction of registration time to 1st E or 1st E2

B. Percentage of total time in np and ABC and frequency of non penetrations corrected for pattern E. Total time in E is subtracted from total EPG time

PARAMETER	RESISTANT			SUSCEPTIBLE			
A.							
Pot. E index(%)	11.5	±	4.0	48.5	±	9.3	***
Pot. E2 index(%)	7.4	±	2.3	55.0	±	10.0	***
B.							
%Time in np	31.7	±	4.1	30.0	±	3.4	
%Time in ABC	62.9	±	3.8	66.9	±	3.5	
Freq/h of np	4.3	±	0.3	5.7	±	0.6	

Mean ± standard error. Mann-Whitney tests for comparison of 2 samples, *** = $p < 0.001$.

duration of the E2 pattern on the susceptible line was longer than we have calculated. For other patterns the errors are less important because their frequency is usually higher and their mean duration much shorter.

Another method to overcome this problem is to calculate a "potential E index" and a "potential E2 index", this is the percentage of time spent in E or E2 after subtraction of the time needed to reach E or E2 for the first time, in other words the total time spent in E (or E2) divided by the total length of the EPG minus the time to first E or E2 (table 4a, Formula: Potential E index = 100 * Total time in E/(Total EPG time - time to 1st E from start registration)). In fig. 1 the potential E index can be calculated as the sum of all arrows IV divided by arrow V. These indices take into account that it takes considerable time to reach an E pattern (Tjallingii & Mayoral, 1993) for the first time. So E can never occur at the start of the registration and the time available for E is shorter than the total time of the experiment. Theoretically it is possible to find a shorter total time in E on a susceptible line than on a resistant line just because it takes longer to reach E pattern on the susceptible line. In that

case most of the E pattern would occur at the end and after the experimental period, leading to the false conclusion that there is less feeding. The potential E and E2 indices showed very clear significant differences. The indices were much larger for the susceptible line (48.5 and 55.0% respectively) than for the resistant line (11.5 and 7.4%). Noticeable is the fact that if only E2 was concerned the difference was clearer than with pooled E1 and E2, which again suggest that the E2 pattern is more important.

During the analysis of the results one has to bear in mind that nearly all parameters influence each other. If the E2 pattern is 40% of the experimental time on the susceptible line and only 5% on the resistant line, then the number of penetrations will be higher on the resistant line. Careful interpretation of the data of these dependent variables is therefore necessary to reveal causal relationships. In this case the longer total time spent in np and ABC pattern on the resistant line was simply due to the "extra" time available for patterns other than E. When the percentage of time in np and ABC was calculated with the total time in E subtracted from the total EPG time there was no difference between the lines (see Table 4, Formula: X = 100 x Total time in np (or ABC)/(Total EPG time - Total time in E)). This also holds for the number of non penetrations per hour after the same correction (Table 4b, Formula: X = Total number of np/(16 hours - Total time in E)). Therefore, these parameters indicated a strongly reduced feeding period but did not show the origin of this reduction during the EPG. To determine the origin of this reduction the sequence of the EPG patterns had to be studied in detail.

Start of the EPG (Table 3). The duration of the 1st non penetration period of the EPG can give information on the influence of the plant phyllosphere on the aphid (volatiles, leaf structure, colour etc). No differences were observed in this experiment. However, we consider the use of this parameter in EPG recording rather limited because the 1st np period is strongly influenced by the aphids pretreatment and aphids tend to probe on any leaf surface to determine the possibility of feeding (Moericke, 1955). However, large differences in the 1st non penetration period may indicate differences observed by the aphid before penetration. This can not be investigated further by EPG's but requires separate behavioral studies under more natural

conditions. The other two parameters (duration of 1st penetration period and duration of 2nd np period) showed no difference, indicating that the unsuitability of the resistant plants was not yet discovered by the aphid.

1st E. During an E pattern, either E1 or E1 followed by E2, the stylets are located in a sieve element (Kimmins & Tjallingii, 1985; Tjallingii & Hogen Esch, 1993). The occurrence of an E pattern is the first clear proof of a sieve element puncture. Nearly all aphids (32 out of 35) reached a sieve element during the EPG as shown by the presence of an E pattern. However, during the preceding ABC pattern brief sieve element punctures can occur as potential drops which are indistinguishable from potential drops of intracellular punctures in any other type of cell (Tjallingii & Hogen Esch, 1993). Though likely, it is not clear whether cells are sampled by the aphids during potential drops. It seems as if during these cell punctures sieve elements are not immediately recognized as such.

The time needed for the aphid to reach an E pattern for the first time was 2.7 to 5 hours and not significantly different between the two lines. This period contained a number of penetrations (not different between lines) showing mostly ABC pattern with numerous potential drops (cell punctures) (see fig. 1). Therefore we conclude that the sieve elements were not harder to locate on one of the lines and no chemical or mechanical factor inhibiting penetration or feeding was present outside the phloem.

The time to the 1st E pattern within the first successful penetration (see fig. 1, arrow II; Table 3) was significantly different ($p < 0.05$), being twice as long on the susceptible line (22 min versus 41 min). However, when this parameter was extended to all penetrations reaching pattern E (see fig. 1, arrows III) no difference was found (Table 3: Time to E from start of penetration). Therefore, the difference found in this parameter for the first successful penetration was considered an artefact and it was concluded that there was no mechanical barrier present which made it harder to reach the phloem. A resistance mechanism outside the phloem, is suggested in other aphid-plant combinations where the resistance was related to the time needed by the aphid to reach the phloem (Dreyer and Campbell, 1984; Niemeyer, 1990), but this seems unlikely here. Niemeyer (1990) suggested a chemical factor outside the phloem in the case of *Rhopalosiphum padi* on

barley (possible role of hydroxamic acids). In the resistance of sorghum against greenbugs (*Schizaphis graminum*) a mechanical barrier (e.g. pectin composition) outside the sieve elements was suggested (Dreyer & Campbell, 1984). In both cases the authors did not distinguish between registration time, from start of the experiment, and penetration time, from the start of the successful penetration. It is possible that an increased registration time before reaching a sieve element occurs with an equal penetration time in which case there are simply more or longer penetrations preceding the successful one. If this occurs and the penetration time from the start of the successful penetration is not different then mechanical differences can be excluded as a resistance mechanism.

The duration of the 1st np period after an E pattern could be influenced by the uptake of feeding stimulants or deterrents with the food, signalling to the aphid that the plant is a host or a non-host. Our results did not show any difference between the lines in this respect.

1st E2 (Table 3). The E2 pattern is known to be related to passive phloem sap ingestion (Tjallingii, 1985). The duration of the 1st E2 pattern on the susceptible line (Table 3) was 2 hours, showing clear food uptake. The mean duration of 1st E2 on the resistant line showed that there was some food uptake but much shorter (20 min, Table 3). So the longer duration of the E2 patterns in general (Table 2) was also reflected in the 1st E2. This was the first occasion during the EPG where a difference occurred between the lettuce lines. Apparently during the 1st E2 (the first feeding) the host plant suitability was perceived by the aphid. This suggested a resistance factor in the phloem vessel, presumably related to characteristics or composition of the phloem sap.

E1 versus E2 patterns. The irregularity in the E pattern on the resistant line also indicated that during that stage of behaviour the unsuitability of the plant was interfering with feeding. Nothing is known about the activities of the aphid during E1. It is possible that during E1 the aphid is sampling the phloem sap or preparing the sieve element for feeding. No data on food uptake, saliva excretion or muscle activities during E1 are available so far.

More detailed knowledge of E1 is necessary since this phase of the EPG seems to be of crucial importance in this case of resistance.

Duration of EPG's. The duration of the EPG's in this experiment (16 h) was considerably longer than the length in earlier reports (4 h) (Van Helden, 1990). We decided to make longer EPG's because in the short EPG's not all patterns could be seen. For most *N. ribisnigri* aphids it took more than four hours to reach an E pattern for the first time. In a natural situation an aphid may leave the plant when it has noted that it is not a suitable host plant. In that case the aphid would leave after a sieve element puncture. However it is still possible that a free aphid responds to more subtle stimuli which occur before it reaches the phloem which are masked in the EPG due to the tethering. A good comparative experiment with free aphids is therefore necessary. A minimum EPG length of 8 hours seems necessary in this case. From this experiment it could not be made clear whether the second part of the EPG yielded much extra information. Normally one would expect a equilibrium in the distribution of the time over the different patterns to be established by then. However, between hour 9 and 12 of the EPG (clock time ± 22.00 hour) a clear change in the distribution of the different patterns occurred. This could be due to a diurnal rhythmicity in the plant or the aphid, in spite of the continuous light provided during the experiment. The length of the EPG's in this kind of experiments should include a reasonable amount of phloem feeding on the susceptible line and ample time to reach the phloem on both lines. A good way to decide on the duration of the EPG's would have been to determine the time an aphid needs to start the production of honeydew on the susceptible line. The EPG should then be recorded for a period of about twice as long. This was not done in this case.

Correlation with bionomics. Van Helden *et al* (1992a) described the bionomics of *N. ribisnigri* on these same lines. Their experiments suggested a total absence of feeding on the resistant line but no toxic effect of the resistance on the aphid. The EPG results did agree with their conclusions. The possibility of a chemical resistance factor as suggested in Van Helden *et al* (1992a) is consistent with the EPG's. In this case a relation between the amount of feeding and the growth rate (Van Helden *et al*, 1992a) could not

be established because the EPG reflected only the initial phase of aphid plant interaction while the mean relative growth rate was determined over a period of several days.

Correlation with behaviour. The behaviour of the aphid during the EPG could be influenced by the treatment. Especially the tethering can influence the aphid (Tjallingii, 1985). It is likely that the aphid would leave a resistant plant after it has classified it as such, in the case of *N. ribisnigri* on lettuce after the 1st E2 pattern. Tjallingii (1985) showed a decreased sensitivity of the aphid to plant suitability in a host versus non-host situation. In order to evaluate the possible disturbance of the aphids behaviour an additional behavioral study is needed (Van Helden *et al*, 1992b).

Resistance mechanism. A resistance factor in a phloem vessel can be either a mechanical or a chemical barrier. A mechanical barrier in the form of sieve element or aphid stylet blocking after puncturing still is a possible resistance mechanism. However, the resistance gene is specific against *N. ribisnigri* and gives no clear resistance against other lettuce attacking aphids like *Myzus persicae, Macrosiphum euphorbiae* or *Uroleucon sonchi* (Van Helden, unpubl.; Reinink & Dieleman, 1989). This suggests a very species specific mechanism. It is possible that the stylet penetration activities of the aphid induce a resistance mechanism in the sieve elements by elicitors in the saliva of the aphid or alternatively elicitors may be released from by plant in response to the aphids salivary enzymes. A purely mechanical mechanism seems not very likely. A possible mechanical barrier during the penetration and before the sieve element is reached can be excluded based on our results. The possible uptake of small volumes of plant sap during penetration which can then be tasted by the chemoreceptors in the pharyngeal cavity (Tjallingii, 1985; Wensler & Filshie, 1969) may provide information for the aphid and influence its behaviour (Niemeyer, 1990). Though it seems likely, no clear proof exists for sap uptake during penetration and before the phloem has been reached. In this case no difference was observed during the penetration to the phloem. This suggests that the aphid did not gain any information about the resistance in this stage. The phloem sap may contain many clues about the host plant suitability. During the E2 pattern uptake of

phloem sap takes place and contact with the pharyngeal chemoreceptors occurs and we therefore hypothesize that the chemical composition of the phloem was responsible for the resistance. As shown earlier (Van Helden *et al*, 1992a) there was no proof for intoxication of the aphid on the resistant line, which suggests a feeding deterrent (or absence of a feeding stimulant) as a possible mechanism. The absence of long E2 patterns supports this theory and therefore our studies will now concentrate on the phloem sap composition of the lettuce lines.

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4. FEEDING BEHAVIOUR OF *FREE* N. RIBISNIGRI

ABSTRACT

The feeding behaviour of *Nasonovia ribisnigri* (Mosley) alates was studied to find the behavioural phase where rejection of an absolutely resistant lettuce (*Lactuca sativa* L.) line occurred. No differences were observed between landing percentages or behaviour during the first 30 minutes on resistant and susceptible plants. Longer observations revealed that aphids were more mobile on the resistant line and started to leave after 4 hours. Most aphids stayed for more than 48 h on the susceptible plants.

In a previous study, using electrical penetration graphs, we showed that differences in probing behaviour occurred during the first attempted phloem feeding. The first observable sieve element puncture usually occurred after 5h. Through comparison of free and tethered aphids we showed that tethering did affect probing behaviour. Rejection of the plant by free aphids occurred after 4 to 24 hr, which corresponded well with the time after which free moving aphids started to leave the plant. We therefore conclude, that rejection of the resistant plant occurs after ingestion from a phloem sieve element.

INTRODUCTION

Absolute resistance in lettuce to the aphid *N. ribisnigri* (Van Helden *et al.*, 1993) is based on a single dominant gene (Nr gene, Eenink & Dieleman, 1982; Eenink *et al.*, 1982; Reinink & Dieleman, 1989). The resistance mechanism is still unclear. Electrical Penetration Graphs (EPG's) (Tjallingii, 1988) from *N. ribisnigri* on resistant and susceptible lettuce showed that probing behaviour differed only after the aphid had reached the phloem (Van Helden & Tjallingii, 1993). During the EPG the aphid is attached to a gold wire which hampers its movements and will probably influence its behaviour (Tjallingii, 1985, 1986). The experiments presented here aim to study at what point free moving aphids reject the resistant plant and whether free aphids and tethered aphids behave similarly.

MATERIALS AND METHODS

Aphids and plants. Mass-rearing of *N. ribisnigri* on lettuce (cv. "Taiwan") and culture of plants were performed as described by Van Helden *et al.* (1993). Alates were produced as described by Van Helden & Tjallingii (1993). Recently-moulted alate adults were used. The plants were two nearly isogenic lines (RES and SUS) which differed only in the *Nasonovia* resistance gene (Van Helden *et al.*, 1993). Plants were about 4 weeks old (6-8 fully expanded leaves). In the flight chamber another susceptible lettuce line ("Taiwan") and a non-host plant Chinese cabbage (*Brassica cernua* (Thbg) cv. "Granaat") were also used.

Landing behaviour

Pretreatment. Teneral alate virginoparae were collected with a brush from their food plant and transferred to a small piece of leaf (3 x 3 cm) in a Petri dish at 22 °C for 24 hours, after which the leaf fragment was removed. The aphids were used after another 24 hours of starvation when they showed much flight activity in the Petri dish.

Flight chamber. A 1 x 1 x 1 m flight chamber was constructed comparable to the one described by Legge (1962) and Halgren & Rettenmeyer (1967).

The walls, floor and ceiling were covered with white cloth. Wind speed ranged from 0.50 m/s (directly under the air inlet) to 0.05 m/s on the bottom. Light intensity from HF Fluorescent tubes was 36 $\mu E.m^{-2}.s^{-1}$ (ceiling under funnel) to 6 $\mu E.m^{-2}.s^{-1}$ (floor). Temperature was 25 ± 3 °C.

Protocol. Aphids were released individually from a small platform (5 mm in diameter) on the tip of a white cone at 20 cm from the floor of the chamber and 20 cm from the left wall of the chamber. A plant was placed at 20 cm from the right wall with a white paper cover (11 cm high) around the pot. When the aphid stayed on the platform for more than two minutes the observation was discarded. Most aphids flew off soon and landed either on the plant (end of observation), on the walls, or on the pot cover. If they stayed there for more than two minutes the observation was ended. Some aphids walked to the plant after landing nearby. About 100 aphids were released in the flight chamber for each plant type.

Reflection-spectra. Reflection spectra (350-1100 nm) of both the adaxial and the abaxial leaf surfaces were measured at two different dates with a spectrophotometer.

Behavioural observations

Experiments were performed in the laboratory at 22 ± 1 °C under HF fluorescent tubes at 48 $\mu E.m^{-2}.s^{-1}$ Lux.

Pretreatment of the aphids. Teneral alate virginoparae were collected with a brush from their food plant (lettuce cv "Taiwan") into a Petri dish between 8.30 and 9.00 am. They were marked with water soluble paint in different colours using a vacuum device (Van Helden & Tjallingii, 1993; Van Helden & Tjallingii, 1994) and placed on a fresh Taiwan plant for at least 3 h to recover (Pretreatment identical to Van Helden & Tjallingii, 1993).

Continuous observations (0.5 h). One aphid was carefully picked up with a fine brush and transferred to a paper platform (8 mm in diameter) on the adaxial side of the fourth leaf of the plant (from the growing point). Observation started when the aphid walked off the platform. The behavioural sequences observed (Table 1a) were comparable those described for *Aphis fabae* I. (Scop.) (Ibbotson & Kennedy, 1951, 1959). Behaviour was recorded

Table 1a. Events recorded during continuous behaviourial observations (0.5 h).

EVENT CLASS	EVENT	DESCRIPTION
ACTIVITY	Walking	Aphid walking, proboscis not extended
	Penetration	Stylet penetration: Proboscis in continuous contact with leaf surface without moving, antennae backwards
	Tapping	Tapping leaf with proboscis, antennae upright
	No activity	None of the previous activities
LEAF POSITION	Adaxial	Adaxial (upper-)side of leaf
	Abaxial	Abaxial (under-)side of leaf
	Stem	Stem or main vein near to the stem
	Growing tip	Smallest still folded leaves (1 or 2)
PENETRATION SITE	No penetration	No proboscis contact (occurs only when ACTIVITY is other than penetration)
	Main vein	First order (central) vein of leaf
	Side vein	Second and third order veins which were clearly raised from the abaxial leaf-surface
	Lamina	Lamina or very small vein (not raised from the abaxial surface of the leaf).

for 30 minutes using a portable computer and "THE OBSERVER"™ (Noldus, 1991) software.

Interval observations (48 h). The start of the experiment was identical to the previous observations. 8 aphids, each on a different plant, were observed at 0.5, 1, 2, 4, 8, 16, 24, 32, 40, and 48 hours. The events are listed in Table 1b. To prevent aphids walking off the plant and then returning to it, a ring of plastic tape with Fluon™ was placed around the base of the plant. Aphids walking on the Fluon™ dropped down into a dish filled with water and were recorded as having left the plant.

Table 1b. Events recorded during interval behaviourial observations (48 h)

EVENT CLASS	EVENT	DESCRIPTION
RELOCATION	No relocation	Aphid in same position as last observation
	Relocation	Aphid shifted to new position
PLANT POSITION	Growing tip	Smallest still folded leaves (1 or 2)
	Younger leaf	Leaf younger than starting leaf
	Start leaf	First fully extended leaf (usually nr.4 from tip)
	Older leaf	Leaf older than starting leaf
LEAF POSITION	Adaxial	Adaxial (upper-)side of leaf
	Abaxial	Abaxial (under-)side of leaf
	Stem	Stem or main vein near to stem
	Growing tip	Smallest still folded leaves (1 or 2)
PENETRATION SITE	No penetration	No proboscis contact
	Main vein	First order (central) vein of leaf
	Side vein	Second and third order veins which were clearly raised from the leaf-surface
	Lamina	Lamina or very small vein which did not raise from the surface.

Statistics. All parameters were first calculated per aphid and then averaged over all aphids. Landing and behavioural data were tested for significance with Mann-Whitney rank-sum tests or Fisher's exact test.

RESULTS

Landing behaviour. Aphids took off readily and flew in the chamber for periods of up to ten minutes before landing. Direct landing frequencies (Table 2) on Chinese cabbage and Taiwan lettuce were comparable (25.7 and 27.6%) and lower than on RES and SUS (42 and 35%). Many aphids walked

Table 2. Results of the flight experiment.

Plant	N	% ARRIVING AT THE PLANTS Landing	Walking	Total
Taiwan	105	27.6	47.6	75.2
RES	100	42.0	38.0	80.0
SUS	100	35.0	48.0	83.0
Chin. Cabbage	105	25.7	32.4	58.1

to the plants after landing nearby. All together the number of aphids arriving at the plant was comparable for all lettuce lines and somewhat higher than for Chinese cabbage (Table 2). The colour spectra of the plants were almost identical with a peak in the visible spectrum at 550-560 nm. The abaxial side allways showed a higher reflection. RES had a slightly higher total reflection than SUS (e.g. 34 versus 31% at 555 nm) and both were somewhat higher than Chinese Cabbage and Taiwan which were almost identical (20-23% at 555 nm).

Continuous observations (0.5 h). Only the duration of the first stylet penetration showed a significant difference, being almost twice as long on the resistant line (43.1 versus 23.9 s) (Table 3a; "free"). The aphids were probing almost 75% of the time.

These visual observations of "free" aphids could be compared with results of tethered aphids in EPG recordings (Table 3a) derived from earlier experiments (Van Helden & Tjallingii, 1993). Significant differences were apparent especially in the duration of the first non-penetration period (the time before the first penetration) which was over three times as long for the tethered aphids. The total time spent in stylet penetration (Table 3a: total time sp) was a little longer (and consequently the total time in non-penetration shorter) for the free aphids. Although significant only for the resistant line, the mean duration of the individual non-penetration periods (interval between penetrations, table 3a: mean duration np) was somewhat

Table 3. Results of continuous observations of free aphids (0.5 h) and comparison with tethered aphids (extracted from earlier EPG data)

A. CONTINUOUS OBSERVATIONS (0.5 h) AND EPG DATA

	SUSCEPTIBLE				RESISTANT			
	FREE		tethered		FREE		tethered	
Nr. of observations	33		15		34		20	
Total time np	458 ± 34	*	777 ± 105		504 ± 41	*	746 ± 83	
Number of np	4.2 ± 0.2		5.1 ± 0.4		4.9 ± 0.3		4.9 ± 0.4	
Mean duration np	118 ± 10		149 ± 17		116 ± 11	*	164 ± 24	
Total time sp	1342 ± 34	*	1023 ± 105		1296 ± 41	*	1054 ± 83	
Number of sp	4.1 ± 0.2		4.8 ± 0.3		4.8 ± 0.3		4.8 ± 0.4	
Mean Duration sp	365 ± 23	*	248 ± 40		307 ± 22		252 ± 34	
Dur. first np	55.5 ± 6.8	*	179.9 ± 32.2		68.5 ± 12.7	*	205.2 ± 49	
Duration first sp	23.9 ± 1.8#		115.6 ± 54.8		43.1 ± 7.2#		47.4 ± 10	

B. INTERVAL OBSERVATIONS (48 h) AND 16h EPG DATA

Nr. of observations	36	15	33	20
% time in sp	-	77.0	-	74.2
% of obs. in sp	92.7	-	81.1	-
% aphids leaving	33.3	0	97.4	0

Time in seconds. Mean ± standard error. np = non-penetration, sp = stylet penetration. Total time is sum of all individual periods per aphid (e.g. Σ(time probing)), mean duration is average of duration individual periods (e.g. Σ(time probing)/nr of probes)). * = Significant difference between free and wired aphids on the same line ($p < 0.05$).
= Significant difference between aphids on resistant and susceptible plants ($p < 0.05$).

longer for the tethered aphids. The mean duration of the individual stylet penetrations (Table 3a: mean duration sp) was a little longer for the free aphids but only significant on the susceptible line. Nearly all free aphids changed from adaxial to abaxial of the leaf in the first minutes of observation, often without penetrating first ("Seitenwechsel", Klingauf, 1972) (Table 4).

Table 4. Aphids switching to abaxial side of leaf in continuous observations.

PARAMETER	SUSCEPTIBLE	RESISTANT
% showing side switch	100	94
mean time to switch (s ± SE)	67 ± 10	73 ± 9
% performing a switch without penetrating first ("Seitenwechsel")	55	53

Interval observations (48 h). On the susceptible plants 66.7% of the aphids stayed on the plant for 48 h. (Table 3b, Fig. 1a). On the resistant plant 97.4% left the plant (Table 3b, Fig. 1b), i.e. only one aphid stayed. The aphids left between the 4th and the 24th hour of the experiment (Fig. 2).

Over the whole observation period aphids on the resistant line consistently showed significantly higher mobility (80-90% of the aphids relocated between two observations) than on the susceptible line (50-60% relocation). The aphids remained longer on the 4th (start) leaf on the susceptible line whereas they moved earlier to the growing tip on the resistant line (Fig. 1a,b). Stylet penetrations were mostly on the main vein or the larger side veins (Fig. 1c,d). Compared with the percentage of time in non-penetration from EPG registrations (Table 3b) free aphids seemed to probe a slightly larger percentage of time although comparison of (continuous, 16 h) EPG and visual (interval, 48 h) observations is difficult. Aphids were almost exclusively found on the abaxial side of the leaves.

DISCUSSION.

Landing behaviour.

Aphids are attracted to green-yellow surfaces, often irrespective of the plant species (Moericke 1955, 1962; Müller, 1962). In our experiment the small differences in landing percentages on the different plants probably reflect the small differences in brightness (Müller 1964). Although the experimental arrangement is not really comparable to a natural situation no indication was found that the aphids were able to distinguish between

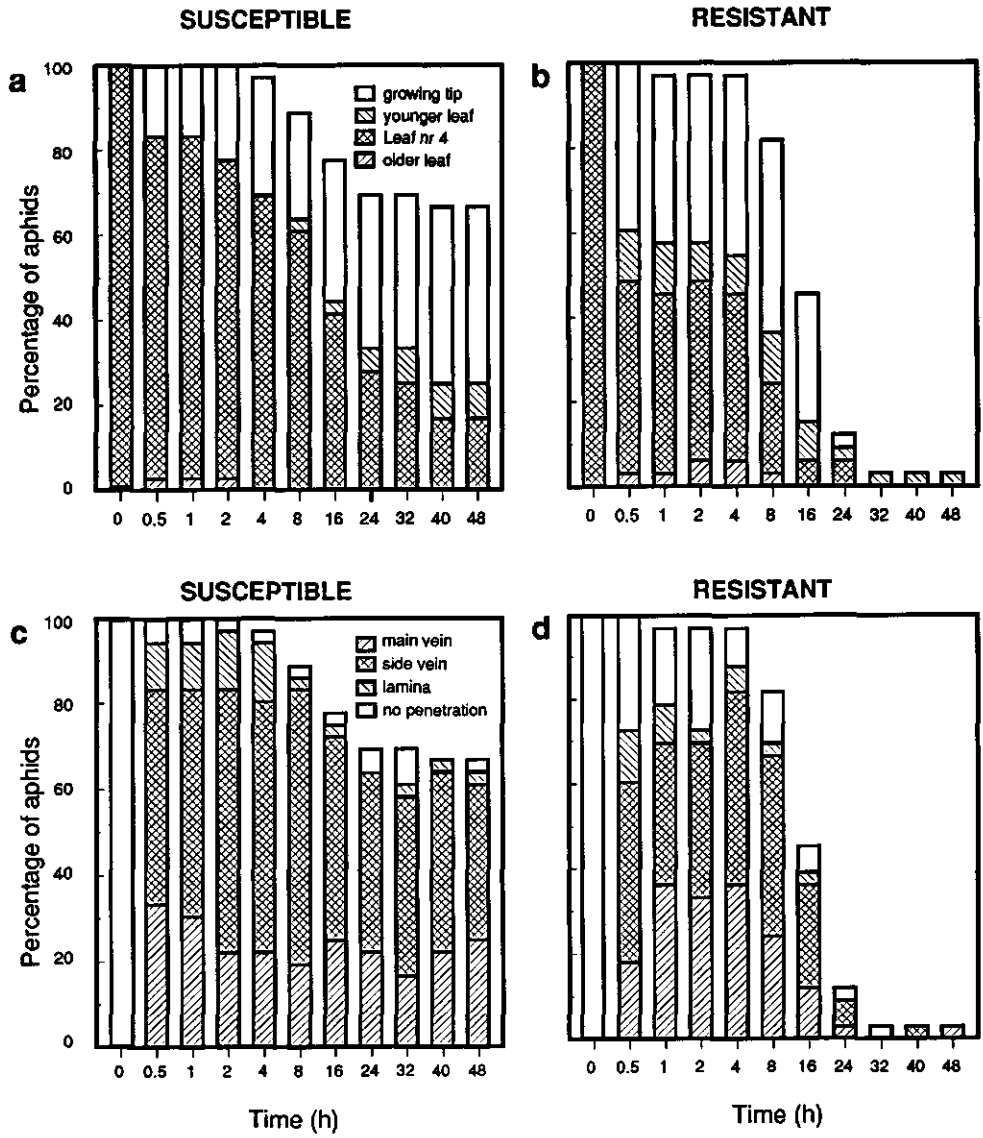

Figure 1. Interval observations (48h). Aphid position on susceptible (a) or resistant (b) plants and penetration sites on the leaves of susceptible (c) and resistant (d) plants at different observation times. Decreasing column lenghts show aphids leaving the plant.

susceptible and resistant lettuce before alighting. The same result has been shown for several other aphid-plant combinations (Müller, 1962).

Continuous observations (0.5 h)

Resistant versus Susceptible. The behaviour of the free aphids on RES and SUS lettuce showed very little difference during the first half hour. The duration of the first non-penetration period (time before probing) was not different, so effects of host-plant differences in this stage were not shown. Several authors have reported significant differences in this parameter in a host/non-host situation, always with a longer time before probing on non-host plants (Klingauf, 1972, Goffreda *et al.*, 1988). Similarly, a longer first non-penetration period was reported on resistant lettuce lines with the same Nr-gene as we used here (Harrewijn, 1990), but his lines were not near-isogenic so differences might be attributed to differences not related to the resistance gene.

The longer *duration of the first penetration* on the resistant plants suggests that the aphids did encounter some difference in plant stimuli during stylet penetration, before phloem contact. Klingauf (1972) even suggested that a longer first probe reflects better plant acceptance. However, apart from this single parameter we observed no important effects on the subsequent behaviour of the aphids (see also interval observations).

After landing on the adaxial side of the leaf aphids walk to the edge and change to the abaxial side, often without a first probe ("Seitenwechsel", Klingauf, 1972). Suppression or delay of this behaviour was considered a sign of plant deterrence (Klingauf, 1970; 1972). No differences were observed in this behaviour on SUS versus RES lettuce.

Thus, although some behavioural differences showed between aphids on resistant and susceptible plants before reaching the phloem this did not lead to host-plant rejection (as measured by leaving the plant).

Free versus EPG. Many differences were observed when free and tethered aphids were compared. The pretreatment (handling, paint marking, starvation etc.) may influence the results, especially the parameters *duration of the first non-penetration* (time before probing) and *duration of the first penetration* (Van Helden & Tjallingii, 1993). The pretreatment of the free and tethered (EPG) aphids was almost identical, since even the free aphids were

given a spot of paint on their thorax, similar to the wired aphids in EPG recordings. However, several causes may explain the observed differences in these parameters:

a. The transfer of the aphids to the test plant was slightly different. The "free" aphid was picked up with a brush and placed on a paper platform. Observation started only after the aphid walked off the platform. In the EPG experiment the aphid was lifted from the Taiwan lettuce plant by the gold wire and lowered directly on the leaf of the test plant. The pretreatment can have an important effect on subsequent behaviour (Van Helden & Tjallingii, 1993).

b. Behavioural observations started on the adaxial side of the leaf, free aphids often walked several cm and more than 50% even changed leaf surface before probing (Table 4). Tethered aphids were clearly hampered in their movements, often pulling the gold wire for a while before probing for the first time. Surface changes were impossible for these aphids and the surface exposed to them was limited by the length of the gold wire.

The EPG results showed no significant difference in the duration of the first penetration period on resistant versus susceptible plants. However, variation in the results is large and the number of replicates is small (Van Helden & Tjallingii, 1993). It is possible that the tethering reduces the impact of a subtle stimulus in this phase on the aphid, thus reducing the difference in behaviour on RES versus SUS, as was also concluded by Tjallingii (1985). The larger percentage of time spent in non-penetration (and consequently a shorter time in stylet penetration) by tethered aphids indicates a general "restlessness". The total time in non-penetration (or stylet penetration) is a combination of *frequency* and *mean duration*, which did not always show a significant difference individually (Table 3a). The difference in these parameters can be attributed to the direct physical effect of the tether on the insect which acts not only during non-penetration periods (when the aphid cannot move further because of the length of the wire) but also during probing, constantly pulling the aphid.

We conclude that the difference in the length of the first non-penetration period is attributable to both differences in the pretreatments and tether effects whereas variation in other parameters was mainly caused by tether effects.

Interval observations (48 h)

Resistant versus Susceptible. Already at the first observation (after 0.5 h) the aphids showed a higher mobility having moved more to the growing tip on the resistant plants. This confirms our observation that a difference in plant quality affected aphid behaviour as found in the previous experiment. However, this did not result in a rejection of the resistant plants in any form. Though generally a higher mobility is considered a sign of lower plant acceptance (Müller, 1962) the reaction almost resembled a faster acceptance and subsequent relocation to the preferred feeding site (growing tip) on the resistant plant. However, the stimulus which caused this small difference might play a role in the many (poorly understood) sequential steps leading to final plant acceptance or rejection (Klingauf, 1987). It is also possible that the stimuli involved are related (genetically or chemically) to larger differences encountered during phloem contact. This requires more knowledge on the nature of the resistance and host-plant evaluation by the aphids during different phases of penetration.

In a host versus non-host situation one would expect the aphids to leave the plant quite soon after the first plant contact (Müller, 1962; Klingauf 1970, 1987). While comparing two lines of *Vicia faba* l. Müller (1958) found that nearly all aphids left the more resistant line after 1-10 minutes (clearly before contacting the phloem bundle). In our experiment the aphid initially seemed to accept the resistant plant.

Although the level is quite different, the time course (shape of the curve) of those aphids leaving was similar for both lines suggesting that the decision moment (time after plant access) was the same (Fig. 2).

Free versus EPG.

Using EPG recordings Van Helden & Tjallingii (1993) found no difference in penetration behaviour on RES versus SUS before the phloem was reached. As shown in Fig. 2 most aphids reached the phloem within 1-8 hours in the EPG study. This is well within the range of time that the free aphids left the plant, corroborating the conclusion that phloem contact is necessary before rejection of the plant. No ultimate proof can be obtained that the aphids do reach a phloem sieve element without an EPG recording and even then not every sieve element contact can be recorded since brief

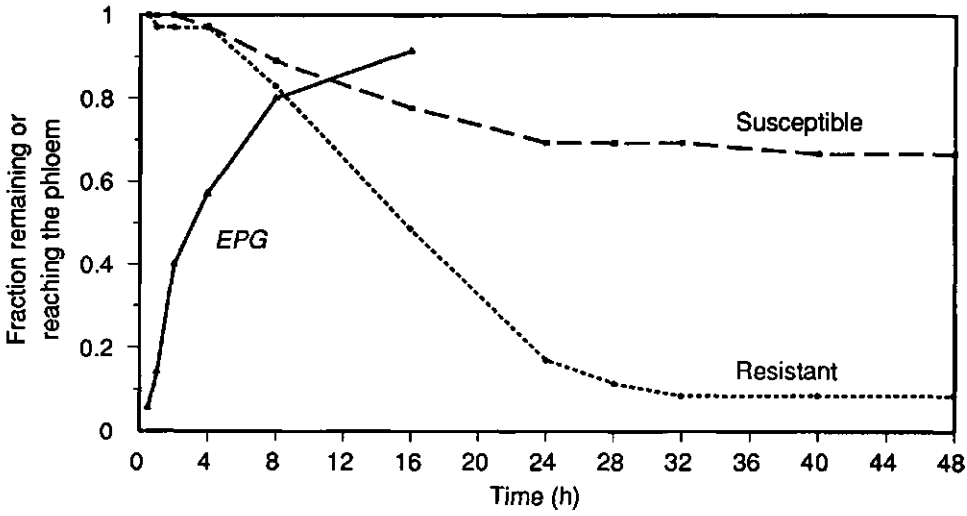

Figure 2. Time course of the departure of free aphids on RES and SUS compared to the aphids showing E pattern (stylets located in a sieve element) in EPG experiments. (EPG data on RES and SUS were pooled since no differences were found)

punctures of phloem sieve elements do occur intermixed and indistinguishable from other cell punctures (Tjallingii 1990; Tjallingii & Hogen Esch, 1993). Interval recording of free aphids (Montllor & Tjallingii, 1989) was impossible because the aphids moved to the growing tip where they could not be reached by an electrode.

Since tethered aphids cannot leave the plant there were large differences between free and tethered aphids due to free aphids leaving after several hours.

In EPG experiments the aphids had access to a limited surface area on the abaxial side of fully expanded leaves (Van Helden & Tjallingii, 1993) which is not the preferred feeding site (Mackenzie & Vernon, 1988). What determines this preference is unknown but both plant structure, phloem composition, microclimate and shelter will be different in the growing tips or in the heads of lettuce. Tjallingii & Mayoral (1992) showed differences in the time needed to reach the phloem on different plant parts.

CONCLUSIONS

1. Aphids encounter differences between resistant and susceptible plants before the phloem is reached but this does not result in plant rejection.
2. Rejection of the resistant plants occurs only after phloem contact. Therefore, further research should focus on differences in the phloem (either chemical or mechanical) between resistant and susceptible lines.
3. Tethering effects occur during EPG's, mainly due to locomotion restraints.

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5. PHLOEM SAP COLLECTION

ABSTRACT

Three methods to collect phloem sap on different lettuce lines were optimized and described in detail. Success ratio for *stylectomy* (1) of aphids was over 80% through the combination of a specially designed setup and Electrical Penetration Graphs to monitor phloem sap ingestion. On some lettuce lines stylets never showed sustained exudations for unknown reasons. Clear differences in stylet exudation showed between two aphid species on the same lettuce line. *Honeydew* (2) collection in hexadecane made accurate quantitative analysis possible, samples were large and clean but biotransformed. The *EDTA chelation* method (3) produced large samples but dilution, oxidation and impurities from the wound surface reduced the reliability.

INTRODUCTION

Lettuce resistance: The absolute, monogenic resistance of lettuce (*Lactuca sativa* L.) to the aphid *Nasonovia ribisnigri* (Mosley) (Nr-gene, Eenink *et al.*, 1982; Reinink and Dieleman, 1989; Van Helden *et al.*, 1993) is based on an early interruption of phloem sap uptake after the aphid-stylets have reached the sieve tubes of the plant (Van Helden and Tjallingii, 1993; Van Helden *et*

al. 1992). Possible resistance mechanisms are (1) a mechanical blocking of the aphid stylets or the sieve tube after puncturing by the aphid, or (2) a difference in chemical composition of the phloem sap between susceptible and resistant lettuce (Van Helden and Tjallingii, 1993).
Phloem sap collection: Several collection methods of phloem sap have been described in literature. Direct collection of phloem sap after incisions in the plant as described for trees (Zimmerman, 1957), cucurbits (Richardson *et al.*, 1982) or legumes (Pate *et al.*, 1974) is not possible for lettuce because of the presence of large quantities of lactiferous ducts around even the smallest veins, which immediately exude latex at wounding. The remaining methods therefore are amputation of aphid stylets (stylectomy, Downing and Unwin, 1977; Unwin, 1978; Fisher and Frame, 1984), collection of honeydew (Banks and Macaulay, 1964) and "facilitated exudation" through EDTA chelation (King and Zeevaart, 1974; Girousse *et al.*, 1991; Groussol *et al.*, 1986).

The objectives of this study were: (1) the optimisation of these three methods for lettuce, (2) a qualitative and quantitative comparison of the samples and (3) comparison of the results for different lettuce lines and aphid species in relation to aphid resistance.

The detailed technical description of all three different phloem sap collection methods and their results may be useful in research on plant physiology and aphid-plant interactions in relation to host-plant resistance.

MATERIALS AND METHODS

Plants. The plants used in the experiments were lettuce plants of the lines "Taiwan" (susceptible, genotype nrnr), "411" (resistant, genotype NrNr) and two set of isogenic lettuce lines RES (genotype NrNr) and (SUS, nrnr) (Van Helden *et al.*, 1993) and RES2 (NrNr) and SUS2 (nrnr). The second set is different from the first set (apart from the source of the resistance gene) and was selected for partial resistance to *Myzus persicae* (Sulz.) (Reinink and Dieleman, 1989; Reinink *et al.*, 1988). The culture of plants was as described by Van Helden *et al.* (1993). Plants were used in a 4-5 leaves stage (stylectomy) or in a 6-8 leaves stage (honeydew and EDTA chelation).

Aphids. Mass-culture and synchronized culture of adults of the aphids *N. ribisnigri* (biotype WN1), *M. persicae* (biotype WM1) and *Macrosiphum euphorbiae* (Thomas.) (WMe1) (Reinink and Dieleman, 1989) were performed as described by Van Helden *et al.* (1993). Aphid rearing plants were *L. sativa* cv. "Taiwan" for *N. ribisnigri*, *Brassica napus* L. cv. "Olymp" for *M. persicae* and *L. sativa* cv. "Snijsla" for *M. euphorbiae*. For stylectomy we also tried *Aulacorthum solani* (Kltb.) and *Uroleucon sonchi* L. from a mass-culture on *L. sativa* cv. "Taiwan" and originating from a local field population.

Stylectomy

Setup. Experiments were performed in the lab at 22 ± 1 °C, RH ± 60% and 4000 Lux from HF fluorescent tubes. In some cases the humidity was raised to over 95% RH using a ultrasonic humidifier. Plants were potted in small (5 cm) square pots and mounted in a specially designed setup. The abaxial side of the fourth (fully expanded) leaf of the plant was fixed over a cylindrical perspex support in the centre of the setup (fig. 1) which made it possible to turn the aphid "target" in every direction (arrows) while keeping it in focus of a horizontally viewing stereomicroscope (not shown).

Electrical penetration graphs (EPG, Tjallingii, 1988) were used to monitor the feeding behaviour of the aphids. This method enables recording of different waveform patterns during penetration which can be related to different aphid activities and stylet locations in the plant tissue (Van Helden and Tjallingii, 1993). Phloem sap ingestion occurs during waveform pattern E2 (Tjallingii, 1990). Six aphids were wired and via a choice switch each aphid could be connected to an EPG amplifier (DC system, 1 GΩ input, Tjallingii, 1988) coupled with a storage oscilloscope to monitor its feeding-behaviour. Stylets were cut the following day when aphids showed phloem sap ingestion pattern E2.

Stylectomy was performed with a high frequency microcautery unit (48 MHz, circa 25 Watt, Syntech). Output of the microcautery unit was reduced to around 50% of the maximum output (output circa 10-15 Watt) , using a 0.2 s pulse. Needles were prepared from 0.2 mm tungsten wire and electrolytically sharpened to produce a needle with a long (1.5 cm) tapered thin tip (Brady, 1965).*Needle position.* The aphid was approached frontally, carefully moving the tip a little beyond the labium. Just before the amputation

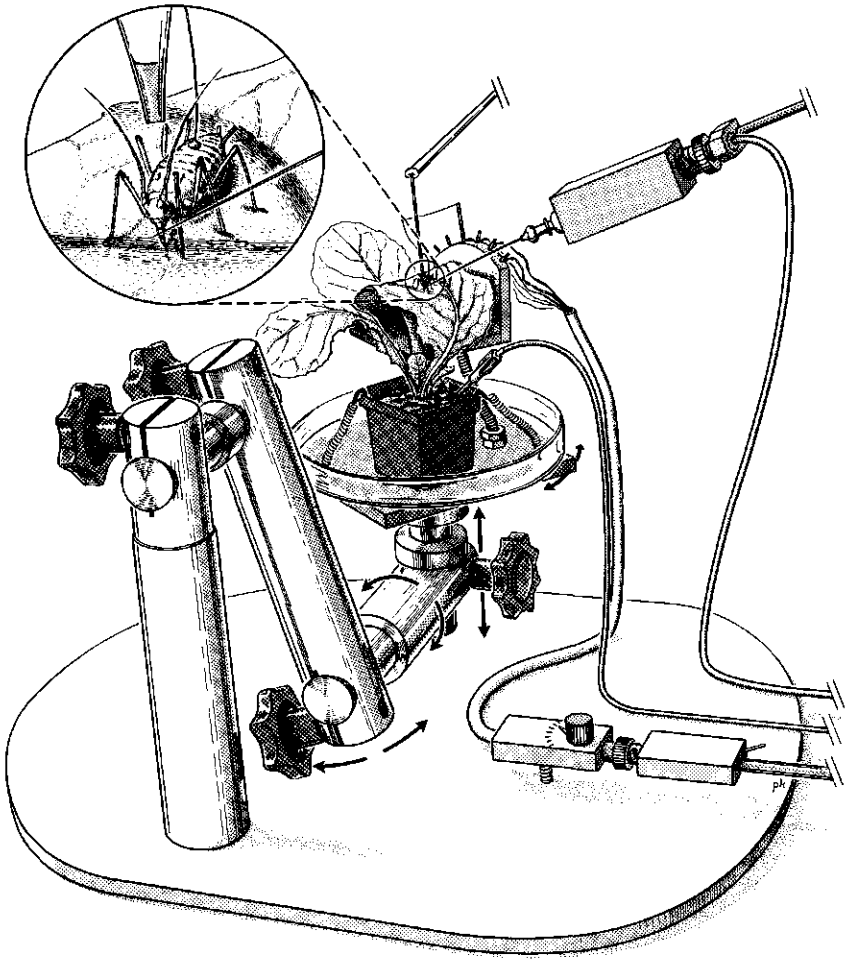

Figure 1. Design of the stylectomy setup and microcautery unit. The plant can be turned in every direction (arrows). Six aphids can be connected over a choice switch to an EPG amplifier to record the EPG signal. Detail shows needle position and microcapillary just before amputation.

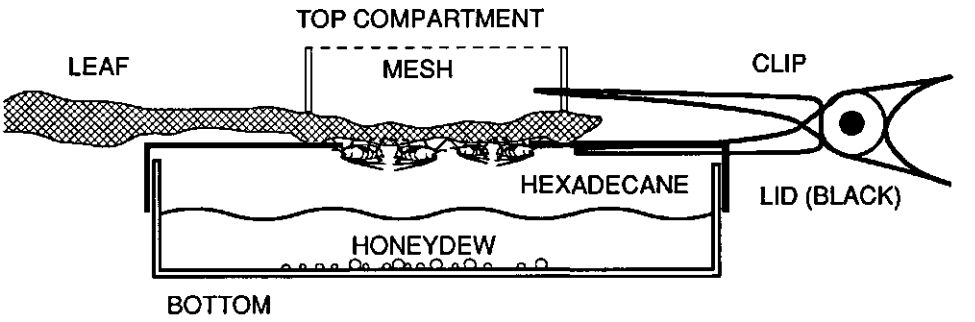

Figure 2. Design of the honeydew collection cage.

the needle was slowly moved laterally against the narrowest part (at the second labial segment) of the proboscis (fig. 1, detail), firmly touching the proboscis, and causing a slight deflection of the needle tip and the proboscis. Then a foot-switch activating the microcautery pulse was pressed immediately. In some cases is was necessary to bend the needle to reach the labium. Other needle locations on the proboscis were tried.

Phloem sap collection. A capillary (20 µl Microcaps™, 52 mm long, outer diameter 0.97 mm, inner diameter 0.69 mm, with a tapered tip, broken at outer diameter 0.15 mm, filled with silicon oil) was placed over the exuding stylets, such that these were positioned in the oil filled part. The position of the upper oil meniscus was determined every 5-15 minutes. Volumes were estimated by the extension of the fluid-column (precision 0.04 mm or 15 nl using a measuring Eyepiece). An overestimation of volume and rate appeared when the oil did not completely fill the tapered end. When exudation stopped the capillary was emptied in 100 µl of 50% methanol for sugar analysis. Samples were frozen at -80 °C until analysis.

Statistics. Comparison between aphid species were made by Mann-Whitney U tests ($\alpha < 0.05$). Comparison among veins for each species were made using the Kruskal-Wallis test for multiple comparison ($\alpha < 0.05$). For tables and statistical tests concerning duration, sample size and exudation rate only

Table 1. Overview of the stylectomy results

Result of attempt	N	%
Total number of attempts	337	100.0
E2 continued until microcautery	322	95.5
Succesfull amputation	284	84.3
Exudation from stump	245	72.7
Long exudation (> 1 min)	124	37.7
Samples > 1 µl	48	14.2

values larger than zero were used and some discordant values were excluded with duration > 200 min (5 cases excluded), sample size > 15 µl (1 case) or rate > 1 nl/s respectively (5 cases).

Honeydew collection

Honeydew was collected in the greenhouse at 22 ± 2 °C and RH 70% under continuous illumination. Honeydew collection cages were prepared from 5 cm Petri dishes modified after Eenink *et al.* (1984) (fig. 2). 5-10 adults were placed in the bottom compartment (an empty base of a Petri dish), and left there for 24 hours to allow settling and reproduction of the aphids on the leaf piece. The next day the bottom part was replaced by a fresh one filled with two ml of n-hexadecane ($C_{16}H_{34}$, sg 0.77 g/ml, mp 18 °C). At 24h intervals the honeydew was collected in a capillary and stored at -80 °C under n-hexadecane. Different aphid species were used, depending on the lettuce line (see results). The top ring of the clip-on cage was made into a second cage by closing it with nylon mesh (fig. 2, top compartment). Before and during a few collections on the lines RES, RES2, SUS and SUS2 the top compartment was filled with 5-10 *N. ribisnigri* adults.

EDTA chelation

Leaves were cut close to the base and the cut surface was rinsed in water for a few seconds. Leaves were weighed and placed in 1.5 ml vials containing 0.8 ml of an EDTA solution buffered with 5 mM phosphate buffer (pH 6). All vials were placed in a large translucent container (RH > 95%, 22

Table 2. Overview of the results per aphid-plant combination

Aphid Species	*M. persicae*		*N. ribisnigri*		Other		Total
	N	S. size	N	S. size	N	S. size	N
Lettuce line							
TAIWAN	147	1.3 ± 0.4(54)	88	2.8 ± 0.4(47)	1		236
RES	19	##	**		27	##	46
SUS	13	##	12	##	6	##	31
RES2	0		**		0		0
SUS2	0		11	##	2	##	13
411	10	2.0 ± 1.5(2)	**		1	##	11
TOTAL	189		111		37		337

N = number of attempts, S. size = sample size of long exudations (> 1 min) in µl ± SE(number of samples), ** = incompatible aphid-plant combination, ## = No long exudation observed (Sample size < 1 nl).

± 2 °C, and artificial illumination) for 18-24 h . Different EDTA concentrations and pH's were tried. The time course of the sugar yield and the yield per gram of leaf and its relation to leaf age determined. This method was compared to collection in darkness or collection with only a short (2h) EDTA exposure after which leaves were transferred to a vial containing water only. Final collection was in 8 mM EDTA and 5 mM phosphate buffer at pH 6. Sodium metabisulfite $Na_2S_2O_5$ (5 mM) was used as an antioxidant.

Sugar concentration. Total sugar concentration of the EDTA samples was determined using a colorimetric reaction with anthrone. After heating for 10 minutes at 90 °C the reaction mixture shows equal extinction per gram sugar for different sugars (Van Handel, 1967) and therefore is a good measure of total sugar content.

Results of a more detailed chemical analysis of the samples are given elsewhere (Van Helden *et al.*, 1994)

RESULTS

Stylectomy

A summary of the stylectomy is given in tables 1 and 2. The results of 337 attempts were recorded, mainly on "Taiwan" with *N. ribisnigri* and *M. persicae*.

Needle positioning. The optimal approach of the needle was from the front side of the aphid, amputating at the second labial segment. When the needle accidentally touched the aphid it sometimes ended the penetration, but more often disturbance was only shown in the EPG signal which temporarily changed from waveform E2 (phloem ingestion) to E1 (stylets in sieve element, salivation but no ingestion, Prado and Tjallingii, 1994) (table 1: E2 continued until microcautery). Usually E2 was resumed after a few minutes and the attempt could be continued. Sometimes the aphid raised and swayed its abdomen and moved its legs without a change in the EPG waveform.

Amputation. The overall success ratio for amputation was 84.3% (table 1). Usually the proboscis was partially, and the stylets completely cut allowing the aphid to retract the severed proboscis and walk off leaving only the stylet stump. In one third of the amputations the proboscis was also cut. The remnants could usually be removed with a fine brush, if not removable the capillary was simply placed over them. In around 10% of all cases the mandibulary stylets separated from the maxillary ones, bending away. When a pulse was fired but amputation failed this was usually attributable to inadequate contact between needle and proboscis.

Other places of the proboscis than the third segment were either harder to reach (proximal segments) or gave a lower success ratio of amputations (distal segments). Bending the needle with smooth curves did not affect the efficiency of the microcautery but made the positioning difficult. Stronger and/or longer pulses caused damage to the plant surface.

Exudation success. Nearly all successful amputations were followed by some exudation from the stylet stump (245 of 284). When amputation was accidentally performed during an EPG waveform other than E2 exudation was never observed.

Duration of exudation. 50% of all exudations (124 of 245) stopped after around 10 seconds, when a only tiny droplet (diameter 0.05-0.15 mm, i.e. volume ± 1 nl) had been formed. When exudation continued for more than a minute, so beyond this one droplet (further referred to as "long exudation") total sample volume was mostly smaller than 1 µl, with durations of 5 to 120 minutes (mean 53 minutes, median 30 minutes). Exudations producing volumes of 1-5 µl occurred irregularly (48) with durations of 30-200 minutes. One exceptionally long exudation of three days occurred.
Exudation rate. The exudation rate (table 3) of long exudations was very variable (range 0.05 to 0.8 nl/s). Exudation rate usually decreased with time but sometimes increased temporarily e.g. when the capillary was changed.
Miscellaneous observations. The phloem sap flowing out of the stylet stump adhered to the wall of the capillary, quickly forming a complete disk with menisci at the oil interfaces and pushing the upper meniscus upwards when exudation continued. When the phloem sap droplets did not touch the wall of the capillary they could be observed sinking to the lower oil/air interface. Exudation from levels below the top of the stump occurred in a few cases. Sometimes a few seconds delay preceded the appearance of the phloem sap at the tip of the stump. Increasing the humidity did not increase exudation rate or duration. When the mandibular stylets separated from the maxillary ones exudation seemed to stop earlier than when they remained joined.
Differences among lettuce lines. Success ratio for amputation or exudation did not differ among lines (table 2). "Taiwan" lettuce showed long exudations quite often while the lines RES, SUS, RES2 and SUS2 never exuded phloem sap for more than a few seconds. Line 411 (which contains the resistance gene) showed long exudations on two (out of ten) occasions.
Differences between aphid species. Tables 3 and 4 summarize a number of parameters to compare *N. ribisnigri* and *M. persicae* on "Taiwan" lettuce. Stylet stumps of *N. ribisnigri* were longer, showed higher exudation rates and yielded larger samples. The success ratio for long exudations seemed somewhat higher for *N. ribisnigri* (47 out of 88 versus 54 out of 147 for *M. persicae*). *N. ribisnigri* preferred the larger veins while *M. persicae* often probed in the lamina (table 4).

Table 3. Differences between *Nasonovia ribisnigri* and *Myzus persicae* during stylectomy on Taiwan lettuce.

Parameter	*M. persicae*			*N. ribisnigri*	
Nr of attempts	147			88	
Nr disturbed	5			7	
Nr of successful amputations	135			79	
Nr of stylets exuding	128			68	
Nr exuding > 1 min	58			48	
Stump Length (m)	154 ± 4	84	*	304 ± 10	65
Dur. of exudation (min)	44.6 ± 6.2	57	*	73.5 ± 8.2	44
Sample size (l)	0.84 ± 0.12	53	*	2.75 ± 0.38	47
Estimated speed (nl/s)	0.33 ± 0.22	52	*	0.46 ± 0.04	43

Values are mean ± standard error. Some discordant values excluded (see text). * = significant difference between species (α = 0.05, Mann-Whitney test).

Differences among veins. The size, exudation rate and duration of samples collected on different veins of "Taiwan" lettuce leaves showed no statistically significant differences (table 4) but the number of replicates was low.

Honeydew

The honeydew collection method yielded 10 to 50 µl per cage per day. Adult aphids produced large quantities of offspring which contributed to the honeydew. On a successful aphid-plant combination many droplets of very different sizes were collected. Honeydew droplets dropping in the oil sank to the bottom and fusion of individual droplets falling on top of each other occurred frequently. A minimum temperature of 21 °C was necessary to avoid solidification of the hexadecane (mp 18 °C). Contamination by the aphids and exuviae which had dropped into the hexadecane was avoided by the manual collection. Due to the affinity between the glass capillary and the aqueous honeydew the droplets were automatically sucked into the capillary together with some oil. Other methods like filtration or centrifugation of the oil/honeydew suspension were not tried, both for the risk of contamination and the need to wash the droplets from the Petri dish (dilution). Dry matter content of the collected honeydew was around 11.5%.

Differences among aphid-plant combinations. Combination of aphid species and lettuce genotype gave different yields, due to differences in number of larvae produced in the cage and number of aphids dropping into the oil. On the lines RES and RES2 *N. ribisnigri* could not be used. However also *M. persicae* showed a lot of aphids dropping in the oil on these line as it did on the line SUS2, resulting in small yields. On these lines *M. euphorbiae* gave better yields. No difference in aphid behaviour or honeydew production was observed when *N. ribisnigri* was introduced into the adaxial top compartment (see fig. 2) on the resistant lines.

EDTA chelation.

Optimisation. The highest sugar yield was reached at 8 mM EDTA and decreased at higher concentrations (fig. 3A). A linear relationship appeared between leaf-fresh weight and the total amount of sugar in the extract (sugar yield (mg) = 1.33 * leaf weight (g) - 0.06, r = 0.57). The young, not fully expanded leaves yielded 30 to 60% lower quantities of sugar per gram of fresh weight compared to fully grown leaves. The time course of sugar yield showed that the rate of sugar exudation rose until the sixth hour of EDTA exposure and remained constant until the end of the collection period (fig. 3B). No relation was shown between pH and yield. When exudation was performed in the dark yields were reduced by 50%. In experiments with a short exposure (2h) to EDTA and subsequent collection in buffered water no sugar could be found in the extract after 20 h. Adding CO_2 (1%) to the container did not increase sugar yield. Sodium metabisulfite ($Na_2S_2O_5$) suppressed the visible browning of the extracts at 5 mM without affecting sugar yield.

Yield. EDTA chelation yielded samples of 0.2-0.6 ml per leaf. The sugar content of the samples was 0.3 to 0.9% (w/v, sucrose as standard) or around 1.3 (0.3-2.4) mg per gram of leaf. Large leaves yielded small samples with high sugar concentrations.

Differences among lettuce lines. Two differences were observed between resistant and susceptible plants. 1. The sugar yield (per gram of leaf) from the resistant plants was on average around 30 percent lower than on susceptible plants. 2. Leaves of resistant plants remained turgescent during the EDTA chelation while leaves of all susceptible plants lost turgor.

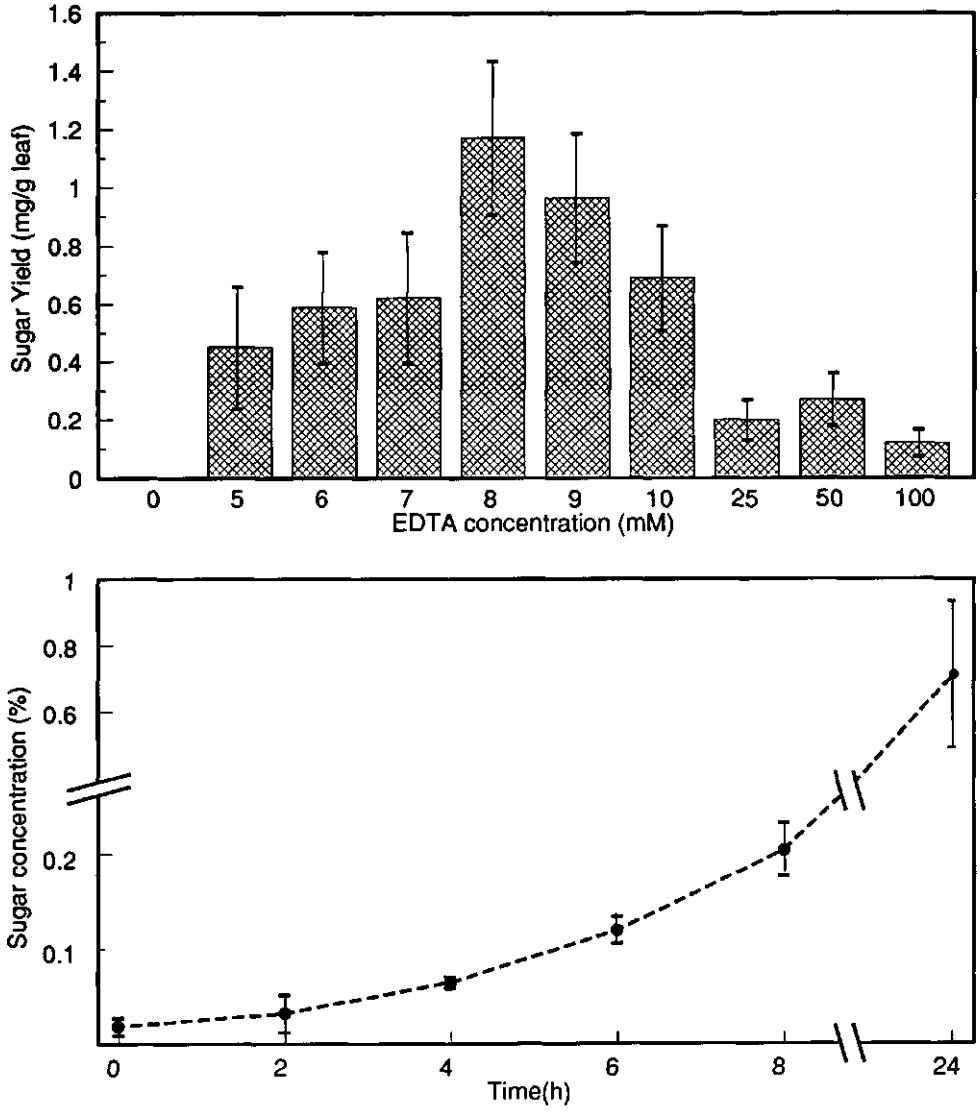

Figure 3. Results of the EDTA chelation experiments on Taiwan lettuce.
A. Sugar yield per gram of leaf material at different EDTA concentrations.
B. Time course of sugar exudation.

DISCUSSION

Stylectomy

Setup. The high success ratio of the stylet amputations can be attributed to the special setup with the aphid and the plant located in the centre of the setup where they can be turned in every direction without moving them out of the focus point of the microscope. Disturbance of aphids during the positioning of the needle, which is considered a drawback of radio-frequency microcautery (Fisher and Frame, 1984) rarely occurred. The use of laser microcautery (Fisher and Frame, 1984) was not considered because the anatomy of lettuce makes it impossible to avoid damage to the plant. The observed behaviour of some aphids moving their body and antennae (e.g. when the needle accidentally touched the aphid.) was very similar to the effect of a larva walking by and touching the adult.

The thin wall of the Microcaps™ capillary reduced refraction problems during the positioning over the stylet stump. The thin tip makes it possible to move the stylet stump slightly to an optimal position with oil layers on both sides of the exuded phloem sap. The oil avoided evaporation and enabled estimation of volumes. Weighing could not be used because of the oil. Silicon oil does not interfere with the chemical analysis and its density (0.98 g/ml) was expected to be close to that of the phloem sap. However it appeared that the phloem sap is heavier than the oil.

Amputation. The success ratio for amputation is much higher than any result published so far using microcautery (Girousse *et al.*, 1991, Rahbé *et al.*, 1990, Chino *et al.*, 1991) and comparable to the results of Kawabe *et al.* (1980) who used laser equipment, even though the aphids we used are smaller than the insect species they used (*Acyrtosiphon pisum* Harris, *Macrosiphum albifrons* Essig and *Nilaparvata lugens* Stal.).

The narrowest part of the proboscis at segment 2 appeared the best place for amputation because its cuticle is less chitinised than the distal (darker) proboscis segments as used by Mentink *et al.* (1984). Amputation at these distal proboscis segment required higher a output power from the microcautery and increased the risk of plant damage. The electrical contact between the needle and the proboscis should not be broken during the

microcautery pulse. This was achieved by using needles with a long flexible tapered point and the firm lateral contact against the proboscis just before amputation. When only the very tip of the needle was positioned against the proboscis (Downing and Unwin, 1977) the electrical contact was often broken during the pulse resulting in insufficient damage to the stylets. Sometimes little visible damage to the proboscis (low electrical resistance) still gave successful stylet (high resistance) amputation. The bleeding of the stylet stump below the top indicates that the maxillary stylets were sometimes cut or damaged at a lower level than the mandibulary stylets.

Differences among aphid-plant combinations. The success ratio for amputation depended on the aphid species and host-plant suitability. Some species were more restless (*U. sonchii*) or had a very short proboscis when feeding (*A. solani*). Some aphid-plant combinations could not be used because of complete resistance (*N. ribisnigri* on RES and RES2) or had a low amount of aphids showing E2 (e.g. *M. persicae* on SUS2 and RES2), apparently because of partial resistance (Reinink *et al.*, 1988).

Exudation success. The high success ratio for exudation from a successful amputation (over 90% on "Taiwan") can only be achieved by the use of the EPG method to ascertain phloem feeding before amputation (Mentink *et al.*, 1984). Without EPG recording exudation success ratio's of 0.05 to 55% have been reported (Winter *et al.*, 1992; Girousse *et al.*, 1991, Rahbe *et al.*, 1990, Hayaschi and Chino, 1986, Fisher and Frame, 1984). The extra work involved with the use of EPGs pays off by the certainty that the "target" is indeed ingesting phloem sap and that feeding is continued until the amputation. We never observed exudation when waveform E1 (which always precedes the actual ingestion waveform E2, Tjallingii, 1990) was present, confirming the hypothesis that no ingestion occurs during this waveform (Prado and Tjallingii, 1994). Even when the waveform E2 continued until the moment of amputation some stylets did not produce phloem sap. It is possible that the stylets were translocated or were mechanically blocked during the amputation. The latter possibility seems to be confirmed by the short delay in the exudation which sometimes occurred after amputation as if an obstacle had to be expelled with the first outflow.

Duration of exudation. Many exudations stopped after about 5-20 seconds as was also reported by Girousse *et al.* (1991). Apparently the amputation itself

or the outflow of the phloem sap caused a plant reaction, blocking the outflow very soon after amputation. This reaction could be triggered by the sudden decrease of turgor pressure in the sieve element cell (Peel, 1975) or by energy generated during the pulse which is transported down the stylets into the plant, either as electrical discharge or as heat. Callose formation or P-protein gelation (Girousse *et al.*, 1991) have been mentioned but callose formation seems unlikely because of the speed of the reaction (< 10 seconds). Tjallingii and Hogen Esch (1993) observed fibrous structures in the very tip of the food-canal of cauterised stylet (which had shown only a short exudation) similar to P-protein bodies in the sieve elements.

Long exudations apparently occur only when this short term blocking mechanism fails completely or is incomplete. It is not clear wether these same mechanisms stop the outflow from long exudations (see hereafter).
Exudation rate. The mean exudation rate of long exudations was 0.33 and 0.46 nl/s for *M. persicae* and *N. ribisnigri* respectively (range 0.2-0.8 nl/s). Earlier reports show very variable results (Rahbe, 1990: 0.1-0.3 nl/s from *M. albifrons* Essig on lupine; Peel, 1975: > 1nl/s from *Tuberolachnus salignus* Gmelin on willow; Tjallingii, 1994: 0.09 nl/s for *N. ribisnigri* on lettuce; Girousse *et al.*, 1991; 0.07 nl/s of *A. pisum* on *Medicago sativa* L.; Fisher and Frame, 1984: 0.3 nl/s for *T. salignus* and 0.03 nl/s for *Quadrapidiotus astraeformis* Curtis both on willow). These differences are thought to be caused by variation in the length and diameter of the food canal in the stylet stumps (Fisher and Frame, 1984). However, the diameter of the food canal of a large aphid like *T. salignus* (1-2 µm i.d., Peel, 1975) is not very different from *M. persicae* and diameters varied from 0.6 - 1.4 µm among 16 measured species (Forbes, 1977) which cannot explain all the variation in exudation rate. Large differences in exudation rate for the same species on different plants of the same genus (*T. salignus* on willow, Peel, 1975, Fisher and Frame, 1984; *N. ribisnigri* on lettuce, Tjallingii 1994, our data) show that plant quality plays an important role.

The exudation rate of long exudations was comparable to the estimated exudation rate directly after amputation (estimate based on exudation of the first droplet). This suggests an absence of blocking reactions. The slow decrease of the exudation rate over time can be caused by plant blocking reactions but also by evaporation from the air-exposed part of the stylet

stump between the leaf surface and the capillary. This seems to be confirmed by Downing and Unwin (1977), who found a higher osmotic strength of the sap when collected in an oil filled capillary (identical to our method) compared to sap collected in an oil droplet covering the whole stylet. The increase in exudation which sometimes occurred after changing the capillary and the fact that separation of the mandibular stylets from the maxillary ones caused exudation to stop sooner support this hypothesis and show that blocking can occur higher up the stylet stump, above the plant surface. Closing the air gap by placing the capillary on the plant surface failed because the oil flowed out of the capillary onto the leaf surface.

The mean exudation rate is much larger than the estimated feeding rate of around 25 pl/s for *M. persicae* (Tjallingii, 1994). It shows that aphids actively reduce the flow rate during feeding, possibly to avoid plant defence (blocking) reactions.

Differences among lines. Unfortunately, we were unable to obtain usable stylectomy samples from the two sets of isogenic lines due to immediate blockage of the outflow. Since resistant and susceptible plants of both sets of near isogenic lines posses this characteristic it is possibly linked with the resistance gene, e.g. located on the same chromosome. Apart from the source of resistance gene (*L. virosa*), the two sets are genetically different. From line 411, which contains the resistance gene, some samples could be collected. Mentink *et al.* (1984) also reported amputated stylets "producing sap" (duration not specified) on a resistant lettuce line.

Differences between aphid species. N.ribisnigri is bigger than *M. persicae* and produced longer stylet stumps. The food canal of *N. ribisnigri* seems somewhat larger than reported for *M. persicae* (0.7 µm halfway the stylets, Forbes, 1977). The diameter of the food canal (TEM pictures of *N. ribisnigri*, Tjallingii and Hogen Esch, pers. comm) varied from 0.5 x 0.8 µm (oval) near the tip to 1.1 µm (round) just above the leaf surface, and salivary canal from 0.3 to 0.4 µm at the same locations. Data are incomplete and exact comparison is difficult. This might explain the differences in exudation rate, duration and sample size but aphid-plant interactions can play a role. Salivation into a sieve element during E1 (Prado and Tjallingii, 1994) could prepare the sieve element for sap flow (e.g. injection of anti-coagulants) by suppressing plant defense (blocking) reactions. Efficacy could differ among

Table 4. Relation between penetration site on the leaf and exudation parameters for *Nasonovia ribisnigri* and *Myzus persicae* on Taiwan Lettuce.

Vein class	Total N	Duration(min)	N	Sample(µl)	N	Rate(nl/s)	N
Myzus persicae							
Main Vein	13	17 ± 4	5	0.6 ± 0.2	4	0.5 ± 0.1	4
Second order	38	33 ± 6	15	0.7 ± 0.1	14	0.3 ± 0.0	14
Third order	21	69 ± 20	8	1.4 ± 0.4	6	0.3 ± 0.1	6
Smallest visible vein	12	51 ± 35	5	0.8 ± 0.3	5	0.3 ± 0.1	4
Lamina	47	49 ± 14	16	0.8 ± 0.3	16	0.3 ± 0.0	16
Nasonovia ribisnigri							
Main Vein	21	115 ± 22	9	4.0 ± 1.0	11	0.4 ± 0.1	10
Second order	36	75 ± 10	20	2.9 ± 0.5	21	0.5 ± 0.1	18
Third order	18	51 ± 14	11	1.5 ± 0.5	10	0.5 ± 0.1	10
Smallest visible vein	5	50 ± nd	1	3.9 ± 3.2	2	0.3 ± 0.1	2
Lamina	7	25 ± 5	2	0.7 ± 0.5	2	0.5 ± 0.2	2

Values are mean ± standard error. Some discordant values excluded (see text). No significant differences among veins (Kruskall-Wallis multiple comparison, $\alpha = 0.05$)

species or aphid-plant combinations. *N. ribisnigri* might be better adapted to "Taiwan" lettuce than *M. persicae* because it was reared on this plant. This is supported by the fact that the higher outflow rate found with *N. ribisnigri* apparently did not cause exudation to stop sooner which would be expected if plant reactions play a role in the termination of long exudations.

N. ribisnigri preferred the main veins and larger side veins whereas *M. persicae* penetrated more often on the lamina (table 4), presumably into minor veins. This might indicate a difference in suitability or chemical composition (Rahbé *et al.*, 1990).

Differences among veins. The sieve elements of different veins might react differently to penetration by stylets. However, for each aphid species, exudation rate, samples size and duration of exudation did not differ

significantly among veins (table 4). Chemical composition can be different among different plant parts (Rahbé *et al.*, 1990).

Summarizing, it seems that the differences observed between the two species on the same plant in exudation rate, duration and sample size are caused by a difference in aphid-plant interactions rather than plant characteristics or stylet dimensions.

Honeydew

The collection method described here is comparable to that of Banks and Macauley (1963) and Fisher *et al.* (1984). Although manual collection makes the method rather time consuming, it yields large quantities of honeydew (ml), making quantitative analysis of chemical compounds possible. Hygroscopic uptake or evaporation of water was prevented by the oil layer. Fisher *et al.* (1984) suggested a decrease of volume due to evaporation of 10% in 2 hours during collection in hexadecane, but this was concluded from osmolality measurements, ignoring possible osmolality changes due to continued enzymatic activity in the droplets (Eschrich and Heyser, 1975, Van Helden *et al.*, 1994). Though unlikely, it is possible that some apolar components did dissolve in the very apolar hexadecane. Banks and Macaulay (1964) reported a specific gravity of 1.040 g/ml for the honeydew. It is therefore not clear why the droplets floated just under the surface of their Castor motor oil (0.89 g/ml), but in the lighter hexadecane (0.77 g/ml) sank immediately. The dry matter content of the honeydew was much lower than measured for phloem sap (Van Helden *et al.*, 1994) indicating important changes made by the aphids.

Differences among aphid-plant combinations. The number of aphids which dropped into the oil differed among species and lettuce lines. *N. ribisnigri* does not produce honeydew on the resistant lines (Van Helden *et al.*, 1993) but even the partial resistance to *M. persicae* which is present in the lines RES2 and SUS2 (Reinink *et al.*, 1988) caused an increased restlessness and therefore more aphids dropped in the oil and honeydew yield was very low.

When studying aphid resistance, finding a suitable aphid-plant combination can be difficult, and different aphid species will surely bio-process the phloem sap differently or feed on other sieve elements.

EDTA chelation

The method of EDTA chelation, as described by King and Zeevaart (1974), is applicable for lettuce. However the optimal conditions for lettuce are obviously different from those for *Perilla crispa* Thunb. Light exposure of the leaves during collection almost doubled the sugar yield. A temporary exposure to EDTA was apparently not enough to establish exudation for long periods. Adding carbondioxide as used by Tully and Hanson (1979) to reduce evaporation from the leaves did not increase the sugar yield.

It took a few hours to reach the highest rate of exudation which then continued for a long time with no noticeable decrease (Fig. 3b, Groussol *et al.*, 1986). The influence of the uptake and evapotranspiration of the collection fluid through the leaf on sugar yield is unclear. This method produces large quantities of sugar with very little work involved. One plant with five or six leaves (total mass 6-8 g) yielded around 9 mg of sugar thought to be equivalent to at least 45 µl of phloem sap (assuming 20% sucrose). Disadvantages are the strong dilution of the samples and their unknown contamination with substances from the cut surface of the petiole or the submerged part of the petiole which showed some tissue degradation. No large scale contamination of the samples by lactiferous ducts was observed. Latex was released immediately after excision but stopped soon and was washed off by the rinsing in water. It is unknown wether major physiological changes of phloem sap occur due to the excision of the leaves.

Difference among lines. The lower sugar yield on the resistant lines may be caused by the fact that the resistant plants were slightly smaller. In addition to their lower weight, these smaller leaves may have been physiologically younger and therefore give a lower yield per gram of leaf material. Wether the absence of turgor loss on the leaves of the plants 411, RES and RES2 compared to "Taiwan", SUS and SUS2 has any direct relation with the mechanism of aphid resistance is not clear. However, it may have a direct relation with the lower sugar yield.

CONCLUSIONS

The three methods produce very different samples, both in quantity and in quality. *Stylectomy* is very laborious. Although the success ratio of amputation and exudation was exceptionally high it yielded only very small (but presumably pure) samples, often too small for analysis. The number of cut stylets yielding a usable sample is very unpredictable and certain plants show no long exudations, in spite of the fact that the aphids show sustained phloem sap ingestion. *Honeydew* collection produces relatively large samples. A good aphid-plant combination is necessary. Collection in hexadecane makes quantification of compounds possible. *EDTA chelation* produces large but very diluted samples with unknown contaminations. Excision might influence the phloem sap composition.

Which method is preferred depends largely on the intended use of the samples. For major compounds of the phloem sap for which sensitive micro-analysis methods are available (sugars, amino acids, Van Helden *et al.*, 1994) the very pure stylectomy samples are optimal. For identification of (unknown) minor compounds stylectomy samples are simply too small. The other methods yield larger samples (in terms of phloem sap equivalents) but their reliability is unclear. None of these methods is ideal when studying aphid resistance caused by differences in phloem sap composition as in our model. When resistance is induced by aphid attack, collection without using aphids (EDTA chelation) may not show any significant differences. Using other aphid species (honeydew or stylectomy) might not induce the defence reaction. To gain more insight in the differences among samples from the three collection methods a chemical comparison was performed which will be reported in Van Helden *et al.* (1994).

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6. PHLOEM SAP CHEMISTRY

ABSTRACT

The chemical composition of phloem sap from lettuce, collected by three different methods, was compared. Phloem sap from stylectomy samples contained sucrose and 14 amino-acids. Honeydew and EDTA chelation samples showed considerable breakdown of sucrose into fructose and glucose, several additional amino-acids and large differences in relative concentrations of amino acids, when compared to stylectomy samples. Honeydew contained considerable amounts of other oligosaccharides, and few proteins in low amounts, while EDTA samples showed many proteins. HPLC chromatograms showed numerous unidentified secondary plant compounds in honeydew and EDTA samples. Comparison of phloem sap samples from near-isogenic susceptible and resistant lines showed no relation of phloem sap composition with monogenic resistance to the aphid *Nasonovia ribisnigri*.

INTRODUCTION

The absolute, monogenic and dominant resistance (Nr-gene) of lettuce to the aphid *Nasonovia ribisnigri* is based on the interruption of phloem feeding (Van Helden and Tjallingii, 1993, Van Helden *et al.*, 1992, 1993) probably

caused by a difference in chemical composition of the phloem sieve element sap.

The composition of the phloem sap of lettuce (*Lactuca sativa* L.) can be derived from samples collected by stylectomy (Downing and Unwin, 1977), from aphid honeydew (Banks and Macaulay, 1965) and from samples collected by "facilitated exudation" through EDTA chelation (King and Zeevaart, 1974). In a previous paper we reported on the optimisation of these methods for maximum yield (Van Helden *et al.*, 1994), without comparing their chemical composition. The collection of phloem sap by stylectomy failed on two sets of (near isogenic) resistant and susceptible lettuce lines (lines in one set differ only in the *Nasonovia* resistance gene, Van Helden *et al.*, 1994). On a susceptible lettuce line "Taiwan" stylectomy was successful and samples of three collection methods are compared in this paper.

Chemical comparisons between stylectomy and EDTA chelation sap samples are available for several plant species (Weibull *et al.*, 1990, Girousse *et al.*, 1992). So far only Rahbé *et al.* (1990) compared these three methods simultaneously, concentrating on sugars and amino acids. Though stylectomy samples are considered as the best representation of real phloem sap, this technique does not work on all plants ((Van Helden *et al.*, 1994) and samples size is very limited (usually < 1 µl) (Fisher and Frame, 1984, Van Helden *et al.*, 1994). The reliability of samples from other methods is unclear. EDTA chelation samples from excised leaves are contaminated with compounds released from the wound surface, and honeydew is a phloem sap which is bio-transformed by the aphid used for collection.

The objectives of this study were: (1) Qualitative comparison of three collection methods (stylectomy, EDTA chelation and honeydew) for sugars, amino acids, proteins and UV absorbing secondary metabolites, (2) comparison of phloem sap samples of (near isogenic) resistant and susceptible lines.

MATERIALS AND METHODS

Samples. In an earlier paper (Van Helden *et al.*, 1994) we reported on the methodology and yield of phloem sap collection methods. Samples were col-

lected from lettuce line "Taiwan" for comparison of collection methods, and two sets of near-isogenic lettuce lines: RES (genotype NrNr) with SUS (nrnr) and RES2 (NrNr) with SUS2 (nrnr) respectively, for comparison of resistant and susceptible plants (Van Helden *et al.*, 1993). These two sets have distinctly different genetic backgrounds apart from the source of the resistance gene which originates from *L. virosa* L..

Stylectomy samples (phloem sap volume 0.1 - 1 µl, stored in 50% MeOH or 2% sulfosalicylic acid) were available from lettuce cv. "Taiwan" using two aphid species, *Nasonovia ribisnigri* and *Myzus persicae* (Sulzer). *Honeydew samples* were collected in hexadecane. Samples from lettuce line "Taiwan" were collected from the aphids *M. persicae, N. ribisnigri* and *Macrosiphum euphorbiae* (Thomas). On the other lines honeydew was collected from *M. persicae. EDTA samples* were collected in 8 mM EDTA, pH 6, yielding 0.2 - 0.6 ml/leaf, total sugar content 0.3 - 0.9% or around 1.3 mg/g of leaf. Samples were stored at -80 °C (honeydew and stylectomy) or -20 °C (EDTA) before analysis. All tests with 2-4 independent replicates per treatment.

Sugar analysis was performed with a high performance anion-exchange chromatography system (Dionex CarboPac PA-1 Column, PED/PAD-Detector) as described by Gruppen *et al.* (1992), concentrating on sucrose, glucose and fructose. Samples from stylectomy (± 0.5 µl in 100 µl 50% MeOH) were lyophilised and redissolved in 1 ml H_2O shortly before analysis. EDTA samples were diluted 50 fold, honeydew samples 1000 fold before analysis. Injection volume 20 µl.

Amino acid analysis was performed with an ion-exchange amino acid analyzer (Beckman). Proteins were removed by adding of (for the EDTA samples), or dilution in (for stylectomy and honeydew), 2% sulfosalicylic acid and centrifugation. Injected samples (100 µl) contained ≈ 2.5 µl stylectomy samples (several collections pooled), 5 µl of honeydew, or undiluted EDTA samples. Some other products (NH_3) were also detected.

Protein analysis. Proteins in EDTA samples (5 ml) and honeydew (0.5 ml) from the lines RES and SUS were precipitated by addition of acetone up to 80% (Joosten, 1991) and centrifugation. SDS-PAGE was performed on a 12% mini-slab gel with a 4% stacking gel. Proteins were silver-stained according to Morrissey (1981).

Secondary plant chemicals. EDTA and honeydew samples were separated using a 250 x 4 mm C_{18}RP-HPLC (nucleosil 120-5 C_{18}) column (Macherey-Nagel) with a 30 mm pre-column using water/acetonitrile or water/methanol gradients, or isocratic mixtures with tri fluor acetic acid (TFA, 0.1%, pH 2) or tetra butyl ammonium ions (added as chloride or hydrogen sulphate, 0.5%, pH 5). Flowrate 1 ml/min, runtime 35-40 min. UV detection at 200-400 nm was performed with a diode array detector (Waters 911). Injection volumes were 20 µl of undiluted EDTA samples or 10 fold diluted honeydew. Stylectomy samples were not available.

RESULTS

Sugars.

The main sugar in most samples was sucrose (fig. 1A and B), with the exception of some honeydew samples. Fructose and glucose were identified in nearly all sample types from all lines.

Differences among collection methods. The total sugar concentration in the stylectomy and EDTA samples was 13.9 to 18.0% (mean 16.2%, n=4) and 0.19 to 0.71% (mean 0.41%, n=28) respectively. In honeydew the combined sucrose, fructose, glucose quantity was 3.4 to 6.2% (mean 5.1%, n=24).

In the stylectomy samples nearly all sugar was sucrose and only traces of glucose, fructose occurred (fig. 1A, left columns), while the EDTA collected samples showed fructose and glucose in roughly equal shares of 0 to 15% of the total sugar content each (fig. 1A,B). Variation in profiles between replicates was larger than among lines. Honeydew samples showed little variation between replicates but variation among aphid-plant combinations was considerable (fig. 1A, right bars, Fig 1B). Fructose concentrations were 3-8 times higher than glucose (fig. 1A,B). Large amounts of other (unidentified) oligosaccharides (possibly representing polymerisation series of glucose containing oligosaccharides, Fisher *et al.*, 1984, Ammeraal *et al.*, 1991), were found (not quantified but up to 50% of total peak area of chromatogram). A small unidentified peak (probably a monosaccharide with a retention time between those of glucose and fructose) was sometimes observed.

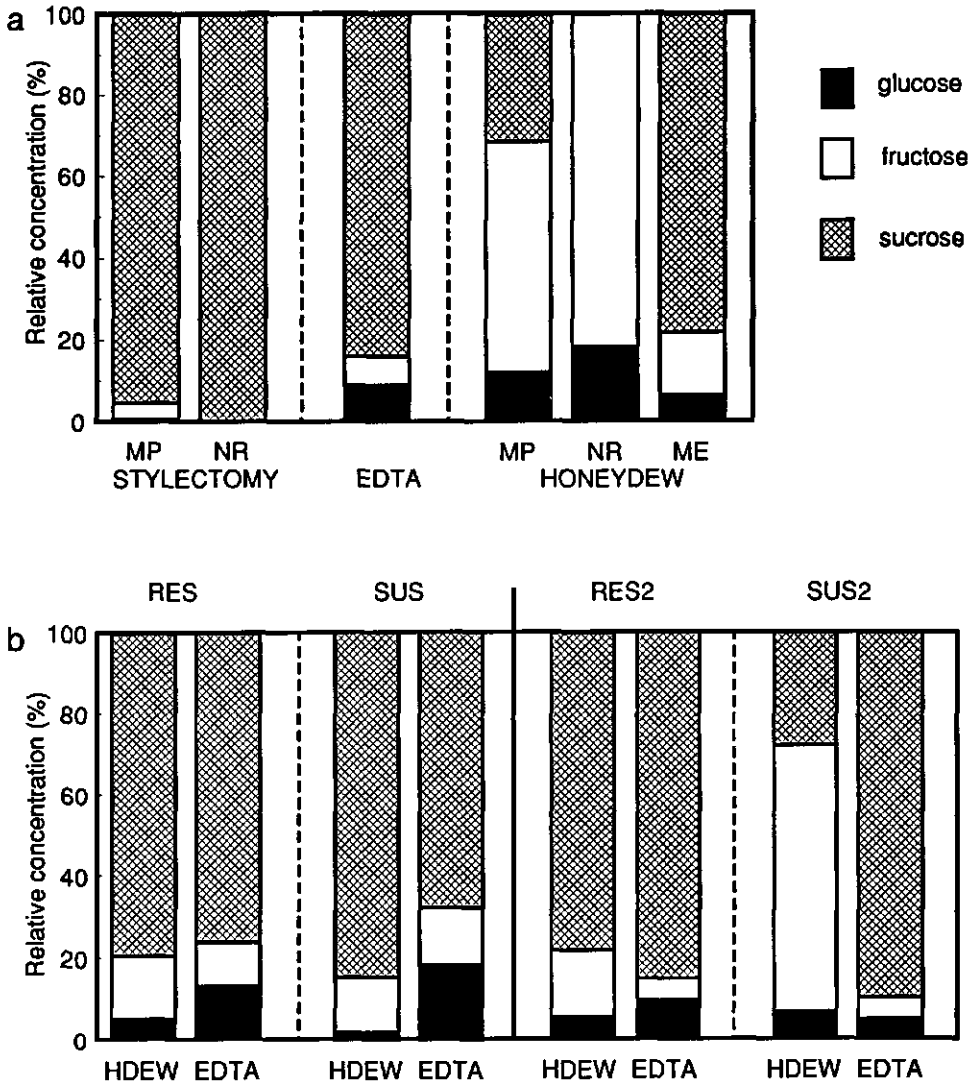

Figure 1. Relative sucrose/fructose/glucose concentrations of samples from different collection methods. a. Comparison on "Taiwan" lettuce using stylectomy of *Myzus persicae* (MP) and *Nasonovia ribisnigri* (NR), EDTA chelation, and honeydew of *M. persicae* (MP), *N. ribisnigri* (NR) and *Macrosiphum euphorbiae* (ME). b. Comparison of two sets of isogenic lines, using *M. persicae* honeydew (HDEW) and EDTA chelation.

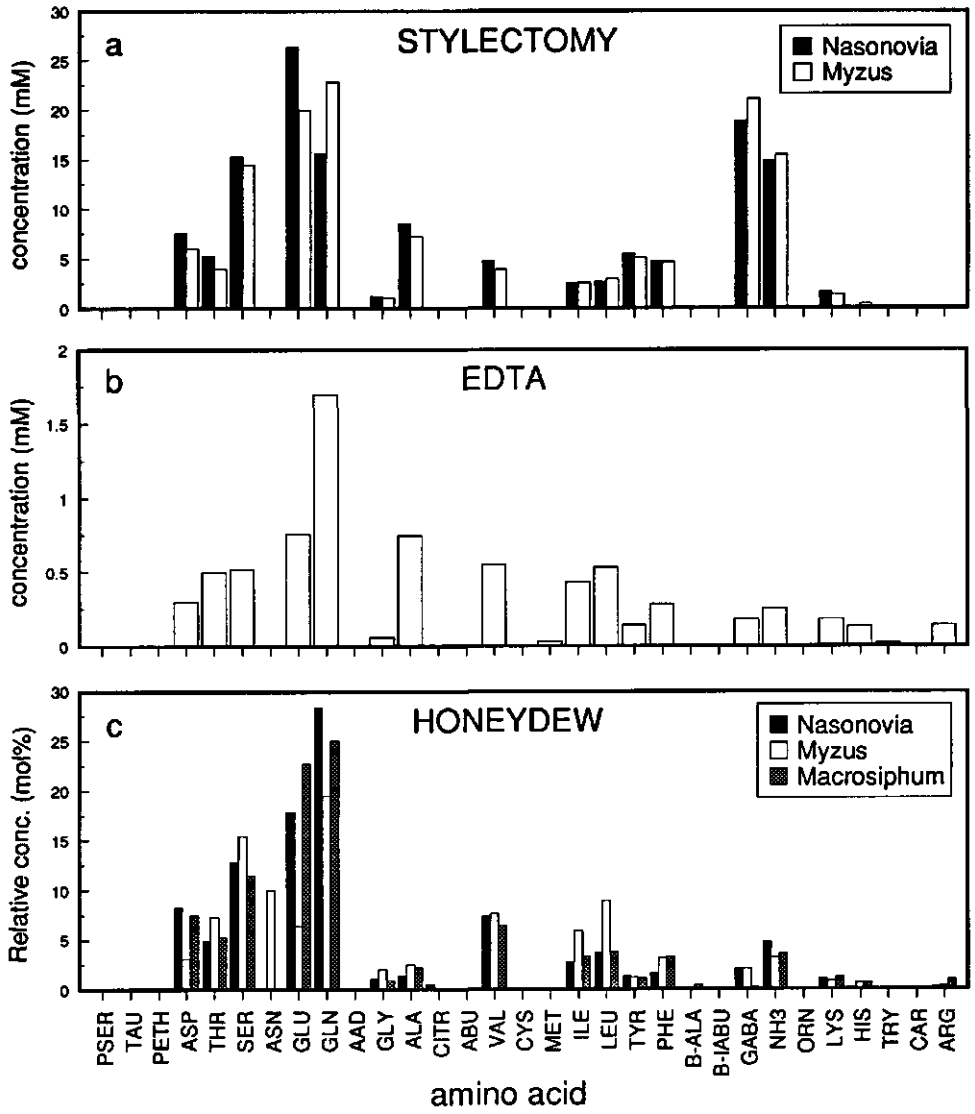

Figure 2. Amino acid composition of phloem sap samples from "Taiwan" lettuce using different collection methods. a. Absolute concentrations of stylectomy samples of *Myzus persicae* and *Nasonovia ribisnigri*. b. absolute concentrations of EDTA chelation samples. c. Relative concentrations of honeydew of *M. persicae*, *N. ribisnigri* and *M. euphorbiae*.

Differences among aphid species. Stylectomy samples of *M. persicae* and *N. ribisnigri* showed near equal concentrations and profiles of sugars. Differences in sugar profiles among honeydew samples of aphid species were considerable (fig. 1A, right bars). *N. ribisnigri* honeydew from "Taiwan" showed no sucrose at all. The combined sucrose, fructose, glucose quantity in honeydew from *N. ribisnigri* on "Taiwan" (5.3%) was higher than for *M. persicae* (3.5%) and *M. euphorbiae* (3.9%).

Differences among lettuce lines. Fig. 1B shows the results of a comparison of the two sets of isogenic lettuce lines, using EDTA samples and honeydew of *Myzus persicae*. Stylectomy samples were impossible to obtain from these lines (Van Helden *et al.*, 1994). The sugar profiles of the EDTA samples of all lines were identical. The lines RES and RES2 showed total concentrations of 0.19% and 0.22% respectively against 0.34, 0.52 and 0.49% on the lines SUS, SUS2 and "Taiwan". The honeydew samples showed a high proportion of fructose on line SUS2 (and "Taiwan", fig. 1A). The combined sucrose, fructose, glucose quantity in honeydew from isogenic lines was comparable (mean 5,2%, range 4.2 - 6.2%) a little higher than on "Taiwan" (4,2%)

Amino acids

Differences among collection methods. The total concentrations of amino acids in the original samples were 54 mM for honeydew (range 32-72 mM, n=24), 5.4 mM for EDTA samples (range 2.2-8.8 mM, n=30) and an estimated 130 mM (range 125-168 mM, n=4) for stylectomy samples. Amino acid profiles of stylectomy samples (fig. 2A) showed 14 amino acids. Main amino acids were glutamine, glutamic acid, serine and γ-isobutyric acid (GABA). Ammonia was also present in fairly high concentrations (6 mM). Proline was not detected. No unexpected peaks were detected.

EDTA samples on "Taiwan" (fig. 2B) consistently showed substantial quantities of one extra amino acid (arginine, rel. conc. 2%) and trace quantities of α-aminobutyric-acid, methionine and tryptophan. Compared to stylectomy samples they showed an increase in the glutamine/glutamic acid ratio, lower relative quantities of serine, tyrosine, and especially of GABA and ammonia.

Honeydew samples of different aphid species on "Taiwan" (fig. 2C) showed the same amino acids as the stylectomy samples plus traces of citrulline and arginine. Relative amounts of alanine, tyrosine, GABA and

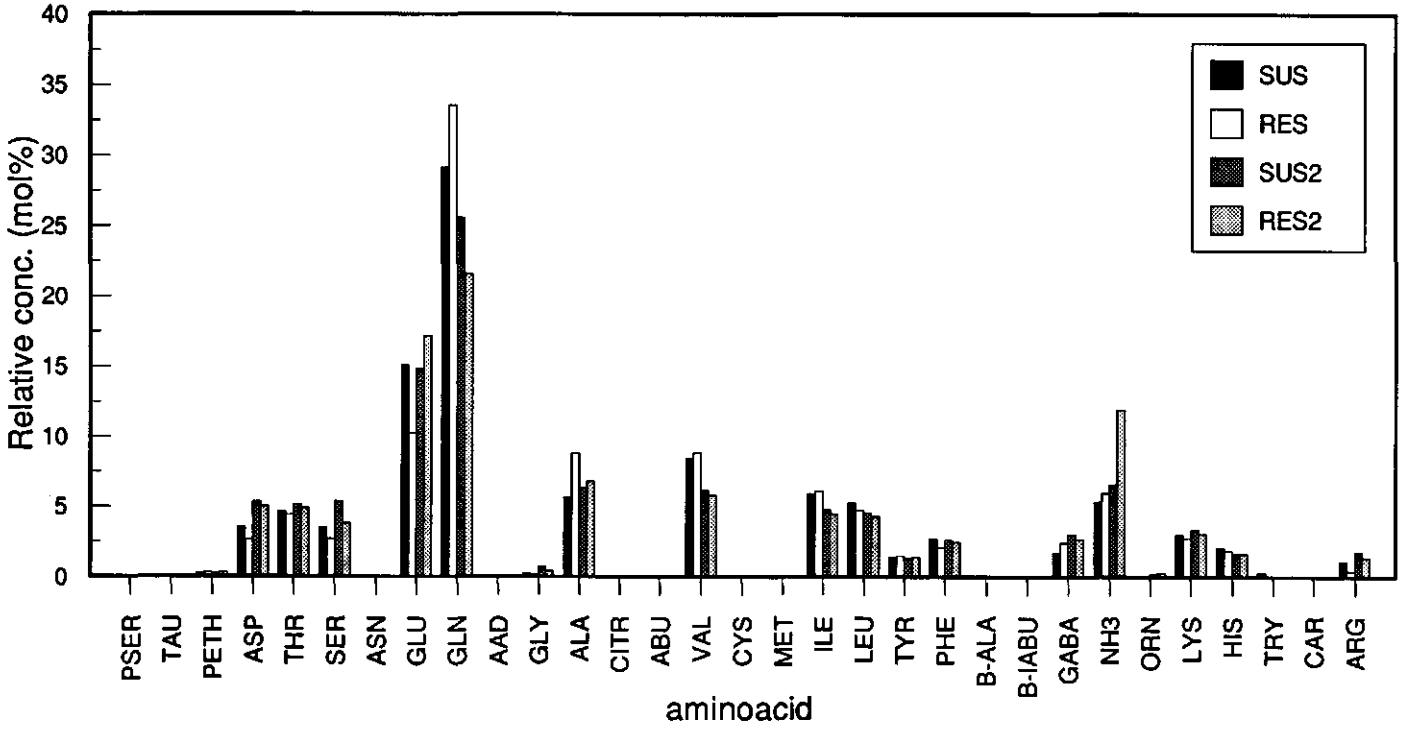

Figure 3. Relative concentrations of amino acids in phloem sap samples, collected by EDTA chelation, from two sets of isogenic lines.

ammonia were strongly reduced, and phenylalanine was somewhat reduced, for all aphid species.

Differences among aphid species. Amino-acid levels and profiles in stylectomy samples of *N. ribisnigri* and *M. persicae* on "Taiwan" were almost identical (fig. 2A). Honeydew amino-acid profiles for three aphid species on "Taiwan" were similar (fig. 2C) though the total concentration varied from 32.4 (*N. ribisnigri*) through 46.9 (*M. persicae*) to 72.8 mM (*M. euphorbiae*). Honeydew of *M. persicae* showed asparagine (2 out of 3 replicates) and lower relative quantities of glutamic acid and higher concentrations of leucine.

Differences among lettuce lines. Comparison of EDTA samples of isogenic lines showed similar amino acid profiles for all lines (fig. 3) apart from the presence of trace amounts of ornithine in RES2 and SUS2. Sometimes minor compounds were not detected (glycine, methionine, tryptophan) but no relation with resistance appeared. The lines RES, RES2, SUS and SUS2 differed from "Taiwan" by the presence of traces of phospho-ethanolamine. The total concentrations of amino acids in the EDTA samples were 50% lower on the resistant lines (RES 2.3 mM, RES2 3.6 mM) compared to the susceptible lines (SUS 6.3 mM, SUS2 7.1 mM, "Taiwan" 7.4 mM).

Proteins

EDTA and *M. persicae* honeydew samples of the lines RES and SUS showed very low amounts of protein (EDTA: 20 µg/ml, Honeydew: 80 µg/ml). SDS-PAGE showed a large number (>30) of proteins in the range of 50 to 200 kD in the EDTA samples. Honeydew contained only a few proteins (5-10), most of which were also found in the EDTA samples. No difference was observed between resistant and susceptible plants.

Secondary Plant compounds

The results from our analysis from honeydew and EDTA samples showed many peaks which were not primary compounds (sugars, proteins or amino acids) as judged by their UV spectrum and retention times. A number of them probably represented secondary plant compounds. No attempt was made to identify individual compounds but we concentrated on differences between resistant and susceptible lines. Differences between EDTA samples and honeydew samples from "Taiwan", SUS and RES were mainly in the

ratio of different peaks. No consistent differences were observed between resistant and susceptible plants.

DISCUSSION

Sugars

Stylectomy. The stylectomy samples show that sucrose is the only sugar present in the phloem sap of lettuce, as expected for most plants (Ziegler, 1975). The small quantities of fructose and glucose occurring in the stylectomy samples of *M. persicae* are probably the result of a contamination of the sample by micro-organisms (invertase) during collection. Invertase is not present in the phloem sap (Ziegler, 1975). The size of the stylectomy samples was difficult to determine exactly (Van Helden *et al.*, 1994). Therefore, our measurements are only an estimate of the exact quantity of sugars. Our findings (16.2%) correspond well with an expected value of 15-20%.

EDTA. The near equal amounts of fructose and glucose in the EDTA samples show the result of sucrose breakdown. Invertase activity in the sample can occur due to microbial contamination or release of plant enzymes from the wound surface of the leaf. Though the sugar concentration in the EDTA samples is much lower than in honeydew, EDTA samples are a better representation of the phloem sugars (see below), and they are also easier to collect. Assuming 16% sugar (as sucrose) in the real phloem sap EDTA samples are approximately diluted 40 fold.

Honeydew. Honeydew sugar composition is very different from phloem sugar due to biotransformation by the aphid, the combined activity of invertase from the aphids alimentary tract (Srivastava and Auclair, 1962b), and biotransformation. The resulting total sucrose/fructose/glucose content of the honeydew (5%) is low compared to the stylectomy samples. A higher concentration was expected (Lamb and Kinsey, 1959; Peel, 1975). Sometimes a total breakdown of sucrose in the honeydew occurred with nearly all glucose "missing" (e.g. honeydew of *N. ribisnigri* from "Taiwan"). Reports on honeydew sugars, from "dry" collections nearly all show sucrose, but a total absence of sucrose has been reported as well (Auclair, 1963;

Lamb, 1959). Enzymatic activity (invertase) in the honeydew has been reported (Srivastava and Auclair, 1962b; Rahbé *et al.*, 1994). Fisher *et al.* (1984) suggested that after invertase action a large proportion of the glucose is transformed by the aphid to glucose containing oligomers for osmoregulation. This is in line with the observed predominance of fructose over glucose, and the presence of large quantities of higher order oligosaccharides in honeydew samples (polymerization series of 5 to 7 peaks, retention times suggest one glucose unit extra for each peak). Identification and quantification of these oligomers is difficult but the peak area of these compounds suggests total concentrations of up to 5% (w/v) (Ammeraal *et al.*, 1991) or 50% of all sugars present. It is not clear whether the sucrose breakdown and glucose polymerization occurred exclusively in the aphids alimentary tract or continue after excretion. The increase in osmolality of honeydew droplets as observed by Fisher *et al.* (1984) (see also Van Helden *et al.*, 1994) suggests that invertase activity does continue. Enzymatic activity in the honeydew could also be caused by microbial contamination which is prevented by the hexadecane cover, but might occur in the aphid alimentary tract (Grenier *et al.*, 1994, Srivastava and Auclair, 1962a).

Differences among lettuce lines. The observed difference in sugar concentration between EDTA samples of resistant and susceptible plants was not reflected in the honeydew samples. This suggests that the difference is caused by variation in the efficiency of the EDTA collection method rather than a different sugar concentration in the phloem sap (also confirmed by the equally lower amino-acid concentration (see hereafter). However, a genetic coupling seems to exist since resistant lines of both sets possess this trait (Van Helden *et al.*, 1994), possibly as a leftover from the introduced part of the *L. virosa* genome (Eenink *et al.* 1982a,b).

Differences among aphid species. The differences in honeydew sugars among aphids on "Taiwan" are remarkably large, as are the differences between lettuce lines. This suggests an extensive difference in "biotransformation" of the phloem sugar, by differences in invertase activity (Srivastava and Auclair, 1962b) and glucose utilization (for assimilation and by polymerization) among aphid species. Another possibility is that the amount of phloem sap ingested, and therefore, the degree of utilisation is

very different which could be checked by a comparison of honeydew production.

Summary: For sugars only stylectomy gives a good representation. EDTA samples are an acceptable substitute, though collection efficiency can differ among lines. Honeydew sugars cannot be compared to real phloem sap. Sugar composition or concentration in samples from different the lettuce lines cannot explain the resistance.

Amino acids

Stylectomy. The estimated 125 mM of amino acids in the phloem sap is in the same range as found for most other plants (Ziegler, 1975; Rahbé *et al.*, 1990, 1994; Riens *et al.*, 1991). The resemblance in amino acid profiles from stylectomy samples of the two different aphid species seems self-evident. Still, differences between species could have occurred due to different "preparation" of a sieve element prior to sap ingestion and thus stylectomy (Prado and Tjallingii, 1994), or different aphid species selecting different sieve elements for feeding (Van Helden *et al.*, 1994). More sensitive methods for amino acid analysis (Riens *et al.*, 1991) would have allowed analysis of individual stylectomy samples and better quantification of trace amounts. Differences between samples may have been reduced by the pooling of samples.

The presence of relatively large quantities of GABA in the stylectomy samples is somewhat surprising. Chino *et al.* (1991), Rahbé *et al.* (1990) and Girousse *et al.* (1991) claim GABA to be an artifact of the EDTA extraction method. GABA has been found in honeydew on many occasions (see Auclair 1963). So far, the relative amount of GABA reported in stylectomy samples has always been lower than in EDTA samples. Our findings suggest that GABA is one of the major amino acids present in lettuce phloem sap, though our identification is only based on retention times. The concentration of ammonia in the stylectomy samples is higher than expected, other reports showed lower amounts (Kuo-Sell, 1989; Weiner *et al.*, 1991). This may be explained by our collection method, which prevents evaporation. Contamination by atmospheric ammonia was prevented by the oil layers, so the ammonia is either present in the phloem sap or a result of breakdown of nitrogenous compounds during collection and storage.

EDTA. It is not evident from our results that the amino acids found in EDTA samples are of phloem origin and not from other plant parts. The relative concentrations of amino acids in phloem sap is usually not more than a factor 2 different from the cytosol (Riens *et al.*, 1991; Winter *et al.*, 1992; Weiner *et al.*, 1991). However, it seems unlikely that (except for macromolecules like proteins) some compounds present in the phloem sap would exude (sugars) and others not. This is confirmed by the fact that concentrations of both sugars and amino-acids are 50% lower in the RES and RES2 samples. The presence of arginine and traces of 3 other amino-acids, which were not found in stylectomy or honeydew samples suggest that contamination occurs, though amounts might have been under the detection limit in the stylectomy samples.
Increase in the glutamine/glutamic acid ratio in EDTA (and honeydew) samples has been reported by several authors (Weibull *et al.*, 1990; Girousse *et al.*, 1991). The EDTA samples might differ from the other collection methods because the phloem sap is collected at other locations on the plant, which might have a different amino acid profile. This was shown for oat (Kuo-Sell, 1989), but not for lupin (Rahbé *et al.*, 1990).
Honeydew. Honeydew amino acids are the result of utilisation and biosynthesis of new amino acids (Sasaki *et al.*, 1990). Total concentrations in the honeydew are 50 to 75% lower than in the phloem sap. The volume of the excreted honeydew is supposedly less than the actual ingested volume. Tjallingii (1994) suggests around 50%, but the difference, mainly due to evaporation, will be variable. This shows that a very large proportion is utilised by the aphid. Surprisingly, the ammonia concentration in the honeydew is lower than in the stylectomy samples suggesting that the ammonia is of plant origin rather than a result of amino acid breakdown by the aphid (Sasaki *et al.*, 1990).
Differences among aphid species. The amino acid profiles of honeydew of different aphid species showed differences in biotransformation of amino acids among species (e.g. low glutamine and glutamic acid and high leucine and isoleucine concentration for *M. persicae*). However, the differences in total concentration of amino acids were much larger.
Differences among lines. Comparison of the EDTA samples of resistant and susceptible lettuce showed very little differences among two lines of the

same set and little variation between sets, apart from traces of ornithine in the lines RES2 and SUS2 (fig. 2). Differences with "Taiwan" lettuce (fig 1C) are somewhat larger. Resistance is obviously not based on amino acids.
Summary: Both honeydew and EDTA samples are not a very good representation of phloem sap amino-acids. It is difficult to judge which sampling technique is a better representation, and differences in amino acids in honeydew from different aphids are very large.

Proteins

EDTA. Based upon the sugar content, Van Helden *et al.*, 1994 estimated that EDTA samples are a 40 fold dilution of the real phloem sap. The observed protein concentration of around 20 µg/ml would mean that the lettuce phloem sap contains around 0.8 mg/ml of protein, which is in the same range as reported for the phloem sap of several plant species collected by incision (Ziegler, 1975) or stylectomy (Fisher *et al.*, 1992). The number of proteins is very high but comparable to Fisher *et al.* (1992) who reported several hundreds of different proteins in phloem sap from wheat, collected by stylectomy or from broken pedicels.

It is not evident that all these proteins are normally present and mobile in the phloem sap. The loss of turgor pressure upon incision of a phloem strand (and presumably also excision of a leaf for EDTA samples or stylectomy) might cause considerable leakage of enzymes from the surrounding phloem bundle cells into the sieve elements (Eschrich and Heyser, 1975), and proteins which are normally immobile in the sieve elements, incorporated or bound to structural elements (e.g. P-proteins fibrils) can be released by the wounding or EDTA action. Tjallingii and Hogen Esch (1993) observed filamentous structures, probably P-proteins, blocking the food canal of amputated aphid stylets. Finally, contamination of EDTA samples can occur with proteins released from the wound surface.

Honeydew. The honeydew showed low amounts and few proteins compared to the EDTA samples. Aphid alimentary tracts do not contain proteinases (Srivastava and Auclair, 1963) so proteins ingested by the aphid are expected to pass the aphid unchanged or accumulate in the aphid (Rahbé and Febvay, 1993). It seems unlikely that nearly all proteins which were present in the EDTA samples were accumulated. If the aphid would ingest phloem sap with 800 µg/ml (EDTA sample estimate) of protein and excrete it

without metabolising, then the protein content of the honeydew would be higher than the value found in our experiments (80 µg/ml). Normal aphid feeding rates are a factor 10 lower than the outflow from amputated stylets (Van Helden *et al.*, 1994; Tjallingii, 1994) in which case no leaking of proteins from the companion cells to the phloem sieve element is expected (Eschrich and Heyser, 1975) and no wound reaction (P-protein gelation, responsible for the first fast wound reaction) will occur. This confirms the hypothesis that the EDTA samples contain proteins which are normally not present or not mobile in the phloem sap and are therefore not ingested by the aphid. Additional proteins, present in the honeydew but not in the EDTA samples, are produced by the aphid or its symbionts.

Summary. More experiments are necessary to judge which sampling method provides the best representation of the proteins of phloem sap (or aphid food). Honeydew might be the best sample, even better than stylectomy, though some proteins might accumulate in the aphid.

Secondary Plant compounds

The main reason for aphids to use phloem sap as food source is thought to be the absence or low concentrations of secondary compounds in the phloem sap as compared to other plant parts. Many different groups of secondary substances have been reported from lettuce (phenolics; flavonoids; sesquiterpene lactones; alkaloids; sterols, Gonzalez, 1977), but nothing is known about their presence in the phloem sap.

The analysis method using HPLC with a diode array detector is limited, only substances having UV absorption, present in sufficiently high amounts and separated with our column/solvent system do show up in the chromatogram.

The resemblance between EDTA and honeydew samples indicates that aphids do excrete most of the ingested secondary substances unchanged through the honeydew. At the same time it confirms that EDTA samples are mainly of phloem origin, the secondary compounds originating from the phloem sap were not overrated by contamination from the wound surface by high concentrations from vacuoles of damaged cells. The effect of aphid digestion on secondary compounds is unclear although several secondary plant compounds are reported to pass unchanged (Molyneux et al. 1990). Polyphagous aphids might possess detoxification mechanisms breaking

down secondary compounds and leaving no traces in the honeydew while monophagous species do not accept these plants. Hussain *et al.* (1974) reported 18 different phenolic acids present in aphid honeydew feeding on radish, five of which were found in radish seedlings together with four other phenolic acids. Therefore, they suggested that most of the phenolics present in honeydew were breakdown products. However, they used whole plants and not phloem sap samples. Compounds and concentrations in phloem sap are expected to differ widely from whole plant samples.
Difference among lettuce lines. We were unable to show consistent differences between lettuce lines. Therefore a relation with resistance is not clear. Because of the limitations of the analysis method this is no proof that secondary plant compounds are not involved in resistance.

Overall comparison of collection methods.
The outcome of the chemical analysis of different compounds reveals that the question of which collection methods is most reliable cannot be answered easily and differs among chemical groups.
Stylectomy. So far stylectomy has been thought to represent the real phloem sap. However, it is possible that the aphid changes the composition of the phloem sap prior to or during feeding. Auclair (1963) reports on a 10-50% reduction of the amino acid content of the honeydew due to crowded aphid attack. The aphid's penetration behaviour, together with salivary sheath formation and salivation into the sieve element (Prado and Tjallingii, 1994) might influence the plant. Phloem sap collected by stylectomy depicts the phloem sap of an aphid attacked sieve element. Even then, some compounds normally immobile (proteins) might be translocated due to the high outflow rate (or the sudden turgor changes), which is much higher than under normal aphid feeding circumstances. The time-consuming and often rather uncertain yields (Van Helden *et al.*, 1994; Fisher and Frame, 1984) are an obvious disadvantage. The small sample size and shortage of replicates pose a problem for the chemical analysis of many compounds, especially secondary plant substances.

EDTA. EDTA collected samples appear to be a good representation of most compounds of the phloem sap apart from proteins. What exactly happens with the proteins remains obscure. Attention should be paid to

possible reactions in the collection vials (invertase, oxidation) and leakage of compounds from other cells. The quality might be improved by adding more inhibitory compounds (anti oxidants, enzyme inhibitors) to the solution, though these compounds might influence the metabolism of the leaf. Leaking of compounds from the wound surface seems limited, even for secondary compounds. Washing of the surface after cutting is advisable. Cutting the petiole again after a short EDTA exposure to enhance exudation (Girousse *et al.*, 1991) is unnecessary and will cause extra contamination from the wound surface. EDTA collection is by far the easiest method, yielding the largest quantities of phloem sap equivalents.

Honeydew. Honeydew is "bio-processed" phloem sap. The profile and concentration of sugars and amino acids differs profoundly from the real phloem sap. Biotransformation occurs in the aphid and might even be continued in the honeydew. Contamination of the honeydew could also be caused by ingestion from other tissues during the cell-punctures which occur during the penetration towards the phloem. The number of these punctures is high (Tjallingii and Hogen Esch, 1993) and the time spent penetrating towards the phloem is a large part of feeding behaviour (Van Helden and Tjallingii, 1993). If ingestion occurs during these punctures it will be from vacuoles where the concentration of secondary compounds is high. To avoid possible contamination, honeydew should be collected preferably from individuals which have been feeding continuously for a long period. Honeydew collection is much easier than stylectomy and usually a homopteran insect feeding on the phloem can be found. The honeydew collection method covers the possibility of short term or local induction of plant reactions which then might cause differences in honeydew composition.

Comparison of lettuce lines: Variation in phloem sap compounds seems very limited between lettuce lines, though minor differences do occur. No relation with resistance has been observed so far. Comparison of phloem sap for unknown secondary compounds proved not to be a useful method to find resistance factors due to the infinite number of possibilities. The most promising option to show chemical differences related to resistance, would be to attempt to develop a bioassay using honeydew (Dorschner and Kenny, 1993) or EDTA samples added to an artificial diet, guiding chemical isolation and identification techniques.

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7. THE DEVELOPMENT OF A BIOASSAY

ABSTRACT

The resistance of lettuce to the aphid *Nasonovia ribisnigri* (Mosley) is located in the phloem. Since chemical analyses of the phloem sap had shown no differences between resistant and susceptible lines, a bioassay was developed in order to test samples from resistant and susceptible plants on aphid feeding. Whole plant extracts, honeydew, and EDTA collected phloem extracts were tried and a sensitive bioassay was developed using EDTA samples. The EDTA was removed, samples were added to a simple sucrose solution or to a complex artificial diet, and presented in a choice situation comparing extracts from resistant and susceptible plants. Samples from susceptible plants were preferred to those from resistant plants. The resistance is probably based on a feeding deterrent action of the phloem sap in the resistant plant.

INTRODUCTION

Resistance of plants to aphids is difficult to investigate because of the highly specialized feeding behavior of these insects. In many cases the resistance is based on an interruption of the feeding behavior after the phloem sieve element has been reached by the insects mouthparts

(Kimmins, 1989; Padgham *et al.*, 1990; Caillaud *et al.*, 1992; Cole, 1993; Van Helden and Tjallingii, 1993). Elucidation of the (chemical) background of the resistance mechanism is very difficult (Dreyer and Campbell 1987, Harrewijn, 1990, Wink and Witte, 1991). So far investigations into resistance to homopteran insects have often used correlations of total plant levels of certain chemical compounds with performance traits (Todd *et al.*, 1971; Argandoña, 1980; Dreyer and Jones, 1981; Herrbach, 1985; Leszczynski *et al.*, 1985; Niemeyer, 1990; Kanehisa *et al.*, 1990, Luczak and Gaweda, 1993). Phloem sap, the only plant component ingested as food by the aphid, will be only a small part of total plant extracts. When resistance is based on a chemical component of the phloem sap, it will very likely be lost in whole plant preparations.

The absolute, monogenic resistance of lettuce (*Lactuca sativa* L.) to the aphid *N. ribisnigri* (Nr-gene, Eenink *et al.*, 1982; Reinink and Dieleman, 1989; Van Helden *et al.*, 1993) is based on an early interruption of sap uptake after the aphid stylets have reached a phloem vessel (Van Helden and Tjallingii, 1993). Although a mechanical resistance mechanism, like blocking of the stylets or sieve elements during attempted feeding, cannot be excluded as a possible resistance mechanism, our current working hypothesis is that a chemical difference in the phloem sap may interfere with host plant recognition or acceptance in the resistant plants (Van Helden and Tjallingii, 1993; Van Helden *et al.* 1994a,b; Wink and Witte 1991).

Phloem sap samples were obtained by stylectomy, EDTA chelation and honeydew collection, and analysed for sugars, amino acids, proteins and UV absorbing secondary plant compounds, which showed no consistent differences between resistant and susceptible lettuce lines (Van Helden *et al.*, 1994a,b). Lettuce contains many secondary compounds (Gonzalez, 1977) but little is known about their presence in the phloem and their biological activity. Exhaustive analysis of secondary compounds in the phloem sap of lettuce, without knowledge of the nature of the active substances (allomones), would be very time consuming.

The development of a bioassay using EDTA collected phloem sap extracts of the plants as described here, might make a "classical" bioassay-guided-fractionation, isolation and identification of the allomones involved in the resistance feasible.

MATERIALS AND METHODS

Plants and Aphids

Plants were grown and aphids were reared as described earlier (Van Helden *et al.*, 1993, 1994a). Synchronized larvae were used when five days old (L3 stage) unless otherwise stated. Two sets of near isogenic lettuce lines were used: SUS(ceptible, genotype nrnr) and RES(istant, NrNr) (SET1), SUS2 (nrnr) and RES2 (NrNr) (SET2), lettuce line "Taiwan" (susceptible, nrnr) and oilseed rape cv. "Olymp" (*Brassica napus* L.) (Van Helden *et al.*, 1994a,b). The two isogenic sets are genetically distinctly different (except for the source of the Nr-gene, Van Helden *et al.*, 1994a).

Plant and phloem extracts

Whole plant extracts. Whole plants were heated in a microwave oven for 20 seconds. Sap was pressed from the leaves, filtered and used directly. Methanol extracts were prepared from 0.2 g lyophilized leaves and 10 ml of MeOH, followed by ultrasonic mixing for several minutes, centrifugation, filtration and vacuum drying.

Honeydew. Honeydew from oilseed rape and "Taiwan" lettuce was collected using *M. persicae* Sulzer (biotype WM1) and from the lines RES and SUS using *M. euphorbiae* Thomas (biotype WMe1). Honeydew was collected in hexadecane (Van Helden *et al.*, 1994a), stored at -80 °C and lyophilized prior to use. Occasionally honeydew was collected directly on Petri dishes which were placed underneath leaves with large quantities of aphid larvae. This "dry collected" honeydew was dissolved in a water/MeOH (1:1) mixture, MeOH was evaporated *in vacuo* and the aqueous extract was lyophilized.

Sample preparation from crude EDTA extracts. Phloem sap exudate was collected by placing excised leaves in an aqueous solution with 8 mM EDTA and 5 mM phosphate buffer at pH 6 for 20 h (King and Zeevaart, 1974; Groussol *et al.*, 1986; Van Helden *et al.*, 1994a). Initially 5 mM sodium metabisulfite ($Na_2S_2O_5$) was used as an anti-oxidant but later this was omitted because of toxicity to the aphids. The "crude EDTA extract" was stored at -20 °C. To remove the EDTA from the crude EDTA extract the following procedure was used: A weak cation exchange column (Carboxylic acid) was activated with 5 ml ammonium buffer (pH 10), washed with H_2O

and loaded with 10 ml of a 0.01 Mol lead nitrate solution. After washing with H_2O, 10 ml of crude EDTA extract was added and 2 ml fractions were collected. Fractions were tested for the presence of EDTA (see below), EDTA free fractions (usually the first 4) were combined, lyophilized and stored at -20°C until use. These EDTA free samples will be referred to as "Ed-samples" (EDTA derived samples) throughout this paper. After cleaning with 10% formic acid in water, the columns were reused.

Control treatments: Together with the Ed-samples two different control treatments were used. The first was a blank control ("BLANK") (nothing added). The second control treatment ("CONTROL") was used to compensate for the amino-acids and sugars in the Ed-samples, and to counterbalance the possible artifacts introduced by the EDTA extraction procedure. A complex artificial diet (consisting of 15% sucrose, amino acids, vitamins and salts, Harrewijn and Noordink, 1971), was diluted around 30 times in the original EDTA chelation solution (8 mM EDTA and 5 mM phosphatebuffer). This solution was then treated identically to the crude EDTA extracts (Pb-EDTA extraction, lyophilization, storage). The resulting samples are referred to as Ed-Diet. To make the 'CONTROL" treatments, this Ed-Diet was dissolved in the sugar (sugar tests) or in a complex diet (diet tests) in the same amounts as the Ed-samples (see below).

EDTA Detection: EDTA was detected using a color reaction with Eriochrome Black T as an indicator. In a test tube two droplets of indicator (0.1% w/v), 1 ml H_2O and two droplets of ammonium buffer were mixed. Lead nitrate solution was added until the color changed to purple. To test for the presence of EDTA five (or more) droplets of a fraction were added to a test tube. A color change to blue indicated the presence of EDTA (detection limit ± 2 µM of EDTA).

Preparation of tests

All tests were prepared in a laminar flow cabinet. Materials were cleaned with 70% ethanol prior to use. All test solutions were filtered through a 0,22 µm micropore filter.

No choice tests. Test solutions consisted of 15% sucrose in water with one of the following additions: whole plant sap (5 or 10% v/v), methanol extracts (50 mg/ml), crude EDTA extracts (10% v/v), EDTA at 0, 5, 10, 25, 100 or 200 mM, or $Na_2S_2O_5$ at 0, 5, 10, 25 or 100 mM. Test solutions were

presented between two layers of Parafilm, stretched over a 26 mm diameter plastic ring to form a test cage. 8 freshly molted adult aphids were introduced and survival and reproduction were scored after 48 h.

Multiple Choice tests were prepared according to Dorschner and Kenny (1993). Six parafilm sachets with different sucrose concentrations (0, 5, 10, 20% w/v in H_2O) were presented through holes in the lid of a 9 cm Petri dish and a large quantity (>100) of aphids of mixed age were introduced into the dish. Distribution of the aphids over the 6 sachets was determined after 24 and 44 h. Plant extracts were not tested.

Dual Choice tests. For these tests we constructed sachets with two adjacent test solutions separated by only a few millimeters were the two layers were firmly pressed together using a rubber wheel (fig. 1)

Twenty larvae of *N. ribisnigri* were introduced and the tests were placed in a 24 °C incubator with weak illumination from the top. Transparent yellow plastic foil was placed below the light source to stimulate settling on the parafilm surface. Aphid distribution was determined several times (after 8 to 72 hours). Usually at least six replicates were performed (see results).

Honeydew tests. Test solutions were made by adding freeze dried honeydew (10 mg/ml) to a sucrose solution (15% in H_2O). Pairwise tests also included 15% sucrose solution ("BLANK").

Testing of Ed-samples.

Acceptability test. Ed-diet was dissolved at 20 mg/ml in artificial diet, and tested against artificial diet to which 20 mg/ml of lyophylised artificial diet (not treated on the column) was added.

Sugar tests: Tests were performed using Ed-samples from RES and SUS (treatment RES and SUS) dissolved at 5 mg/ml in 15% sucrose in water. These were compared with two controls: a blank control ("BLANK", 15% sucrose with nothing added) and a second control ("CONTROL") consisting of a 15 % sucrose solution to which 5 mg/ml of Ed-diet was added. This makes six possible pairs. The same combinations were tested at 20 mg/ml. The extracts of RES2 and SUS2 were tested in the same combinations at 5 mg/ml and 20 mg/ml.

Artificial diet tests: Same treatments and combinations (RES, SUS, and RES2, SUS2). In this case however, all samples were dissolved in a complex artificial diet. There was no BLANK control treatment and the control

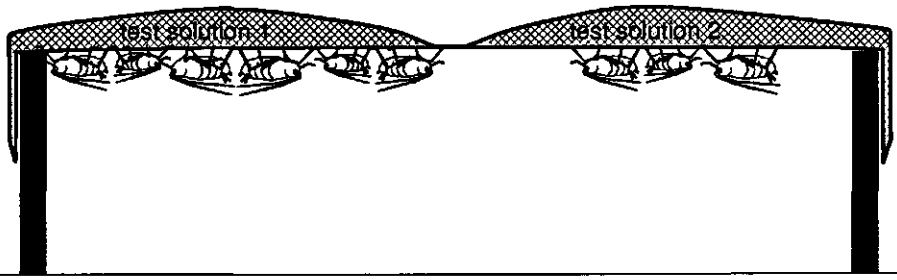

Figure 1. Cross section of the test cage with a dual choice sachet. 20 larvae of *N. ribisnigri* can freely select a feeding site on a test solution.

treatment ("CONTROL") consisted of the same complex artificial diet to which 5 (or 20) mg/ml of Ed-diet was added, so there were only three possible pairs per test.

Statistics

Choice test results were compared by Wilcoxon's signed rank tests at 5% significance level.

RESULTS

No choice tests. Tests with whole plant sap or methanol extracts showed 10 to 50% mortality of the adults and very low reproduction as compared to complex artificial diet. No differences showed between mortality or reproduction on test solutions containing the resistant or susceptible whole-plant extracts.

EDTA affected survival at concentrations over 10 mM, at 100 mM or higher most aphids died within 24 hours. Little mortality occurred at lower concentrations but reproduction was low (< 1 larvae per adult per day). The anti-oxidant sodium metabisulfite caused high mortality, even at concentrations below 10 mM.

Tests with crude EDTA extracts (containing EDTA and anti-oxidant) showed low acceptance of the test solutions, low reproduction and high mortality. When the anti-oxidant was omitted during collection and the EDTA extracted, no mortality was observed and more than 80% of the aphids settled on the test solutions.

Multiple Choice tests gave no discrimination among sucrose solutions of different strengths. Aphids settled poorly and appeared restless.

Dual choice tests.

Observations after 8, 20 or 28 h showed no or less pronounced differences, whereas during later observations (> 48 h) microbial contamination of the test solutions was sometimes visible.

Acceptability test. The acceptability tests suggested that the lyophilized diet was preferred to the Ed-diet (distribution after 40 hr
Diet : Ed-Diet = 10.1 : 6.6, n=11)) but this difference was not significant.

Honeydew tests. Honeydew of host ("Taiwan" lettuce) and non host plants (oilseed rape) was strongly favored over the sucrose treatment "BLANK" (fig. 2a), and lettuce honeydew was preferred to oilseed rape honeydew. When testing honeydew of isogenic resistant (RES) and susceptible (SUS) lines these were both selected over sucrose only and honeydew of resistant plants was preferred to susceptible plants (fig. 2b).

Ed-samples in sugar tests. In the tests based on 15% sucrose, comparing Ed-samples of the SET1, aphids showed a slight preference for SUS to RES at low concentration (5 mg/ml) while all other combinations showed no significant difference (fig. 3a). At the higher concentration Ed-samples of SET1 were preferred to BLANK but not to the Ed-diet ("CONTROL") and SUS was selected over RES (fig. 3b). Ed-diet was selected over BLANK at the high concentration (fig. 3b and d).

The tests of SET2 showed that RES2 was preferred by the aphids at 5 mg/ml (fig. 3c) while none of the other combinations showed discrimination by the aphids at this low concentration, not even Ed-diet against BLANK. At higher concentrations (fig. 3d) RES2 was not favored over SUS2 but all of the other combinations showed a significant deterrence of Ed-samples compared to BLANK or even CONTROL.

Ed-samples in artificial diet tests. The tests based on the addition of extracts to a complex artificial diet (fig. 4) showed very distinct discrimination of

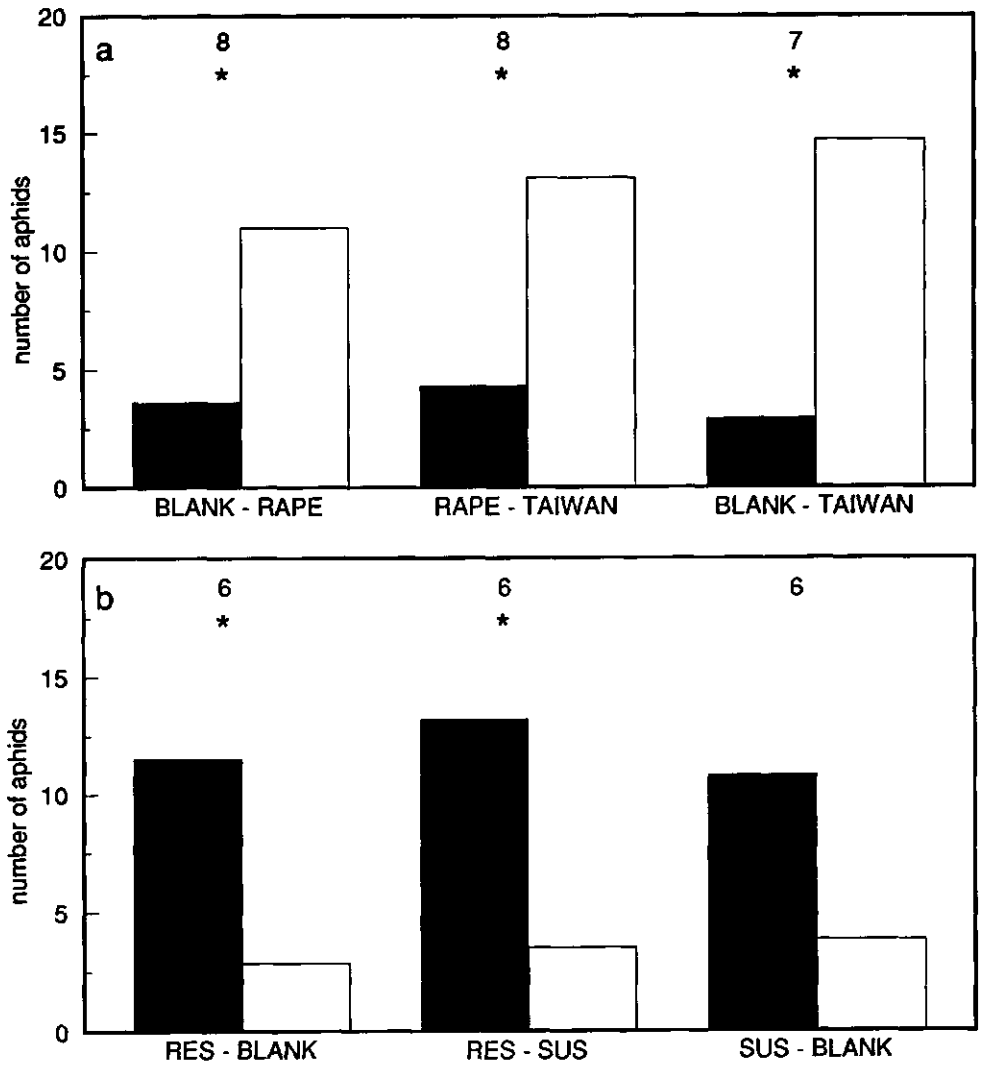

Figure 2. HONEYDEW TESTS. Results of the choice tests using lyophilized honeydew at 10 mg/ml in 15% sugar solution. a. Comparison of different plant species using honeydew produced by *M. persicae*; b. comparison of resistant and susceptible lettuce using *M. euphorbiae*. Bars represent mean number of aphids on each of the test solutions after 44 h. Numbers above bars are number of replicates. * = significant difference at 5% level.

different test solutions by the aphids, except for the low concentration of the SET1 Ed-samples (fig. 4a). At 5 mg/ml samples of SET2 showed a preference for SUS2 to RES2 but SUS2 was not selected over Ed-diet (fig. 4c). At the high concentration Ed-diet was selected over Ed-samples and the Ed-samples from susceptible plants over resistant plants (fig. 4b and d).

DISCUSSION

Whole plant extracts were toxic, probably due to secondary compounds present in cell vacuoles and released or activated upon the crushing of the cells during extraction (Matile, 1984). Plant sap consists mainly of vacuolar fluid, rich in secondary metabolites, which normally is not ingested by the aphid during feeding. We did not succeed in designing a test which resulted in a discrimination of whole plant samples.

A bioassay requires relatively large amounts of phloem sap. Of the methods available to collect phloem sap from lettuce only honeydew and EDTA chelation yield sufficient quantities (van Helden *et al.* 1994a,b). Honeydew is phloem sap that is collected and bio-processed by the aphids. EDTA chelation exudates are considered to be reliable representations of phloem sap. Contamination with substances from other plant parts and from the cut (wounded) surface of the petiole might occur but this has not been shown to be of major importance (Fisher and Frame, 1984; Weibull *et al.*, 1990; Girousse *et al.*, 1991; Van Helden *et al.*, 1994a,b).

The crude EDTA extracts were not accepted by the aphids, because of the presence of EDTA and sodium metabisulfite. Omission of the anti-oxidant during EDTA exudation increased the browning of the extracts but apparently did not affect the biological activity of Ed-samples.

During the processing of the samples the EDTA was retained by the lead ions on the column as an Pb-EDTA complex. It is possible that other components of the crude EDTA extracts may have been retained on the column, some column components may have been released (lead ions or Pb-EDTA complex), and EDTA removal during the EDTA extraction procedure may have been incomplete (below detection limit). In initial experiments traces of lead (precipitation test with KI) could be shown in the

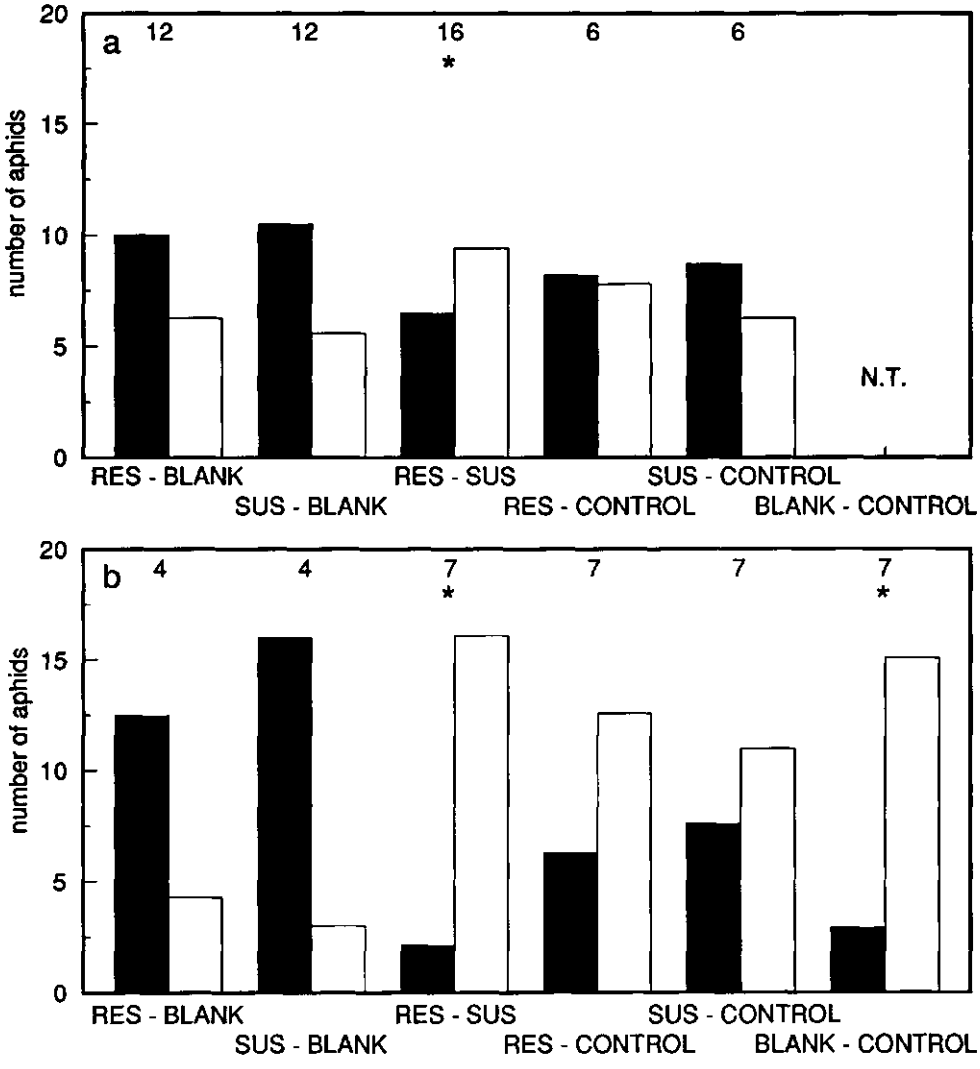

Figure 3a and b. SUGAR TESTS OF SET 1. Results of the choice tests using Ed-samples from SET1 or Ed-diet (CONTROL), all dissolved in 15% sugar solution. BLANK = 15 % sucrose with nothing added. See text for details on processing of extracts. a. RES, SUS or ED-diet at 5 mg/ml and BLANK; b. at 20 mg/ml; Bars represent mean number of aphids on each of the test solutions. Numbers above bars are number of replicates. * = significant difference at 5% level, N.T. = not tested.

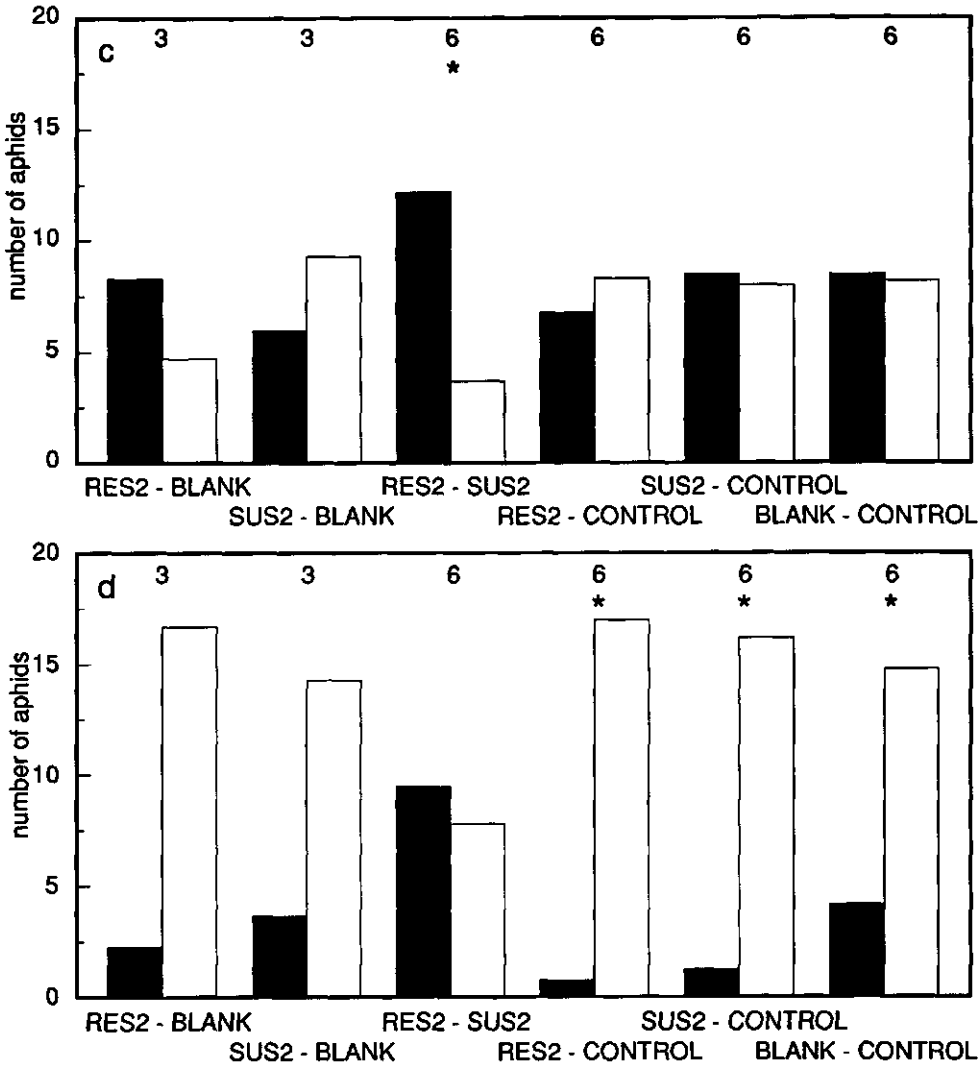

Figure 3c and d. SUGAR TESTS OF SET 2. Results of the choice tests using Ed-samples from SET2 or Ed-diet (CONTROL), all dissolved in 15% sugar solution. BLANK = 15 % sucrose with nothing added. See text for details on processing of extracts. c. RES2, SUS2 or Ed-diet at 5 mg/ml and BLANK; d. at 20 mg/ml (d). Bars represent mean number of aphids on each of the test solutions. Numbers above bars are number of replicates.
* = significant difference at 5% level.

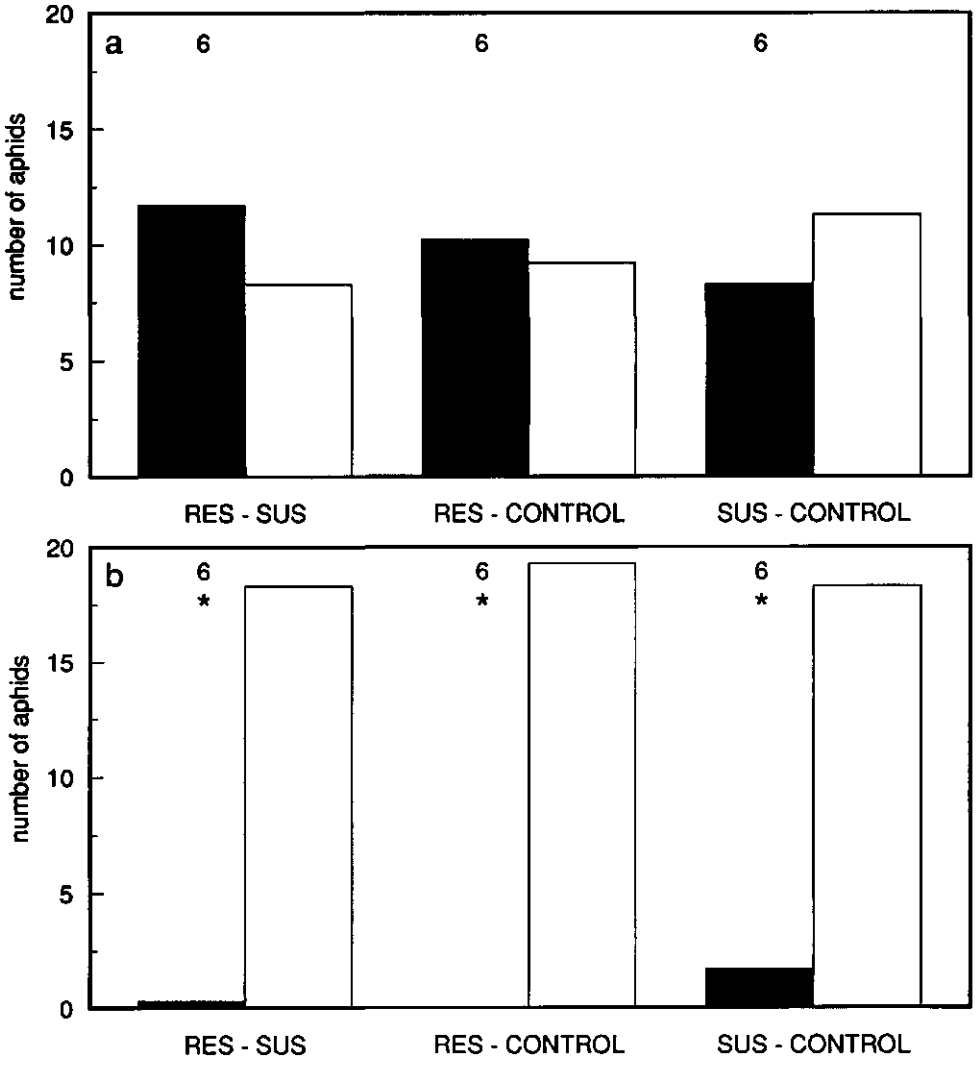

Figure 4a and b. DIET TESTS OF SET 1. Results of the dual choice tests using Ed-samples from SET1 and Ed-diet (CONTROL), all dissolved in complex artificial diet. See text for details on processing of extracts. a. RES, SUS or Ed-diet at 5 mg/ml; b. at 20 mg/ml; Bars represent mean number of aphids on each of the test solutions. Numbers above bars are number of replicates. * = significant difference at 5% level..

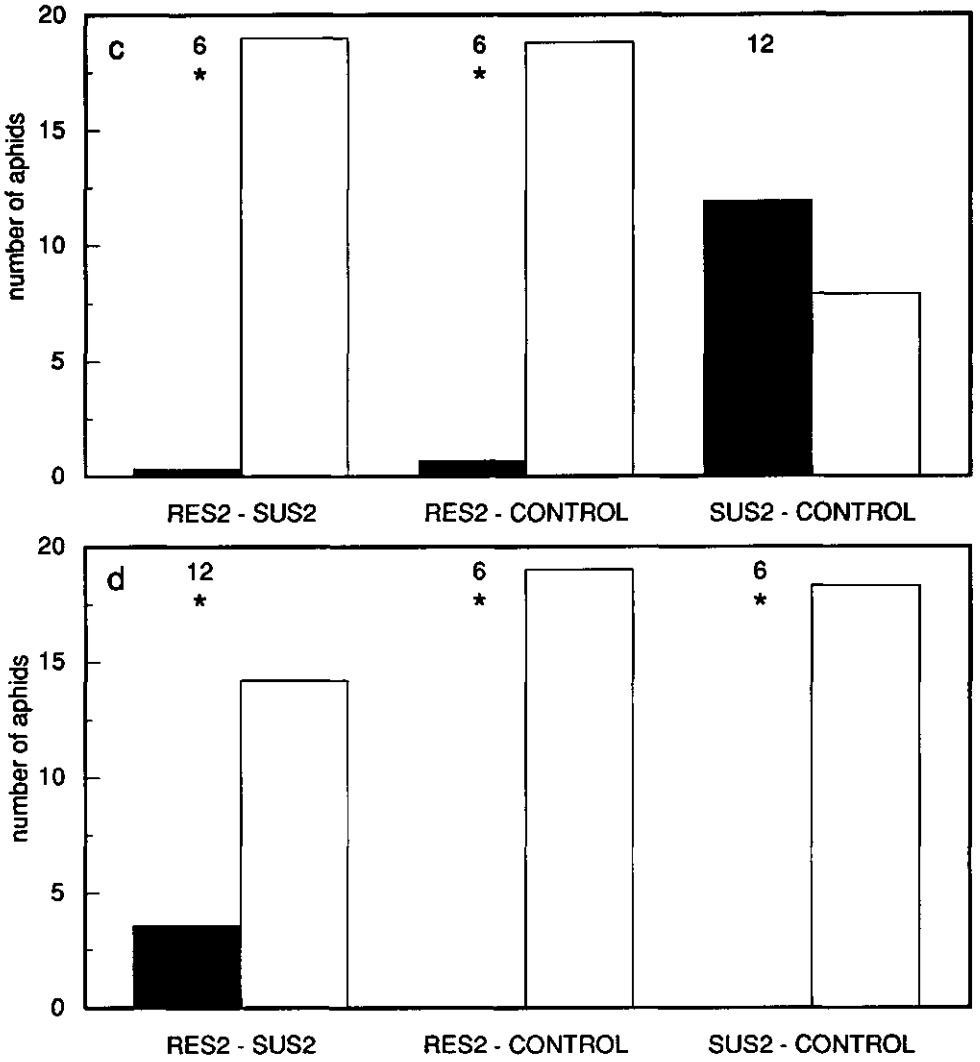

Figure 4c and d. DIET TESTS OF SET 2. Results of the dual choice tests using Ed-samples from SET2 and Ed-diet (CONTROL), all dissolved in complex artificial diet. See text for details on processing of extracts. c. RES2, SUS2 or Ed-Diet at 5 mg/ml; d. at 20 mg/ml. Bars represent mean number of aphids on each of the test solutions. Numbers above bars are number of replicates. * = significant difference at 5% level.

"EDTA free samples" when a pure EDTA solution was processed. In the Ed-samples we sometimes observed a precipitation, presumably of lead-phopsphate. Though we did not observe a significant preference for Ed-Diet over non-treated diet (acceptability test), this may still have affected the aphids. However, since the treatment of all samples included the step of EDTA removal, artifacts will occur in all Ed-samples and Ed-diet in roughly equal amounts, so this does not affect our conclusions

Design of the test

The bioassay of Dorschner and Kenny (1992), which demonstrated preference for hop (*Humulus lupulus* l.) honeydew by the hop-aphid *Phorodon humili* Schrank, turned out unsatisfactory for *N. ribisnigri* and lettuce. Aphids often showed aggregation behavior and accumulated on one or two of the six available sachets (irrespective of the sugar concentration). Therefore we changed to a dual choice test (Mittler and Dadd, 1964; Harrewijn and Noordink, 1971). Our setup, using two parafilm membranes pressed together in the middle, uses very little solution to fill the two compartments and has no border between the adjacent compartments which can act as a barrier (fig. 1). Therefore, the aggregation behavior will not influence the results strongly. Still the individual aphid cannot be considered as independent from the others in the same test. The simplicity of the dual choice test makes it possible to make many replicates, necessary to show statistically significant differences.

Unequal distribution of the aphids over the two sides became apparent only after twenty hours or more. This is much later than observed by Mittler and Dadd (1964) on artificial diet, and also much later than expected from the rejection of the phloem sieve element once this is reached by the aphid (Van Helden and Tjallingii, 1993). The latter may be explained by the level of compounds in the choice tests which is will be around 10 times lower than in the real phloem sap, only 20 mg/ml of Ed-sample is added while normal lettuce phloem sap has a dry weight of around 20% (200 mg/ml). It cannot be excluded that the test solutions did change over time, e.g. because of continued enzymatic activity (Van Helden *et al.* 1994b), but this would also mean that there is a chemical difference between fractions.

Test results

The preference for lettuce honeydew (Taiwan) over the non host plant (oilseed rape) shows that the host-plant honeydew has a feeding stimulant effect (Dorschner and Kenny, 1992). Preference for the honeydew to sucrose only ("BLANK") (fig. 2) is probably largely caused by the feeding stimulancy of the amino acids present in the honeydew (Dorschner and Kenny, 1992; Mittler and Dadd, 1964; Van Helden *et al.*, 1994b). The choice for RES honeydew over SUS honeydew (fig. 2b) shows that there is a chemical difference. The choice for RES honeydew over SUS honeydew is surprising since the inverse would be expected (discussed below).

The sugar tests at 20 mg/ml (fig. 3b,d), just like the honeydew tests, clearly show the attractiveness of a combination of sucrose with amino acids. In the test combinations which include a BLANK treatment, the small quantity of amino acids present in the Ed-samples or the Ed-diet makes that these treatments are strongly preferred to "BLANK", without any amino acids. At the low concentration (5 mg/ml) most choices show no significant preference. The absence of response in BLANK versus Ed-Diet at 5 mg/ml (fig. 3c) suggest that this might be caused by the low quantity of the compounds added.

The comparison of Ed-samples with Ed-diet ("CONTROL") gives more valuable results since little interference due to amino-acid concentration differences (or EDTA extraction artifacts) will occur. Ed-samples (fig. 3b,d) are not preferred to Ed-diet, and in some cases not even to BLANK (fig 3d), suggesting the presence of feeding deterrents in the Ed-samples.

We decided to use a complex artificial diet as a base for the tests in order to decrease the sensitivity to variations in amino acids and sugars in the Ed-samples, which is not related to the resistance (Van Helden *et al.*, 1994b). This could also increase the sensitivity to other compounds which might only show biological activity in a "phloem like" environment. The results of these artificial diet tests (fig. 4) show a profound increase in the response of the aphids. The artificial diet test demonstrated significant preferences for Ed-diet to plant extracts. This suggests that all Ed-samples (of both resistant and susceptible plants) contain a certain feeding deterrence. The preference for SUS and SUS2 to RES and RES2 respectively proves that there is a chemical difference between these extracts, which (at 20 mg of

Ed-sample/ml) causes a stronger feeding deterrence in the Ed-samples from resistant plants. The absence of response in the tests of SET1 at 5 mg/ml (fig. 4a) and the SUS2-CONTROL choice (fig. 4c) suggests that the concentration of the feeding deterrents is under the behavioral threshold for the aphid, just like in in the low concentration sugar tests (fig. 3a,c).

In a few cases extracts from resistant plants were preferred to susceptible extracts (honeydew tests, Ed-samples in sugar tests of SET2 at 5 mg/ml). Feeding deterrents have been reported to act as a feeding stimulants at low concentrations (Bodnaryk, 1991). So deterrence of the resistant extracts at 20 mg/ml could change to feeding stimulance at 5 mg/ml, and these allomones could also be present in the susceptible plant but at lower concentration. To test this hypothesis more test series should be performed, comparing different concentrations of Ed-samples from the same or different plants, and in combination with appropriate controls. However, possible interaction with other biologically active substances , will not make this easy. Also, a feeding deterrent could be metabolized and excreted in the honeydew, its degradation products acting as feeding stimulants, or the reverse in case of a feeding stimulant. Since little is known about the fate of secondary plant chemicals in aphids (Wink and Witte, 1991, Givovich *et al.*, 1992, Van Helden *et al.*, 1994b) this remains speculative.

The sensitivity of the test might be improved by adjustment of the amino acid composition of the complex artificial diet (and the Ed-diet) to that of lettuce phloem sap (Van Helden *et al.*, 1994b), and total removal of possible interfering compounds from the Ed-samples. Using higher concentrations of the samples would require large quantities of plants.

Although the results of the honeydew and sugar tests cannot be explained easily, the diet test results show that there is a clear chemical difference between extracts from resistant and susceptible plants. This allows some optimism that this bioassay can be used to isolate the active substances through bioassay guided fractionation. Still, the physical- and chemical resemblance (pressure, concentration etc.) of the test with the *in vivo* system remains very incomplete and the EDTA sample processing needs to be improved. Direct extrapolations of the *in vitro* test results to *the in vivo* situation are hazardous. The phloem sap origin of any isolated compound needs to be confirmed and the mode of action should be studied in the plant.

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8. SUMMARY AND CONCLUSIONS

The resistance of lettuce to the aphid *Nasonovia ribisnigri* is based on a single, dominant gene, the Nr-gene. On the resistant plant aphids died within a few days, without any honeydew production. Transfer-experiments with a short stay on a resistant plant followed by a relocation to a susceptible plant showed that no weight increase occurred on the resistant plant, but weight gain immediately resumed on a susceptible plant (Chapter 2). So no intoxication seemed to occur on the resistant plant. Behavioral observations of (tethered) aphids using Electrical Penetration Graphs (EPG) (Chapter 3) and comparison with free living aphids (Chapter 4) showed that on the resistant plant the aphid's stylets did penetrate to the phloem sieve elements. On the resistant plants hardly any E2 pattern (phloem sap ingestion) occurred and the aphid finally died of malnutrition, or left the plant (Chapter 4). It was concluded that the resistance is based on a factor in the phloem. This can be either a chemical factor like a feeding deterrent or a mechanical factor which obstructs ingestion. Experiments concentrated on chemical differences in the phloem sap of resistant and susceptible (isogenic apart from the resistance gene) lettuce lines. Amputation of aphid stylets (stylectomy) during feeding can yield small but pure phloem sap samples. However, on the resistant and susceptible isogenic lines no phloem sap samples could be collected because outflow from the stylet stump stopped after a few seconds. Larger phloem sap samples were obtained by EDTA chelation and honeydew collection (Chapter 5).

Chemical analysis of the composition of the phloem sap samples showed no differences in sugars, amino acids, proteins and UV absorbing compounds (Chapter 6). Exhaustive analysis of phloem sap compounds was not feasible, not only because of the number of possible compounds, but also because the sample size and quantity was limited.

In a bioassay aphids were offered a choice between EDTA collected phloem sap samples of resistant and susceptible plants, added to a complete artificial diet (chapter 7). Aphids showed a clear aversion to the extract of the resistant plant. This suggests that the resistance is based on the presence of feeding deterrents in the phloem sap of the resistant plant. Hopefully, these substances can be isolated and identified with the help of the bioassay.

SAMENVATTING EN CONCLUSIES

De resistentie van sla tegen de bladluis *Nasonovia ribisnigri* berust op één dominant gen, het Nr-gen. Op de resistente plant sterft de bladluis binnen 4 dagen, er wordt geen honingdauw geproduceerd. Tijdens een verblijf van twee dagen op de resistente plant vindt er geen gewichtsgroei plaats, na terugplaatsing op een vatbare plant is er direct weer "normale" groei (hoofdstuk 2). Er lijkt dus geen sprake te zijn van een giftige stof in de resistente sla. Observaties van (aangelijnde) bladluizen met behulp van Elektrische Penetratie Grammen (EPG) (hoofdstuk 3), en vergelijking met vrijlevende bladluizen (hoofdstuk 4) tonen aan dat de bladluisstiletten ook op de resistente plant wel degelijk penetreren tot in de zeefvaten van het floëem. Op de resistente plant is echter nauwelijks voedselopname (E2 patroon) waarneembaar, en de bladluis sterft uiteindelijk aan ondervoeding, of verlaat de plant (hoofdstuk 4). De resistentie berust dus op een factor in het floëem van de plant. Dit kan een chemische factor zijn, bijvoorbeeld een smaakvergallende stof, of een mechanische factor die opname verhindert. Het onderzoek heeft zich gericht op het vinden van chemische verschillen in het floëem van genetisch identieke (op het resistentie-gen na) resistente en vatbare planten. Amputatie van bladluisstiletten (stylectomy) tijdens de voedselopname levert normaal zeer kleine -maar erg zuivere- floëemsapmonsters. Op de resistente en vatbare planten konden op deze manier geen monsters worden verzameld doordat de uitstroom uit de

stiletstomp al na enige seconden stopte. Via EDTA chelatie en het verzamelen van honingdauw konden wel sapmonsters van een redelijke volume worden verzameld (hoofdstuk 5).

Analyses van deze monsters liet geen reproduceerbare verschillen zien in suikers, aminozuren, eiwitten en UV absorberende stoffen (hoofdstuk 6). Het is echter ondoenlijk om uitputtende analyses uit te voeren, niet alleen omdat het aantal mogelijke inhoudsstoffen enorm groot is maar ook doordat de hoeveelheid en grootte van de monsters beperkt is.

In een biotoets werd aan bladluizen een keuze geboden tussen EDTA verzamelde floëemsapmonsters van vatbare en resistente planten die waren toegevoegd aan kunstmatig dieet (hoofdstuk 7). Hierbij vertoonden de bladluizen een duidelijke afkeer voor het extract van de resistente plant. Hieruit kan geconcludeerd worden dat de resistentie waarschijnlijk berust op de aanwezigheid van een smaakvergallende stof(fen) in het floëemsap van de resistente plant. Mogelijk kunnen deze stoffen met behulp van chemische fractionering in combinatie met de biotoets geïsoleerd en geïdentificeerd worden.

PUBLICATIONS

The following chapters of this dissertation have been published or submitted as journal articles.

Chapter 2: Helden, M. van, Tjallingii, W.F. & Dieleman, F.L. 1993. The resistance of lettuce (*Lactuca sativa* L.) to *Nasonovia ribisnigri* (Mosley, Homoptera, Aphididae): Bionomics of *N. ribisnigri* on near isogenic lettuce lines. Ent. exp. appl. 66: 53-58

Chapter 3: Helden, M. van & Tjallingii, W.F. 1993. Tissue localisation of lettuce resistance to the aphid *N. ribisnigri* using electrical penetration graphs. Ent. exp. appl. 68: 269-278

Chapter 5: Helden, M. van, Tjallingii, W.F. & T.A. van Beek, 1994. Phloem sap collection from lettuce (*Lactuca sativa*): Methodology and yield. J. Chem. Ecol. 20: 3173-3190

Chapter 6: Helden, M. van, Tjallingii, W.F. & T.A. van Beek, 1994. Phloem sap collection from lettuce (*Lactuca sativa*): Chemical comparison among collection methods. J. Chem. Ecol. 20: 3190-3206

Chapter 7: Helden, M. van, Heest, H.P.N.F van, Beek, T.A. van & W.F. Tjallingii, 1995. The development of a bioassay to test phloem sap samples from lettuce for resistance to *Nasonovia ribisnigri* (Homoptera, Aphididae). J. Chem. Ecol. submitted

OTHER PUBLICATIONS

Other publications on related subjects to which the author has contributed:

Helden, M. van, 1990. Resistance of lettuce to the aphid *Nasonovia ribisnigri*. Are Electrical Penetration Graphs (EPGs) helpful to find the origin of resistance? Symp. Biol. Hung 39: 473-474 (short version) and IOBC/WPRS Bulletin 1990 XIII: 101-104

Helden, M. van & W.F. Tjallingii, 1990. Electrical Penetration Graphs of the aphid *Nasonovia ribisnigri* on resistant and susceptible lettuce (*Lactuca sativa*). Proceedings Symposium Aphid-plant interactions: Populations to molecules, August 12-17, 1990. Stillwater Oklahoma U.S.A.

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Reese, J.C., Tjallingii, W.F., Helden, M. van & E. Prado, 1994. Waveform comparison among AC and DC systems for electronic monitoring of aphid feeding behaviour. In: E.A. Backus & G. Walker, Homopteran feeding behaviour: Recent research advances and experimental techniques. Special issues Ent. Soc. Am.: *in press*

NAWOORD

Toen ik in september 1988, niet geremd door al te veel kennis van bladluis-plant interacties, begon met mijn werk, wist ik niet wat voor moeilijkheden mij nog te wachten stonden. Het onderzoeksvoorstel droeg de optimistische titel "De rol van secundaire plantestoffen in de resistentie van sla tegen bladluizen", maar dat bleek niet zo eenvoudig te onderzoeken. Het idee dat een systematische chemische vergelijking van vatbare en resistente planten verschillen aan het licht zou brengen, bleek iets te optimistisch. Er zijn, in samenwerking met de vakgroep Organische Chemie, vele chemische analyses van totaalextracten en floëemmonsters uitgevoerd die slechts lieten zien dat er geen verschil was in inhoudsstoffen tussen resistente en vatbare planten.

Je zou kunnen zeggen dat ik er in die jaren slechts in ben geslaagd om de uitgangspunten van het onderzoeksvoorstel te bevestigen, namelijk dat secundaire plantestoffen inderdaad een rol lijken te spelen. Die voorstelling van zaken zou echter veel te negatief zijn, ik denk dat het onderzoek zeker wel succesvol kan worden genoemd. Via een systematische benadering is een groot aantal mogelijke factoren onderzocht, veelal door een nieuwe combinatie van bestaande technieken. Zo is duidelijk aangetoond dat de bladluis de resistente plant pas afkeurt als de stiletten tot in het floëem zijn doorgedrongen. De ontwikkeling van de biotoets is een doorbraak voor het onderzoek naar bladluis-plant relaties. Via deze toets hebben we aangetoond dat de resistentie zeer waarschijnlijk op een chemische stof in het floëemsap

berust. Met deze biotoets hebben we bovendien ook meteen een instrument in handen gekregen waarmee de isolatie en identificatie van actieve stoffen uit het floëemsap mogelijk moet zijn.

Het proefschrift is dan wel gereed, het echte werk moet dus eigenlijk nog beginnen. Het afgelopen jaar heb ik mij bezig gehouden met het schrijven van dit proefschrift, gecombineerd met enkele niet entomologische hobby's en geduldig wachten op de uitslag van enige beursaanvragen. Onverwacht is het dan toch nog gelukt en in de komende jaren heb ik de mogelijkheid om, via een STW beurs, als post-doc verder te werken aan dit onderzoek. Hopelijk kunnen we het project succesvol voortzetten.

Hierbij wil ik natuurlijk alle mensen die actief betrokken zijn geweest bij dit onderzoek van harte bedanken: mijn begeleiders Freddy Tjallingii (Entomologie) en Teris van Beek (Organische Chemie); mijn promotor Louis Schoonhoven, de andere leden van de denktank: Frans Dieleman, Paul Harrewijn en Kees Reinink; Hanneke van Heest voor haar prima assistentie bij de experimenten en bladluizenkweek, Paul Piron voor de opkweek van de planten; mijn kamergenoten Jan Janssen en Theo Jetten voor hun aan- en afwezigheid; de instrumentenmakerij, fotoafdeling, tekenkamer en alle andere onderdelen van gecombineerde diensten binnenhaven voor hun onmisbare hulp; alle (sla)zaaddonoren en alle overige medewerkers van entomologie en organische chemie. Ik heb al die jaren met veel plezier onderzoek verricht.

CURRICULUM VITAE

In de jaren na zijn geboorte op 24 september 1963 doorliep Maarten van Helden met succes kleuterschool, basisschool en V.W.O. in zijn geboorteplaats Ermelo. Hoewel een beroepskeuzetest hem geschikt achtte voor de Hogere Hotelschool, Hogere Laboratoriumschool, Academie voor Lichamelijke Opvoeding of Fysiotherapie, verkoos hij toch de Landbouwuniversiteit Wageningen, geïnspireerd door vakantiebaantjes bij familieleden in de land- en tuinbouw. In 1981 begon hij zijn studie Planteziektenkunde. In 1985/86 bracht hij 7 maanden door in Ivoorkust (Afrika) met onderzoek naar virusoverdracht door de witte vlieg *Bemisia tabaci* in cassave. Met afstudeervakken in de Entomologie (insektenkunde), Virologie en Theoretische Produktie Ecologie rondde hij in september 1988 zijn studie af. In diezelfde maand was hij toen als Assistent In Opleiding (AIO) begonnen bij de vakgroep Entomologie met het in dit proefschrift beschreven onderzoek naar de achtergrond van de resistentie van sla tegen de bladluis *Nasonovia ribisnigri*, de eerste twee jaren full-time, later als 0,8 deeltijdbaan. Naast zijn werk houdt hij zich bezig met andere hobby's als doe-het-zelven, tuinieren, sporten en terraristiek.

Momenteel is hij, dankzij een NWO/STW beurs, weer terug in het onderzoek met een project dat voort bouwt op de resultaten van het promotieonderzoek. De komende jaren zal hij bij de vakgroepen Entomologie en Organische Chemie proberen om het vraagstuk van de resistentie van sla tegen *Nasonovia ribisnigri* verder te ontrafelen.