Aphid-plant interactions at phloem level, a behavioural study

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Contents

Foreword

Many approaches are used to control and manipulate insect populations to human benefit. They have been successful to a certain extent, but new demands imposed by the society itself press us to find alternative methods, human and environmentally friendly. I am convinced that many of these new techniques can be found through the fundamental research. It is perhaps enough, but with luck, to find a small and weak link in the insect life or behaviour to change its pest status. It is here where the basic and applied research cannot clearly be distinguished. Under this concept, I was interested to initiate studies in insect-plant interactions. Several years passed between my first contact with Wageningen Agricultural University, through Dr. Tjallingii, and the moment to get the appropriate funding to come here. At this point, I want to thank the support given by the Instituto de Investigaciones Agropecuarias (Inia, Chile) which partially founded my studies and permitted me to be absent from the work during this time.

My gratitude to Prof. Louis Schoonhoven for accepting to be my promotor and to Dr. Freddy Tjallingii, my co-promotor and direct supervisor, for all his patient spent in improving the experimental work and manuscripts. I extent my acknowledgement to the staff and colleagues of the Entomology Department where I always felt a good disposition and made me feel "á l'aise". Special thanks to Maarten van Helden for his practical advises and comments to improve this theses and to Hans van den Heuvel for his assistance in virology techniques. I will have many souvenirs of the friendship of the different visitors that shared the laboratory with me. They came, apart from Holland, from the most diverse places: China, Maroc, Polland, Portugal, Spain, USA. They showed me that we are only inhabitants of this earth without differences.

With Dr. Tjallingii we spent many hours together in different parts of the world, so it remains more than a supervisor - supervised relationship. What a patience he had when any of the device did not work and only a simple command was needed to run it. I apologised for such clumsiness.

Thank to the Dean's Office for International Students which facilitated my stay in Wageningen and help me when ever I requested them. I did not need to worry too much about official requirements.

Be sure that the experience acquired here will help me in my research work and, other students (as already does) and colleagues will profit of my staying in Wageningen. Besides science I have gained other knowledge's, as computer use and

bike repairing, but especially the Wageningen style or way of life. Simplicity together with excellence, freedom with respect and tolerance. The foregoing made me to enjoy and love this city with its "gezellig" atmosphere. I made many friends outside my normal activities. To them my gratitude for their support in the daily life and difficult moments.

1. Introduction

Aphid-plant interactions comprise host plant attraction, plant penetration and sap feeding by the aphid, and reactions to these activities by the plant. Plant reactions can affect plant growth, development, and functioning while the effects can be locally, systemically, and even spread to other neighbouring plants. Plant reactions do also have feedback effects on the aphids themselves and may attract natural enemies of aphids. Besides, aphid-plant interactions are involved in the vector function for many plant viruses, using the subtle stylet penetration events in individual plant cells.

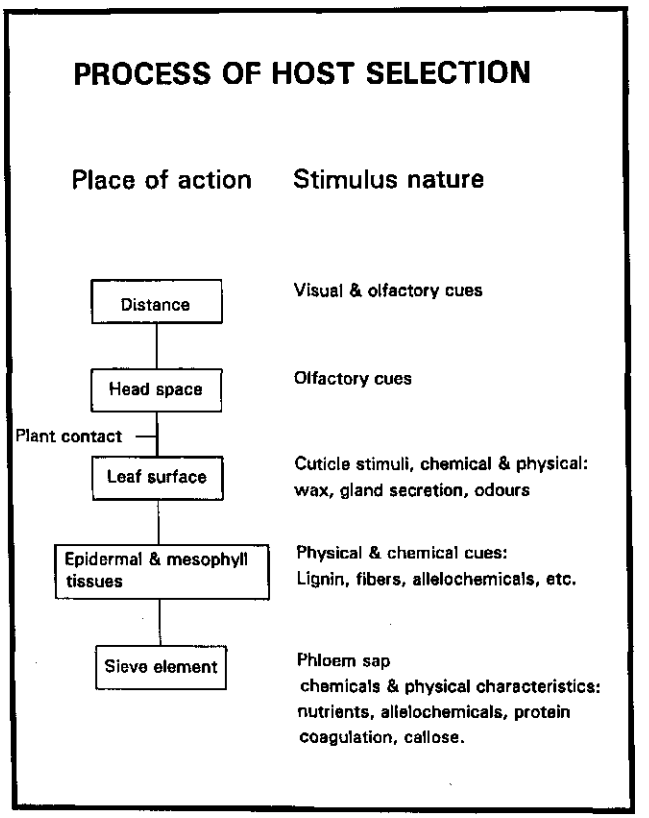
1. ***Host-plant selection***.

Host-plant selection by insects is a chain process of several steps. It starts with orientation activities to find a plant from a distance and it ends with plant acceptance and feeding or with rejection. Aphids often show a complex life cycle with different morphs, some of which show host alternation, i.e. migration to a secondary host for feeding followed by parthenogenetic summer generations, and back to a primary host for sexual reproduction in autumn. Most species have a strong host specificity. In addition, winged and wingless forms coexist during a season (Dixon, 1985). Thus, host-plant selection of aphids shows some special traits in comparison to other insect herbivores (Fig. 1).

Ever since Moericke (1955) colour has been accepted as a major distance factor in aphid attraction to plants and a combination of color and shape has been reported (Åhman *et al.*, 1985; Hodgson & Elbakhiet, 1985; Nottingham *et al.*, 1991). Recently, the discussion on the role of host-plant odours has been renewed on the basis of laboratory studies on aphid olfactory receptors and olfactometer experiments (Pickett *et al.*, 1992; Hardie *et al.*, 1994; Pettersson *et al.*, 1995; Visser & Piron, 1995). However, although responses to sex and alarm pheromones have been demonstrated rather convincingly (Pickett *et al.*, 1992), there are only a few reports on odour attraction (Chapman *et al.*, 1981) or repellence in the field under natural conditions (Campbell *et al.*, 1993; Pettersson, 1996). The role of leaf surface factors, apart from glands (Tingey & Sinden, 1982), remains uncertain in spite of some early references reporting the importance of wax composition (Klingauf, 1971; Klingauf, 1987; Dillwith & Berberet, 1990). As aphids are devoid of external contact chemoreceptors (Tjallingii, 1978b), other than the few hairs on the tip of the antenna, the gustatory organs in the

epipharynx (Wensler & Filshie, 1969) seem to play a major role in the chemical discrimination between plants. Therefore, internal plant factors, encountered during stylet penetration in epidermal, mesophyll, and phloem tissues, are generally considered to be the main cues used by aphids to accept or reject a plant (Pollard, 1973; Montllor, 1991; Pickett *et al.*, 1992; Harrewijn, 1990).

Figure 1. Steps in aphid host-selection.



2. ***Internal plant factors.***

2.1. *Anatomy and plant chemistry.* Contrary to earlier references reporting intra- and intercellular penetration pathways by aphid stylets, the tracks predominantly run intercellularly, although many brief intracellular punctures followed by stylet withdrawal, can often been seen (Tjallingii & Hogen Esch, 1993). The fact that the stylet tracks to the sieve elements are sometimes extensively branched indicates that the length of the stylet is normally not a limiting factor to reach the phloem vessels, which, especially in herbaceous plants, are generally not located very deep in the tissues. Exceptions are young nymphs in some highly pubescent leaves (Carter, 1982). Difficulties to reach the phloem encountered by young nymphs in old stems where phloem depth and tissue fibers increased with plant age, can easily be overcome by moving to more accessible places (Elliott & Hodgson, 1996). Some aphids show preference for primary and secondary veins with a higher density of phloem vessels (Gibson, 1972). The accessibility of phloem seems greater from the abaxial than from the adaxial side of a leaf as the phloem is relatively closer to the abaxial side. However, whether this feature determines the distribution of aphids, remains questionable in accordance to the relation between the stylet lenght and the location of the phloem discussed above. In *Acyrtosiphon pisum* light and gravity are also involved in the choice for the abaxial side of the leaf (Klingauf, 1970). *Aphis fabae* confined to the adaxial side of a *Vicia faba* leaf showed a similar performance as compared to abaxially reared individuals, once they have started feeding. Nevertheless, there is a strong preference to settle on the abaxial side (Prado, unpublished). The spatial distribution of phloem vessels as such can hardly be a constraint to initiate feeding. The selection of a feeding site is determined by numerous other anatomical characteristics and phytochemical tissues properties. One of these which has been reported to affect stylet penetration is the pectin-pectinase interaction (McAllan & Adams, 1961). In one case, *Schizaphis graminum* on sorghum (Campbell & Dreyer, 1990), aphids without the appropriate pectinase appear to be unable to penetrate the pectin of the middle lamella and therefore, either did not accept the plant or penetrated tissues intracellularly. However, it appeared later that the pectin could not be the only resistance factor (Ma *et al.*, 1990) and, moreover, the stylets do not pass through the pectin middle lamella but between the cellulose fibers of the secondary cell walls, thus avoiding any constraint at the middle lamella level (Tjallingii & Hogen Esch, 1993). Nevertheless, physico-chemical properties of tissues seem to account for delayed reaching of the phloem and sieve element acceptance (Gabrys *et al.*, 1997; Lei *et al.*, 1997), but the nature of the delaying factors has so far not been identified.

2.2. *Secondary metabolites.* Secondary metabolites or allelochemicals, e.g. alkaloids, phenolics, glucosinolates, hydroxamic acids, are well-known mediators of behavioural responses in aphids (Niemeyer, 1990). A wide variety of such compounds has been reported to act as deterrent, or phagostimulant (Schoonhoven & Derksen-Koppers, 1976), or to cause antibiotic effects (Pollard, 1973; Montllor, 1991). Before reaching the phloem, aphids and other phloem feeding insects encounter these allelochemicals only if they occur in the tissue compartments that are sampled. Many of them are predominately stored in the cell vacuoles in a reduced toxic form, i.e. as a glycoside or amide (Matile, 1984). Aphids do suck up small samples, presumably from the cytoplasm and vacuole, during brief (5-10 sec.) intracellular punctures (Powell *et al.*, 1995; Martin *et al.*, 1997) of most cells along the stylet track (Tjallingii & Hogen Esch, 1993). Phenolic compounds mainly occur in the cell walls and their influence on aphid behaviour remains unknown (Montllor, 1991; Rahbé *et al.*, 1997) as well as their function as feeding deterrent (Miles, 1985; Leszczynski *et al.*, 1985). Although no experimental data are reported on sampling of intercellular fluids, it is widely assumed that it does occur. The sampled fluids from intra- and intercellular origin can both be tasted by the epipharyngeal gustatory organ.

From the aphid side a protective function of polyphenoloxidases in the saliva against intoxication by ingested phenols has been proposed (Miles, 1965; 1993). Miles' conceptually attractive "redox hypothesis", assumes that the plant's phenolic compounds, released as a wound reaction, are detoxified by salivary enzymes (polyphenoloxidases). Also, the idea is tempting that along the intercellular route of the stylets no apparent damage is caused to the encountered cells, which is in itself a mechanism to avoid activation (mixing separately compartmentised contents) of the chemical plant defences during probing. Nevertheless, the aphid reaction to plant defences is still questionable as only small amounts of sap are ingested (some reports suggested that they are egested, e.g. Harris, 1977) so that there is probably no real threat of intoxication.

Apart from these toxic aspects, the main role of allelochemicals in non-phloem tissue is thought to be their value as token stimuli (Kennedy *et al.*, 1959; Pickett *et al.*, 1992, Cole, 1997).

2.3. *Phloem factors.* When the stylets reach a sieve element another stimulus situation is met. Saliva secretion has been identified now as the first aphid activity immediately after stylet insertion into a phloem vessel, followed by sap ingestion, generally after some minutes (Chapter 2; Prado & Tjallingii, 1994). Presumably

salivary enzymes are injected, counteracting sieve element reactions as phloem protein gelation, callose deposition around the stylet, and plastid inclusions which have been suggested to function as fast wound reactions and to seal individual sieve elements (Dixon, 1975; Walsh & Melaragno, 1981; Evert, 1990; Tjallingii & Hogen Esch, 1993). During sap ingestion the sap composition, e.g. sugars and amino acids concentrations, is also involved in host-plant acceptance affecting ingestion and aphid performance (Dixon, 1985; Rahbé *et al.*, 1997). The presence of secondary compounds in the phloem sap should also not be overlooked (Wink *et al.*, 1982). Detoxification capability in aphids may be of greater importance here than in other tissues. Phloem constraints may be reflected in longer sieve element salivation previous to sap ingestion (Chapter 5; Caillaud *et al.*, 1995) and generally, in shorter sap ingestion (Therasa *et al.*, 1996; van Helden & Tjallingii, 1993).

3. *Dynamical aspects of plant-aphid interaction.*

3.1. *Plant development and age.* The plant as a food source for aphids is not static but changes continuously in space and time. During plant development the tissue quality fluctuates substantially. Young leaves and stems are especially attractive and nutritious for aphids (Kennedy & Booth, 1951) as high amounts of free amino acids are produced and loaded into the phloem. Also, in senescent leaves amino acids are mobilised by breakdown of proteins. On herbaceous plants *Aphis fabae* and *Myzus persicae* highly prefer young growing and old senescing leaves above mature leaves (Kennedy *et al.*, 1950). In trees, for example in *Acer pseudoplatanus,* the content of free amino acids is much lower in mature leaves during summer time than in spring and autumn when leaves are growing and senescing, respectively (Dixon, 1985; Douglas, 1993). The aphid *Drepanosiphum platanoidis* and other aphid species on *Acer* have adapted to this situation by forming summer forms in which development and reproduction is interrupted. Extensive work has been done on artificial diets in order to determine the importance of amino acids and sugars for optimal growth and reproduction of aphids (Mittler & Dadd, 1964, 1965; Harrewijn & Noordink, 1971; Rahbé *et al.*, 1997). Secondary compounds, such as DIMBOA and glucosinolates, are very concentrated in young plants and growing leaves. Levels decrease with age, and are thought to be unevenly distributed between veins (Niemeyer, 1990; Gabrys *et al.*, 1997). Not much is known about phloem concentrations of allelochemicals. Aphid preference for phloem tissues may be due to the fact that allelochemicals do mainly occur in non-phloem tissues. It has also been suggested that specialists using secondary

plant compounds for host-plant recognition and, detoxifying these compounds, prefer young plant parts, whereas generalists would not be able to detoxify (Nault & Styer, 1972).

3.2 *Induced plant reactions.* Any damage to plants, including damage caused by insect herbivores, may induce plant reactions in which metabolic and/or morphological changes are involved. Some plant reactions are very general, but others have been related to specific organisms and to specific chemicals they release in or on the plant. In some cases of infestation by aphid species the impact of the plant's reactions, is worse for the plant than the actual damage caused by probing and feeding itself. *Schizaphis graminum* and *Diuraphis noxia* can "knock down" plants completely. Many of these reactions have feedback effects to the herbivores. Obviously knocked down plants can no longer serve as a food source, but also weaker deleterious responses have been reported which reduce the suitability of the plant as a food plant. Aphids, like other insect herbivores, and many other plant feeding organisms and pathogens, can induce resistance (Wool & Hales, 1996) by eliciting an increased level of secondary metabolites (Pickett *et al.*, 1992). However, some of the induced effects can be beneficial to the inducing organism, like the galls induced in many plants by a variety of insect herbivores, and which provide the inducing insects good feeding sites as well as physical protection. Other plant reactions also point to increased susceptibility, just the opposite of induced resistance (Prado & Tjallingii, 1997; Chapter 3). Recently, some studies have shown that plants may react to aphid infestations by releasing volatiles, behaviourally affecting the feeding activity of the insect itself and attracting aphid parasitoids in olfactometer experiments (Du *et al.*, 1996), similar to what has been found for mites and caterpillars (Takabayashi *et al.*, 1994; Geervliet *et al.*, 1994). Methyl salicylate, a metabolic derivate of salicylic acid, is one of the volatiles involved. It is present in the biochemical pathways that have been related to other cases of induced resistance. In aphids, methyl salicylate was found to repel *A. fabae* (Hardie *et al.*, 1994), *Phorodon humuli* (Campbell *et al.*, 1993) and cereal aphids (Pettersson *et al.*, 1994). Also, induced deterrence in undamaged cereal plants (several species) by volatiles from aphid infested plants was found in laboratory experiments, but the effect decreased when plants were exposed for a longer period (Pettersson *et al.*, 1996). An intriguing question is how aphids, which mostly live in dense colonies, overcome induced deterrence of the plant they feed upon, especially when the plant responses increase with the infesting population, as has been shown for methyl salicylate (Campbell *et al.*, 1993). However, *A. fabae* showed on previously colonised leaves the

opposite reaction, i.e. enhanced feeding (Chapter 3, Prado & Tjallingii, 1997). Similarly, beneficial effects have been shown for *S. graminum* on leaves infested by *D. noxia*, but earlier *S. graminum* infestation had no effect (Formusoh *et al.*, 1992). Pettersson *et al.* (1996) suggest that initially detrimental effects are followed by beneficial effects. Surely, the direct effects of volatiles (from metyl salicylate to terpenes, aldehydes, ethylene, etc., Harrewijn *et al.*, 1993; Hildebrand *et al.*, 1993; Quiroz *et al.*, 1996) on aphid behaviour need not to be similar for each volatile and the induced effects acting via the plant's metabolism need not to have the same impact on the aphid as the direct volatile effect. Some of them may act as attractants and others as repellents. Additionally, the effects may change in time and may differ from one plant tissue to another. As far as induced effects by aphids are concerned, in general, the saliva has been suggested as a elicitor (van Helden & Tjallingii, 1993). However, experimental evidence for the existence of specific salivary components is limited. Also, the role of the phloem salivation or salivation into other tissues or cells in the aphid-plant interaction remains unknown. The whole field of this relationship is rather new and forms a challenge for further studies, especially with respect to the causes of induction and feedback effects. Aphids are surely faced with a wide range of environmental stimuli, but the importance of each should be evaluated under natural conditions.

3.3. *Aphid infestation.* As aphids usually live in aggregates or colonies, this apparently represents some advantage. Several hypotheses have been proposed to explain the benefits of aggregation behaviour for aphids (Table 1).

Disadvantages also exist mainly through density dependent predators, parasitoids and diseases, as well as in terms of nutrient competition especially within overcrowded colonies, but also between colonies (Inbar *et al.*, 1995). The following causes have been proposed as responsible for or to stimulate aggregation: (a) aggregation pheromone (Kay, 1976; Pettersson, 1994); (b) visual and tactile stimuli (Ibbotson & Kennedy, 1959; Ibbotson, 1966; Dixon & Logan, 1972; Hayamizu, 1982); (c) better food quality at the place of the colony, by simple choice of an intrinsically better place to feed, or by inducing a better sap quality (Ibbotson & Kennedy, 1951; Way & Cammell, 1970; Way & Banks, 1967; Dixon & Wratten 1971); (d) host volatiles released after injury (Campbell *et al.*, 1993). However, as reproducing females with their offspring naturally form aggregates, their maintenance can be seen as a lack of dispersion, rather than the result of an active aggregation stimulus.

Apart from an eventual advantage with respect to natural enemies, the question arises how colony life affects aphid-plant interactions. Beneficial changes in plant metabolism, systemically spread, might need many individual aphids acting together. A variety of changes in plant metabolism due to aphid infestation has been reported, from higher photosynthesis (Vereijken, 1979), to early senescence and increased ethylene production (Berberet *et al.*, 1990; Castro *et al.*, 1996). Senescence also seems to improve the suitability of tissue by the breakdown of proteins in amino acids, ready to be assimilated by the aphids (Dixon, 1975). Other hypotheses for changes in suitability come form earlier studies on stylet penetration by individual aphids on non-infested plants. Surprisingly long probing times were shown before aphids started feeding from sieve elements (Tjallingii & Mayoral, 1992) and complicated stylet tracks showed that many sieve elements in a vascular bundle had been punctured before sap feeding eventually started from one of them (Tjallingii & Hogen Esch, 1993).

Table 1. Reported benefits for aphids by living in colonies

Benefits	**References**
Facilitates honeydew collection by ants	Dixon, 1985
Reduces the chances of captures by predators or parasitoids	Kidd, 1982; Völkl & Stadler, 1996
Preserves the host by not killing the whole plant preserving other plant parts healthy	Way & Cammell, 1970
Facilitates mating of oviparous females by sex pheromone concentration	Pettersson, 1993
Improves food quality and availability	Ibbotson & Kennedy, 1951; Way & Cammell, 1970; Way & Banks, 1967; Dixon & Wratten, 1971
Produces a 'sink' effect, attracting more nutrients to the site of the colony	Way & Cammell, 1970

Analysis of stylet penetration in phloem sieve elements suggested two different activities on basis of electrical penetration graphs (EPGs). One activity, recorded as waveform E2 (Table 3; Tjallingii, 1989), is known to reflect sap ingestion from sieve elements and concurrent salivation. According to the results of Chapter 2 (Prado & Tjallingii, 1994) another waveform, labelled as E1, reflects saliva injection into the sieve element, occurring previous to any sap ingestion. The function of this saliva injection is still unknown but as it occurs before ingestion, it seems that its occurrence forms a condition without which no sap feeding is possible. One suggestion is that it counteracts primary wound reactions in the sieve elements, such as coagulation of phloem proteins (Tjallingii & Hogen Esch, 1993). Similarly, saliva may slow down the defence or wound reactions of the sieve elements, like the formation of callose. Whatever it may be, the question can be translated into susceptibility and resistance terms: Would longer or repeated short periods of this activity (waveform E1), without a switch to continuous sap feeding, indicate some resistance mechanism, for example, an inability to suppress a sieve element reaction or property? Data with respect to phloem salivation (E1) from different aphid-plant combinations, some of which were reported to have resistance at the phloem level, have recently become available (Table 2). In relation to the present study (Chapter 3, Prado & Tjallingii, 1997) a next question was: If a single aphid is not, or only with difficulty, able to suppress a sieve element reaction or property, would the presence of an aphid colony on a leaf have a greater possibility for such suppression?

3.4. *Phloem salivation and aphid-plant relationship.* The role of phloem salivation (saliva injection into a sieve element) is rather unknown. Nevertheless, a number of empirical relations involving different aphid-host combinations have been published recently, and salivation duration in various combinations can be compared to each other (Table 2). From the data presented in Table 2 and from the foregoing paragraph it will be clear that the total duration of phloem salivation (summed E1 periods), probably is a rather crude parameter. The duration of sieve element salivation before feeding seems specific for each aphid-plant combination. *A. fabae*, supposed to be well adapted to its host *V. faba*, showed a much longer phloem salivation than other good aphid-host plant combinations. Furthermore, in some cases where host-plant resistance seems to be phloem located, phloem salivation (E1) appeared to last longer (Table 2) than in susceptible plants. However, much care should be taken to use this prolonged phloem salivation (E1) as a reliable indicator for plant resistance. Short phloem salivation might also be interpreted as a consequence of difficulties to start feeding. Phloem salivation

prior to a period of successful sap ingestion would be a better parameter to estimate sieve element constraints.

3.5. *Virus transmission.* The study of aphid probing at the cellular level may reveal the details of many events in aphid-plant interactions that are involved in the transmission of plant viruses. Phloem restricted plant viruses (luteoviruses) need to be ingested from the phloem cells, and added to the saliva, i.e. virus acquisition by the aphid (Sylvester, 1980). Apparently, a shorter plant access time is required for the inoculation (minimum inoculation access period, IAP) than for the acquisition (AAP) of these viruses as found in some aphids (Granados, 1969) and whiteflies (Costa, 1969). The virus circulates in the aphid vector and, therefore, it needs to be ingested first from where it occurs in the plant, the phloem sieve elements. Thus, we might expect a close relationship between the E2 waveforms in EPGs and virus acquisition, which indeed could be experimentally confirmed (Chapter 2). The amount of acquired virus increases with feeding (E2) time is in agreement with AAP studies showing increased acquisition with longer access to the plant (Gray *et al.*, 1991). It was found as well that inoculation was related to E1 occurrence, strongly suggesting that this waveform reflects saliva injection into the sieve element before sap feeding (E2) starts. Waveform E2 is always preceded by E1 in a phloem phase period and the stylet tips are staying in the same sieve element (Tjallingii, 1990; Tjallingii & Hogen Esch, 1993). Thus, inoculation of the persistent virus occurs during the first period of a phloem phase, when the aphid salivates, and acquisition takes place in the second period during sap ingestion. However, in the latter (E2) activity there is also evidence for concurrent salivation by the aphid. The present hypothesis (Chapter 2) states that this E2 saliva, presumably also containing virions, does not reach the sieve element but is ingested together with the phloem sap through the food canal. Under this scenario, prolonged E1 salivation, for example in resistant cultivars (Table 2), would increase the primary infection by viruliferous migrants, but a reduced secondary virus spread could be expected due to less virus acquisition and reduced aphid performance. As a matter of fact, resistance factors against aphids located before the phloem, in epidermis and mesophyll cells, would provide a better protection against persistent virus infection thus preventing phloem access. Irrespective of resistance location, which is in most cases still unknown, cultivars resistant to aphids have shown lower levels of persistent virus infection under field conditons (Syller, 1996).

Table 2. Mean durations of individual E1 occurrence (phloem salivation) in some aphid-host plant combination. (S) = susceptible plant, (R) = resistant plant

Aphid-plant combination	**Mean duration of salivation periods (E1) (min). Standard error in brackets**
Sitobion avenae x *Triticum aestivum* cv Arminda (S) *S. avenae* x *T. monococcum* line TM 44 (R) (*)	9.9 (6.5) 11 (21)
Rhopalosiphum padi x *Triticum aestivum* cv Okapi (S) (**)	2.8 (0.6)
Rhopalosiphum padi x *Hordeum vulgare* cv Agrar (S) (**)	4.7 (1.3)
Brevicoryne brassicae x *Sinapis alba* (S) (***)	1.4 (0.2)
Nasonovia ribisnigri x *Lactuca sativa* line Nr S (S) *N. ribisnigri* x *L. sativa* line Nr R (R) (****)	3.5 (0.8) 10.4 (7.5)
Phorodon humili x *Humulus lupulus*, mean of susceptible cvs (S) *P. humili* x *H. lupulus*, mean of resistant cvs (R) (*****)	29.8 (11.6) 77.9 (31.1)
Aphis fabae x *Vicia faba* cv 3 x wit (S) (**)	14.0 (4.0)

(*) Caillaud *et al.*, 1995; (**) Prado, unpubl.; (***) Gabrys (pers. comm.); (****) van Helden & Tjallingii, 1993; (*****) Therasa *et al.*, 1996.

4. ***Methods and methodology in aphid-plant interaction studies.***

4.1. *Electrical penetration graphs recording (EPG).* In experimental approaches of aphid-plant interactions the electrical penetration graph technique (EPG) has contributed substantially to our present knowledge of stylet penetration events in the

plant tissues. Traditional techniques to study tissues include optical and electron microscopy, both giving a rather static image of what happens in the depth of the tissues. Electrical recording of stylet penetration by aphids started with McLean & Kinsey (1964, 1965; AC system) and was further developed by Tjallingii (1978a, 1988; DC system). By attaching an electrode (very thin flexible wire) to the dorsum of the aphid and inserting a rod electrode in the potting soil of the plant, aphid and plant are made part of an electrical circuit with an amplifier. During probing the system can record electrical waveforms which have been correlated to the stylet position in the plant tissue as well as to aphid activities (Tjallingii, 1985).

So far 8 different waveforms have been identified in the electrical penetration graph (EPG) and experimentally correlated as given in Table 3, which also contains new correlations found in the present study.

The identification and understanding of EPG waveforms and patterns in stylet penetration will contribute to a better comprehension of aphid probing behaviour. Many of the aphid-plant interactions take place at the level of plant tissue and cells which will apparently be reflected in the EPG.

4.2. Phloem-phase studies. As aphids are phloem feeders, acceptance of phloem sap is the ultimate and crucial result of food plant selection. The two phloem related waveforms, E1 and E2 (Fig. 1, Chapter 2; Tjallingii, 1990), have already been introduced in the foregoing. When stylets were amputated (stylectomy) during E2 the stylet's stump in the leaf yielded sap immediately (Mentink *et al.*, 1984; Kimmins & Tjallingii, 1985). Micrographs (electron microscopy) of these amputated stylets showed their tips being inserted into a sieve element (Tjallingii & Hogen Esch, 1993). Waveform E2 can be sustained for very long periods (days) coinciding with honeydew excretion. Thus it has been inferred that the E2 waveform reflects phloem sap ingestion. The E2 waveform has two frequency components which seem rather independent from one another, i.e. a 4-8 Hz wave shaped component at low amplitude, and a 0.5-2 Hz peak shaped and larger component. The latter component coincides with muscle contraction (electromyograms) of the salivary pump, thus suggesting salivation instead of ingestion. However, no saliva injection during these E2 periods could be detected using radio labelled aphids (Tjallingii, 1978a). This paradox was solved when a forgotten anatomical aspect showed that salivation and ingestion could occur without any saliva being ejected. The two canals in the stylets, i.e. the salivary and food canal,

Table 3. Waveform features and correlations on EPG. Amplitude (minimum and maximum) relative to waveform A (=100 %); repetition rate of peaks or waves in Hertz; voltage level as extracellular (e) or intracellular (i); and the main electrical origin as resistance (R) or electromotive force (emf) (after Tjallingii, 1996)

EPG waveform	Features				Correlations	
	rel. amplit.	rep. rate	volt. level	el. orig.	Plant tissue	Aphid activity
A	100	5-10	e	R	epidermis	electrical stylet contacts, on/off
B	75	0.2-0.3	e	R	epidermis/mesophyll	sheath salivation
C	30	0.2-0.3	e	R	all tissues	activities during stylet pathway
pd	-	0.02	i	emf	any living cell	intracellular puncture
E1	-	2-4	i	emf	sieve elements	sieve element salivation
E1e	-	2-4	e	emf	extracellular	puzzling E1 incident
E2 p	5	0.5-4	i	R	sieve elements	watery salivation
w	-	4-7	i	emf	sieve elements	(passive) ingestion
F	5	11-18	e	R emf	all tissues	mechanical stylet work
G w	0-60	4-6	e	emf	xylem	(active) ingestion
p		1-6	e	R	xylem	unknown subactivity

p=peaks; w= waves.

are in fact fused near the stylet's tips (Forbes, 1969; Fig. 3, Chapter 2), so that during ingestion the excreted saliva is immediately ingested instead of injected into the plant. The phloem sap pressure will drive sap and saliva in the common canal lumen into the food canal. Thus, the two components in the E2 waveform could reflect the concurrent salivation and ingestion. Since the E2 peaks reflect salivary pump activity, the low amplitude waves likely reflect ingestion activity (Tjallingii, 1995).

The processes which occur during the waveform E1 are much less clear. Only one waveform component seems present, a sawtooth-like peak with a steep positive leading edge and a slow return to the base level (Fig. 1, Chapter 2). Every phloem phase always starts with E1, which may change to E2 after about one minute, but sometimes sooner or later depending on both plant and aphid species. Waveform E1 has been correlated with sieve element salivation in this study, as described in Chapter 2. Nevertheless, its biological significance is not yet clear. It is tempting, as mentioned in the foregoing, to think about suppression of any sieve element reaction or property. There are two aspects, however, which need further clarification with respect to the occurrence of E1 waveforms. The first is that, though it is often easy to distinguish between the two phloem phase waveforms, E1 and E2, there occur mixtures between the two or, sometimes, characteristics are missing (e.g. either the peaks or the waves of E2, see Table 3) or masked by noise (sometimes behavioural, i.e. waveform "noise" rather than electrical noise, such as 50 Hz). In these cases it is difficult to decide when E1 stops and E2 starts, especially, when periods of E1 and E2 are alternating during long phloem phases. A good criterion to decide what waveform, i.e. activity, is actually displayed in these cases is missing, unfortunately, and this situation needs further studies and new approaches. The other aspect is the choice of an useful parameter with respect to phloem salivation (E1) occurrence. An attempt to clarify this situation is made in Chapter 3 and the following ones.

4.3. *Variation.* Variability of behaviour is intrinsic and well known in plant penetration data from aphids as reflected in EPG results. On one side, variation can result in the uncomfortable situation that mean values may suggest distinct "numerical" differences between plants or treatments without being supported by statistical significance, which is caused by a few individuals behaving exceptionally. On the other hand, the opposite can occur, isolated cases of statistically significant difference within a data set, where no other supporting differences are found between comparable treatments. Both situations lead to confusion. In spite of the fact that the aphids mostly originate from one clone (parthenogenesis), are reared under uniform circumstances

and on plants grown under identical conditions, these experimental conditions still allow a tremendous variation in results. Plants, plant parts (stems, leaves, flowers, etc.) and within these, probing sites (e.g., leaf vein vs. lamina, abaxial vs. adaxial side) can be very different. Even some indications point to differences between individual sieve elements, as will be discussed in later chapters of this study.

Outliers can be eliminated, transformations applied and other levels of confidence can be chosen. Repeating the experiments would be adequate but when intrinsic variability cannot be eliminated, very likely, the same phenomenon will appear again with other parameters.

The statistical and the biological causes for the occurrence of statistical significance (or not), therefore, both suggest that it is wise not to look only for statistical significance, but also to consider numerical differences.

4.4. *Experimental interferences.* Interferences of experimental methods with the phenomena studied is a very general methodological problem, which is also important in aphid-plant interaction studies. Mostly, aphids are transferred from their original rearing plant to a new test plant. This implies that the insects may become stressed by manipulating them, a temporal deprivation of food, and by the experimental circumstances which are different from their original feeding situation. The plant (species, cultivar, age, etc.), the place on the plant, and locomotion restrictions (confining them in clip cages or by wire attachment) of the test situation can all influence the behaviour studied, and thus the results and the conclusions of the experiments. This is certainly true for the EPG and honeydew recordings, which were used in the experimental work reported here. Honeydew production recorded by a "honeydew clock" (Banks & Macauley, 1964) gives a clear reflection of the phloem sap ingestion rate and is very useful for studying phloem acceptance, although, calculation of ingestion on basis of honeydew excretion has a number of calibration pitfalls. Apart from clear advantages of the techniques and experimental procedures used, therefore, their drawbacks should not be overlooked. Some of them have been studied here in order to better evaluate the results obtained (Chapter 4).

5. *Scope and contents of this study.*

The primary aim of this work was to assess aphid activity during waveform E1 (Chapter 2). The finding that E1 is correlated with salivation into sieve elements opened the possibility to test indirectly the hypothesis that sieve element salivation is needed to suppress sieve element wound reactions (e.g. p-protein gelation, callose formation, etc.)

or other sieve element properties (Chapter 3). Previous colonisation of a leaf might enhance such a suppression or improve sap composition so that individuals would be able to accept sieve elements earlier for sap feeding, which was found earlier to take an (unexpectedly) long time (Tjallingii & Hogen Esch, 1993). As the results were not unambiguously supporting the hypothesis a need was felt to evaluate the experimental conditions, especially with respect to phloem activities (Chapter 4). Finally, a well known case of extended sieve element salivation, found by Caillaud *et al.* (1995), was used in order to investigate the suggested plant resistance relation with E1, as well as some intrinsic difficulties in the E1-E2 waveform distinction (Chapter 5).

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2. *Aphid activities during sieve element punctures* [1]

ABSTRACT

Aphid salivation in sieve elements and phloem sap ingestion were linked to waveforms in the Electrical Penetration Graph (EPG). Non-viruliferous *Rhopalosiphum padi* (L.) (Hemiptera, Aphididae) on barley yellow dwarf virus (BYDV) infected wheat could acquire the virus, which was used as an indication for phloem sap ingestion, whereas virus inoculation by viruliferous aphids on healthy plants was associated with salivation in sieve elements or other phloem cells, so far virus particles are present in the aphid's saliva. Probing was monitored and the waveforms recorded were related to the infection of test plants. The EPG patterns A, B, and C are indicative of the stylet pathway phase, whereas patterns E1 and E2 reflect the phloem (sieve element) phase with an unknown activity (E1) or with ingestion and concurrent salivation (E2).

Aphids showing stylet pathway and E1 rarely acquired virus, suggesting that little or no phloem sap ingestion can occur during these patterns, whereas those showing additionally pattern E2 did so substantially, indicating phloem sap ingestion. The main pattern related to virus inoculation was E1, although some aphids were able to inoculate plants during pathway. Pattern E1 clearly reflects the most important salivation into sieve elements. Pattern E2 had no clear contribution to virus inoculation, supporting the present hypothesis that during this pattern the saliva is mixed with the phloem sap in the single canal at the stylet tips and ingested immediately, without reaching the plant tissue. Sustained sap ingestion did not affect virus inoculation. So, BYDV inoculation mainly occurs during the first period of a sieve element puncture which is always formed by E1. Implications on persistent virus transmission are discussed.

INTRODUCTION

Host plant selection by aphids is considered a sequence of several steps including chemo-perception (taste) of plant tissues during stylet penetration (probing) (Klingauf, 1987). In

[1] This chapter has been published in a slighty modified version as: Prado, E. & W. F. Tjallingii, 1994. Aphid activities during sieve element punctures. Entomologia Experimentalis et Applicata 72: 157-165.

this way the aphid can locate possible plant resistance factors at the successive levels of the tissues encountered from the epidermis to the phloem (Harrewijn, 1990; Niemeyer, 1990). Nevertheless, the phloem seems to play a major role, at least in the few documented cases available (Montllor, 1991; van Helden & Tjallingii, 1993).

Aphid activities and stylet tips positions during probing can be monitored electrically by the Electrical Penetration Graph (EPG) technique. To date, seven different waveform patterns (labelled A to G) have been characterized in the aphid EPG (Tjallingii, 1978; 1988), each by specific features. The pathway phase in a probe, identified as patterns A, B, and C, forms the main activity before reaching the sieve elements in the phloem (Spiller *et al.*, 1985; Kimmins, 1986; Tjallingii & Hogen Esch, 1993). During the pathway phase the salivary sheath is formed and also, many brief (5-10s) intracellular punctures are made, recognized in the EPG as potential drops (pd's). The sieve element phase is formed by two waveforms, E1 and E2, both showing an intracellular stylet tip position in a sieve element (Tjallingii, 1990). Pattern E1 is often, but not necessarily, followed by E2 inside the same sieve element. Reversely, E2 is always preceded by E1 of different durations. Though the E2 pattern activities are rather well known, being passive sap ingestion and concurrent secretion of (watery) saliva, the E1 pattern has been only characterized and described but not correlated with particular aphid activities as yet.

Luteoviruses, like barley yellow dwarf virus (BYDV), are restricted to phloem cells, presumably to sieve elements, companion cells and phloem parenchyma cells, which are interconnected by plasmodesmata. Aphids persistently transmit these viruses in a circulative way, i.e. the virus is ingested with phloem sap of an infected plant, passes through the digestive tract and across the hindgut epithelium into the haemolymph. Then it is transferred to the accessory salivary gland, from which it can be injected with the saliva into a healthy plant (Sylvester, 1980; Gildow, 1990). For this reason BYDV seems a good marker for studying aphid salivation into, and phloem sap ingestion from sieve elements. The complication that other phloem cell types are possibly involved in virus transmission is compensated by the unambiguous relationship between the E patterns and the sieve element location of the stylet tips (Kimmins & Tjallingii, 1985; Tjallingii & Hogen Esch, 1993).

The aim of this study is primarily to elucidate relations between E1 and aphid activity. Using BYDV as a marker we may be able to gain information on virus transmission in due time.

MATERIALS AND METHODS

Aphids. The aphids came from a stock culture of non-viruliferous *Rhopalosiphum padi* (L.), reared in the greenhouse at 20 ± 2 °C and L16:D8 photoperiod on wheat (*Triticum aestivum* L.), cv Okapi. Apterous virginoparous adults were used three to six days after the last moult. Viruliferous aphids were obtained by placing just moulted adults in small individual clip-on cages on BYDV-infected wheat for 48-72 hours. In order to check their ability to transmit the virus, 17 aphids were given a one week inoculation access period (IAP).

Plants. Test plants were 10-12 days old wheat seedlings (two leaves), cv Okapi, grown in the greenhouse at 23 ± 2 °C.

Virus. Virus source plants, BYDV-infected wheat, were obtained after three successive transmission steps, using *R. padi* as vector, from field collected infected wheat at Wageningen. ELISA (enzyme-linked immunosorbent assay) of these original plants showed that these plants were infected with the BYDV isolate PAV.

The presence of viral antigen in plants was detected by means of an indirect TAS-ELISA (Converse & Martin, 1990), in which a polyclonal antibody to PAV (MAFF, Rothamsted, U.K.) was used to trap, and the monoclonal antibody WAU-A7 (Heuvel *et al.*, 1990) to detect the virus. Each leaf sample was tested twice.

The EPG technique (DC method; Tjallingii, 1978) was used to monitor aphid feeding behaviour. A 2 cm gold wire (diameter = 20 µm) attached to the dorsum of the aphid by conductive silver paint (Demetron, type L2027) was connected to the amplifier (1 Giga-Ohm input resistance and a gain of 50 x; Tjallingii, 1985a; 1988). The other electrode was put in the soil of the potted plant. The whole set-up was placed in a Faraday cage. All signals were recorded on a PC hard disk and analyzed by Stylet 2.0 software (Tjallingii & Mayoral, 1992). For analysis, the stylet pathway phase (Fig. 1, patterns A, B and C; Tjallingii, 1988) and patterns E1, E2 representing the sieve element phase (Tjallingii, 1990) were distinguished. About one hour was used for getting the aphids from the culture or pre-treatment plants, wiring them, and giving them access to the plant.

Waveform analysis used four categories of aphids: those that had shown (1) pathway phase only, (2) pathway and E1, (3) pathway, E1, and E2 (<10 min), and (4) pathway,

E1, and E2>10 min. All aphids that reached E2 had shown E1 and the pathway phase as well. Durations of the pathway phase, E1, and E2 were also derived.

The access periods to plants were standarized to 2 hours. However, a number of aphids was monitored longer than 2 hours, up to 8 hours maximally. Longer monitoring was needed in order to get sufficient numbers of replicas of each category, especially of aphids reaching E1 only since E1 usually occurs for a short period immediately followed by E2. Thus aphids for the categories 'pathway + E1' and 'pathway + E1+ E2' were lifted during the E1 or E2 pattern, also, in order to get different durations inside the categories already defined.

Virus acquisition experiment. Virus free individuals of *R. padi* were given access to two months old BYDV infected plants while EPGs were recorded. Afterwards the aphids were gently lifted off the plants, the gold wire was cut near the body and the aphids were immediately transferred to test plants. They stayed on seedlings during 7-10 days under greenhouse conditions, using individual plant cages to avoid contamination. After this period the plants were fumigated with DDVP to kill the aphids and the cages were removed. To keep the plants free of aphids the DDVP treatment was repeated every 10-12 days. A total of 117 aphids were tested and only one aphid per test plant was used. ELISA tests on these test plants were carried out approximately three weeks after transferring the aphids.

Virus inoculation experiment. Viruliferous individuals of *R. padi* were given access to healthy seedlings and their EPGs were recorded. In total 140 aphids were tested for inoculation using only one individual per test plant. After recording the probing behaviour, the aphids were removed, the plants placed in the greenhouse and tested three weeks later with ELISA.

Statistics. The relation between the different patterns and the result of the ELISA test (positive or negative), was analyzed by means of the chi square test ($P<0.05$). The ELISA results were related to several covariates by GLIM (Generalized Linear Interactive Modelling; Aitkin *et al.*, 1990). Because the dependent variate was a negative or positive reading in the ELISA test, the error distribution was chosen binomial (with binomial distribution 1). The link function, linking the scale of measurement (0-1) to the linear scale, where the covariates were supposed to act on, was the logit. In this logistic regression the following covariates were studied per aphid: probing time (excluded non-probing), pathway time, numbers and durations of E1 & E2, and the number of

recognized phloem sieve element punctures (any E). Eventually only the logarithm of E1 and of E2 duration (log x+1) were left in the model because all others did not contribute significantly in any combination or sequence. The resulting model was:

$$\text{logit}(p) = \log(p/1-p) = \beta o + \beta 1 * \log(E1d+1) + \beta 2 * \log(E2d+1)$$

with p the probability of virus acquisition or inoculation; E1d= E1 duration, E2d= E2 duration; ßo= probability of plant infection without the presence of any explanatory variable; ß1= slope of the log (E1d+1) line, and ß2= slope of the log (E2d+1) line.

Testing for significant contribution of covariates in GLIM models acts through comparisons of nested models, resulting in changes of deviance, which have chi square distributions (under the null hypothesis).

RESULTS

Since virus acquisition and virus inoculation do not necessarily result in virus transmission to the test plant, both terms refer here to proven acquisition and successful inoculation resulting in a diseased test plant.

Virus acquisition. Virus acquisition by aphids that had shown different combinations of EPG patterns on the source plant is given in Table 1 (top part). Virus was acquired by one in 42 aphids with only pathway phase patterns, and by one in 22 aphids with pathway phase and additional E1. The duration of E1 in this particular case was 22 seconds. E1 durations in the experiment ranged from 19 seconds to 14.4 minutes, with one to six separate periods of E1 per aphid. Acquisition increased with aphids that showed E2, reaching 58% when E2 was sustained for 10 minutes or longer.

The logistic regression of acquisition on the (log of) E1 and E2 duration is summarized in Table 2. It appeared that the order in which the covariates were put into the model had some impact. Adding the log duration of E1 to the model after E2, hardly affected the deviance (change -0.1), whereas adding E2 after E1, significantly changed the deviance (-19.0). Thus, the E2 duration showed a strong relation to the probability of virus acquisition, irrespective of the duration of E1.

Virus inoculation. Test of the viruliferous aphids showed that 94.1% were able to transmit the virus after one week inoculation access period. For test aphids, inoculation

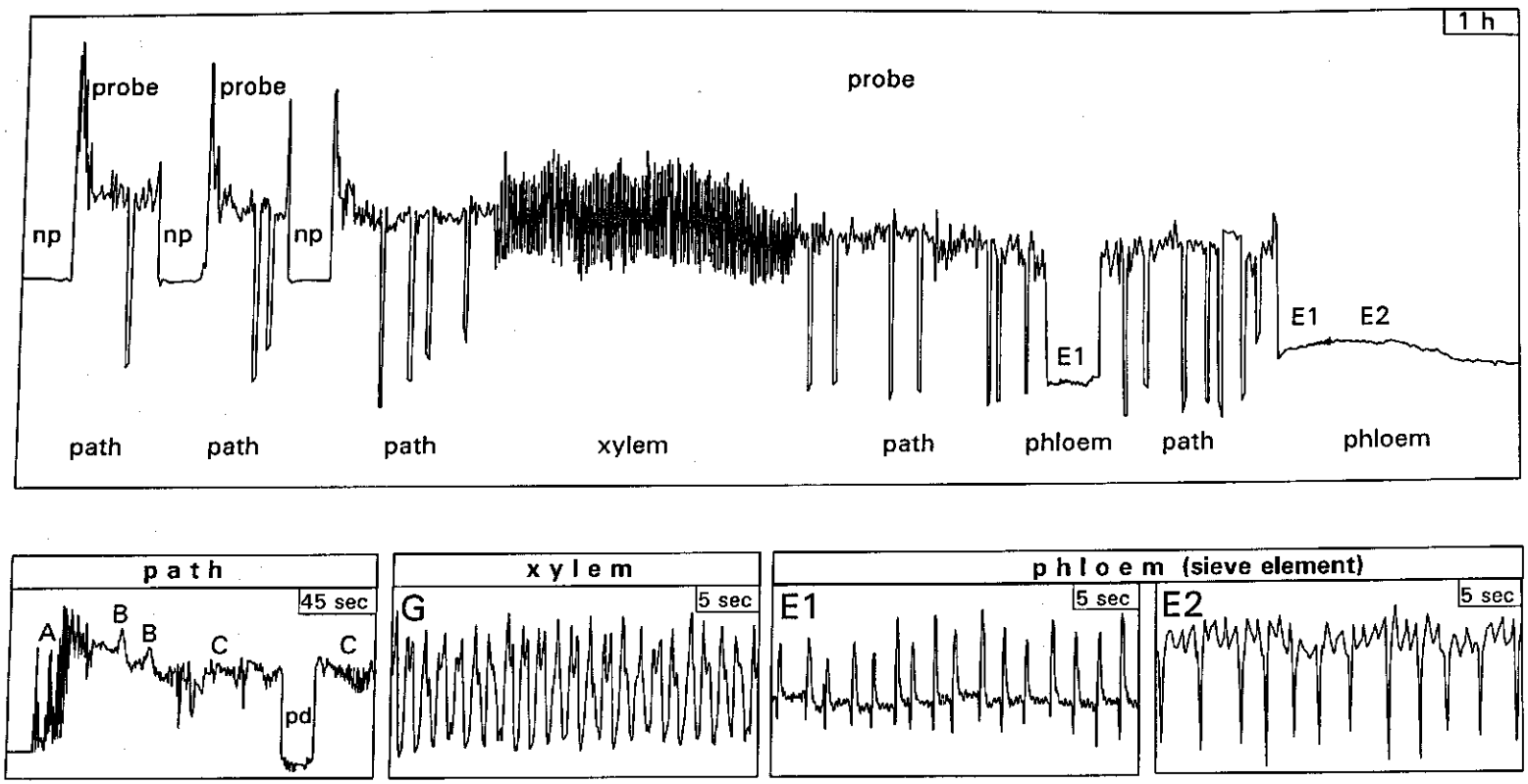

Figure 1. Electrical penetration graph of aphid feeding behaviour. Top trace, probing and non-probing alternation. Probe is stylet penetration period, np is non-probing. First two probes contain pathway phase (path) only, the third probe also includes a xylem phase and two phloem phases: one short with E1 only, the second with E1 followed by E2. Bottom traces, details of each phase with waveform indications; pd, potential drop, i.e. a brief intracellular puncture. Top trace contains many potential drops in the path.

was accomplished by 4 of 31 aphids after reaching the pathway phase only (Table 1, lower part). A higher proportion of aphids (28%) successfully inoculated plants after reaching the pathway phase and pattern E1, and a further increase in inoculation (54%) occurred after reaching E2 additionally. However, aphids that had shown E2 for 10 min or more did not transmit the virus more readily. In respect to those aphids showing E1 and E2, the total duration and the number of E1 periods showed relevance in relation to inoculation. The number of E1 periods increased with the more complete behaviour, i.e. from pathway only to pathway + E1 + E2>10min (Fig. 2), but this parameter explained the virus inoculation significantly less than the E1 duration when analyzed by the multiple regression. The E1 durations appeared to increase considerably in aphids that showed pathway + E1+ E2 as compared to those with E1 only (Fig. 2) but further increase in E2 duration (E2>10 min) did neither result in a further increase of E1, nor in a higher percentage of infected plants.

Table 1. Numbers of BYDV infected test plants in relation to EPG waveforms recorded during acquisition and inoculation probes by *R. padi*

Virus acquisition

Pattern shown	# total plants	# infected plants (%)
pathway	42	1 (2.4) a
pathway + E1	22	1 (4.5) ab
pathway + E1 + E2	29	7 (24.1) b
pathway + E1 + E2>10 min	24	14 (58.3) c
Total	117	23 (20)

Virus inoculation

Pattern shown	# total plants	# infected plants (%)
pathway	31	4 (12.9) a
pathway + E1	21	6 (28.6) b
pathway + E1 + E2	44	24 (54.5) c
pathway + E1 + E2>10 min	44	23 (52.3) bc
Total	140	57 (41)

Treatments followed by the same letter are not different according to the chi square test ($p<0.05$)

The logistic regression of virus inoculation by infected aphids on log durations of E1 and E2 showed that the best explanatory variable was the duration of E1 (Table 2). Adding E2 to the model after E1 did not improve it significantly with a change in deviance of -2.8 but in the reverse sequence, adding E1 after E2, the deviance changed by -6.6, which is significant. Thus, the E1 duration had a much stronger effect (p=0.01) than the E2 duration (p=0.09) and explained better the probability of inoculation.

Table 2. Deviance in the logistic regression model on acquisition and inoculation data. The change in deviance shows the effect of adding E2 (log durations) to the model after E1 (top) versus adding E1 after E2 (bottom) respectively

	Acquisition			*Inoculation*		
Variables	Scaled deviance	Change in deviance	p-value	Scaled deviance	Change in deviance	p-value
empty model	116			191		
model+logE1d +logE2d		-19.0 *	(<0.001)		-2.8 n.s.	(0.09)
model+logE2d +logE1d		-0.1 n.s.	(0.75)		-6.6 *	(0.01)

* = significant. n.s. = not significant. Significance is given by the chi square table.

DISCUSSION

Virus acquisition and inoculation do not depend only on the stylet tip position in the leaf tissue and the activity of the aphid but also on the virus concentration in the sap ingested (Gray *et al.*, 1991) or in the saliva secreted, respectively. Therefore, transmission remains also a matter of probability rather than of the presence of a particular event or

condition only. For this reason, aphids matching the conditions may not be successful in acquisition or inoculation of the BYDV (Table 1).

Acquisition. The clear connection between virus acquisition and EPG pattern E2 supports earlier evidence that E2 represents phloem sap ingestion (Mentink *et al.*, 1984; Kimmins & Tjallingii, 1985). Pattern E1, on the other hand, showing no association to acquisition, is suggested not to be related to phloem sap ingestion. The two aphids showing acquisition without E2 can be explained as caused by some brief (5-10s) cell punctures into any of the virus containing phloem cells within the reported phloem restriction of the virus (Barker & Harrison, 1986; Derrick & Baker, 1992; Shepardson *et al.*, 1980; Heuvel *et al.*, 1989). These brief punctures, the so called potential drops (pd-punctures, Tjallingii, 1985b) in the EPG, frequently occur during the pathway phase (Fig. 1). At least a number of these pd-punctures do occur into phloem parenchyma, companion cells, and into sieve elements as has been demonstrated recently (Tjallingii & Hogen Esch, 1993). Only exceptionally high concentrations of virus particles in these cells would presumably lead to virus acquisition in pd-punctures since the sap samples are minute. This appears in agreement with the general failure to acquire virus in spite of the fact that the majority of aphids presumably must have executed such phloem cell punctures. From the foregoing, therefore, it remains uncertain whether the E1 occurrence had any contribution to the virus acquisition in the single aphid that acquired the virus after reaching E1.

For persistent viruses Gray *et al.*, (1991) found a positive correlation between the acquisition access period (AAP) and virus acquisition. Though this correlation is more indirect and less accurate than the correlation with the E2 duration, it is in agreement with our results. Sieve element acceptance, i.e. sustained phloem sap ingestion (E2>10min), usually does not occur earlier than 3-4 h (mean time) after plant access; in some aphid-plant combinations even 5-7 h were found normal (Tjallingii & Mayoral, 1992).

Inoculation. Inoculation by aphids that had shown only the pathway phase suggests that some saliva can be injected in sieve elements or other infectable cells prior to the sieve element phase. Apparently inoculation has a higher probability of occurring than acquisition during the pd-punctures of these cells. Whether this is due to the minimum number of virus particles needed for a successful acquisition and inoculation during ingestion and salivation respectively, or due to other factors, remains to be studied in further investigations on pd-punctures. In addition to short sieve element punctures, undistinguished in EPG from other cell punctures, periods of pure E2 occur much earlier

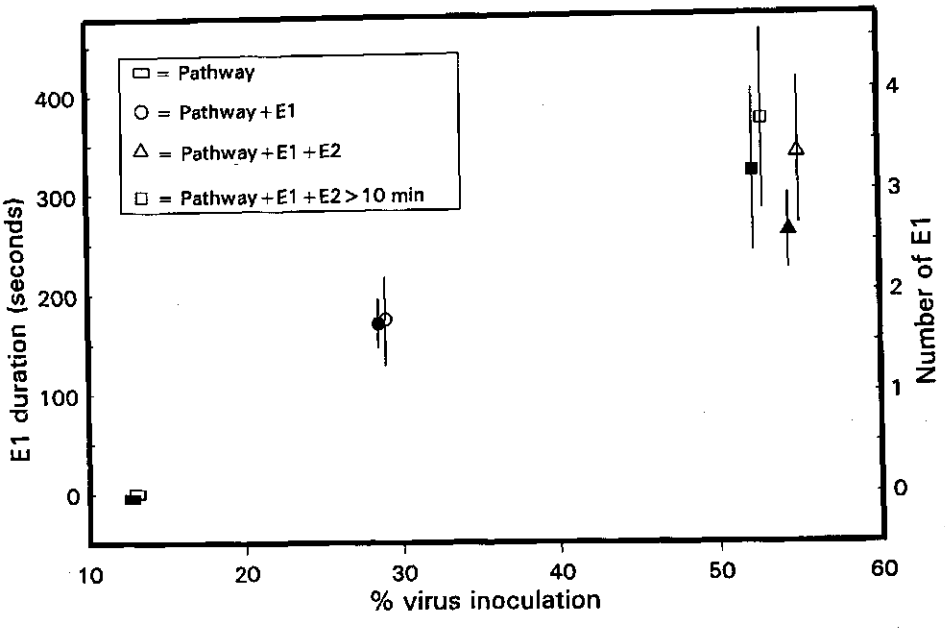

Figure 2. Relation of E1 with inoculation. Durations and numbers of E1 (sieve element salivation) periods (means and SE) in categories of aphids that had shown different waveform combinations in relation to their inoculation success. Open marks, E1 durations; black marks, E1 numbers.

than phloem sap ingestion (E2) suggesting than inoculation could take place before acquisition.

The main pattern related to virus inoculation apparently is E1, which indicates therefore, that it is related to salivation into sieve elements or other phloem cells. Long periods of sap ingestion (E2>10 min) did not increase the inoculation, although E2 is known to include also continuous salivation. This E2 saliva, however, appears unable to contribute to inoculation, thus supporting the hypothesis, based on earlier evidence (Tjallingii, 1978; 1988), that during E2 the saliva is mixed with the phloem sap inside the single canal at the stylet tips (fused food and salivary canal; Forbes, 1969) and is immediately ingested without reaching the plant tissue (Fig. 3).

The relation of E1 numbers and E1 durations with inoculation appears in figure 2 and in the GLIM analysis (Table 2). The occurrence of E1 resulted in a stepwise increase of

16% in inoculation efficiency, and E2 in another 26% (Table 1), which suggests that E2 may play an important role. Figure 2 and the GLIM analysis, however, clearly show that it is the concurrent increase in E1 duration that is responsible for this higher inoculation. This is confirmed by the aphids with E2>10 min , that showed both, no longer E1 and no more inoculation (Fig. 2). Comparably, Leonard & Holbrook (1978) and Scheller & Shukle (1986) found no higher probability of virus transmission with longer periods of their 'phloem ingestion', equivalent to our E1 and E2 together, in the AC monitoring systems they used.

The one-week inoculation access period (IAP) in the control tests for viruliferous aphids resulted in a substantially higher inoculation efficiency (94.1 %) than the relatively short IAP (maximum efficiency 54%) used in our experiments. Power *et al.*, (1991) obtained a 47 % and 87 % inoculation efficiency , using a 2 and 6 h IAP, respectively. Sylvester (1949; 1980) suggested that the transmission efficiency can be improved by allowing viruliferous aphids to make several probes or discontinuous feeding. From our results, we conclude that it is not only the access time or the number of probes which are important, but especially, the duration of the sieve element salivation periods (E1 patterns) within the probes and in less extent their number. Longer plant access will imply longer and more E1 as long as an aphid is not continuously sap ingesting (E2). Any plant resistance increasing E1 durations or numbers (both usually correlated), therefore, would lead to an increase in the inoculation of luteoviruses.

For a phloem restricted virus, an ideal factor of resistance against vectors should be located in the epidermis or mesophyll avoiding the insect to reach the phloem. Whether these effects are common in resistant lines, however, remains unclear (Helden & Tjallingii, 1993). Givovich & Niemeyer (1991), using a 6-h IAP, showed that *R. padi* reduced BYDV inoculation on wheat with high DIMBOA levels due to reaching the sieve elements later. However, the study suggests that the transmission was only delayed since aphids might need more than 6 h to reach the phloem (E1), which would prevent that the reduced inoculation can occur in field circumstances.

E1 appears at the beginning of any phloem phase and is followed by E2 or not (Tjallingii, 1990). Sometimes E1 and E2 may alternate or occur intermixed, especially, during the first hours of a new plant access. However, on resistant plants, E1 or an E1/E2 mixture may occur much more persistently (van Helden & Tjallingii, 1993). In 'phloem ingestion' (PI) waveforms, as defined in the American AC-systems for EPG recording (McLean & Kinsey, 1965; Brown & Holbrook, 1976) no distinction can be made between E1 and E2 patterns, so PI should be referred to as 'phloem position' or 'phloem puncture' rather than phloem sap ingestion (Reese *et al.*, 1997).

A direct physiological relation between the E1 waveform features and the salivary pump activity remains unclear and needs to be studied further. Its saw-tooth like peaks, at a rate of 2 to 4 per second with an electromotive force origin, are in contrast with the E2 peaks having a similar frequency but different shape and a resistance origin (Tjallingii, 1990). These latter peaks have been thought, so far, to reflect the salivary pump contractions. If so, a direct relation of the E1 peaks with these contractions seems very unlikely since both peaks can occur simultaneously during the transient periods between E1 and E2.

Summarizing, we may conclude that E1 represents salivary excretion into the sieve element (Fig. 3, E1) and E2 represents (passive) ingestion of sieve element sap (Tjallingii, 1988) which is mixed with the concurrently secreted saliva (Fig. 3, E2). Further studies will be needed to reveal the functional aspects of these two types of salivary secretion.

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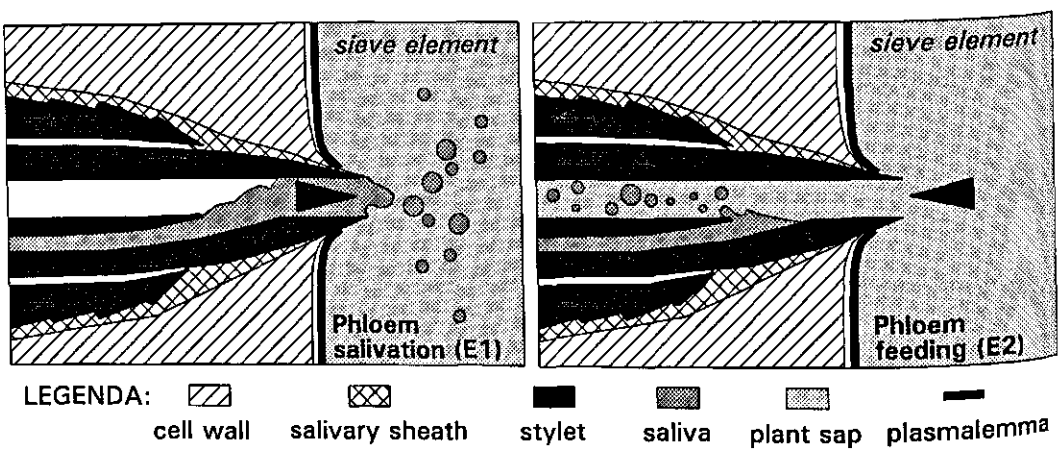

Figure 3. Diagram of main E1 and E2 features. During E1 saliva is secreted into sieve element. The fluid filled food canal does not allow saliva to be ingested. During E2 the sieve element sap is forced (high pressure) into food canal. The secreted saliva, therefore, will not reach the plant but it mixed with the sap. The fusion between the two canals near the tip is an essential anatomical feature in this respect

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3. *Effects of previous plant infestation on sieve element acceptance by two aphids* [1]

ABSTRACT

Probing behaviour as affected by previous infestation was studied in two aphid species on their respective host plants. Probing behaviour by *Aphis fabae* (reported to have beneficial effects when living in colonies) and *Rhopalosiphum padi* (without known beneficial effects) was studied using electrical penetration graph (EPG) technique. In *A. fabae* the effects of the previous infestation were longer and more continuous sap ingestion, and less salivation into sieve elements before sap ingestion. These effects suggest phloem factors. Nevertheless, mesophyll and non-vascular tissues are likely to be involved to a lesser extent, as reflected by fewer non-probing periods before the first phloem phase on previously colonised leaves as compared to uncolonised leaves. Total honeydew production increased on a previously colonised leaf due to the prolonged sap ingestion periods but the excretion rate was not affected, indicating that the ingestion rate remained unaltered. *R. padi* did not show responses to previous colonisation. It is hypothesized that the changes in probing behaviour are due to changed plant properties, chemical contents of sieve element sap and/or physiological changes induced by the saliva from the colony.

INTRODUCTION

Stylet penetration by aphids is an important process in host plant selection, i.e. host plant acceptance and rejection. All tissue layers between the cuticle and the sieve elements are delicately penetrated by the stylets. Many cells are briefly punctured but the track itself stays in an exclusively intercellular position, predominantly going through the secondary cell walls (Tjallingii & Hogen Esch, 1993). Puncturing of cells along the stylet pathway, generally without any visible short-term damage, is very characteristic in probing by aphids. However, it is not completely clear what the

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purpose of these punctures is or what role the other activities during stylet penetration actually play in host plant selection or phloem seeking. The information available is not detailed enough (Pollard, 1973; Montllor, 1991; Pickett *et al.*, 1992) or rather speculative (Dreyer & Campbell, 1987; Miles & Oertli, 1993). The effects of some allelochemicals in plants before reaching the phloem has been demonstrated (Givovich & Niemeyer, 1991) but, ultimately, the phloem sieve element contents seem crucial in host plant acceptance and rejection (van Helden & Tjallingii, 1993; Cole, 1994). Nevertheless, sieve element acceptance, i.e. the onset of sustained sap ingestion from a sieve element, may take hours and often is preceded by many earlier brief punctures of sieve elements and by short periods of stylet tip presence in various sieve elements of one and the same vascular bundle (Tjallingii & Hogen Esch, 1993; Tjallingii & Mayoral, 1992).

Elucidating stylet penetration activities in plants has made a major progress by using electrical penetration graph (EPG) techniques (AC and DC based systems; McLean & Kinsey, 1967; Tjallingii, 1988). Stylet tip locations and aphid activities are directly reflected in the waveforms obtained with these systems. Especially in the DC system, providing more details than the AC systems (Reese *et al.*, 1997), waveforms now can be used to analyse aphid-plant relationships (Givovich & Niemeyer, 1995; Caillaud *et al.*, 1995; van Helden & Tjallingii, 1993). In probing behaviour, especially two 'landmarks' in the sequence of behavioural elements seem important events in host plant recognition and acceptance (Tjallingii, 1994). i.e. (1) the first phloem phase and (2) the first sustained phloem ingestion (Tjallingii, 1995b). These two landmarks can be considered as a first established sieve element access and sieve element acceptance, respectively, thus forming two crucial events in the host plant selection process of the aphid (Tjallingii & Mayoral, 1992).

Before the first phloem phase, the number of probes (or non-probing periods) and the (numerical) proportion of short probes (probes shorter than 2 min; Gabrys *et al.*, 1997) seem important indicators for effects of factors from the leaf surface or from surface cell layers. Additionally, the delay time between the two landmark events, often one hour or more, indicates effects of factors deeper in the plant tissues. TEM (transmission electron microscopy) micrographs combined with EPGs (Tjallingii & Hogen Esch, 1993) showed that sieve elements were not immediately accepted after reaching them during a probe. During the delay, stylets were often withdrawn from the first sieve element (pathway phase) and short punctures into different sieve elements (phloem phase periods) were shown with phloem salivation only. Sieve elements activities became clearer after we found (Prado & Tjallingii, 1994) that the two phloem phase

waveforms E1 and E2 reflect saliva secretion into the sieve element and (passive) phloem sap ingestion with concurrent secretion of saliva, respectively. We hypothesized (Tjallingii & Mayoral, 1992; Tjallingii & Hogen Esch, 1993) that the delay time between the two landmarks, including sheath salivation and sieve element salivation, might be needed to establish beneficial changes in the plant's phloem sap or sieve element properties at the feeding site.

For *Aphis fabae* Scopoli, the aphid used in our EPG and TEM studies above, some beneficial effects of colony life have been reported (Way & Banks, 1967; Way & Cammell, 1970). Aggregates can possibly induce and maintain an improved nutritional quality on their feeding site as compared to the rest of the plant (Dixon & Wratten, 1971; Hayamizu, 1984). As a consequence, an EPG study of probing behaviour and sieve element acceptance in colony conditions or after previous infestation seems attractive in the perspective of the hypothesis mentioned. Aggregation behaviour in *Rhopalosiphum padi* L. has been reported for gynoparae on their winter host (Pettersson, 1993). Apterae of *R. padi* do not seem to benefit living in groups (De Barro, 1992) and conversely, they are apparently very sensitive to intraspecific competition for nutrients (Chongrattanameteekul *et al.*, 1991a, b). For the 'solitary' apterae, an odour has been suggested to mediate a spacing behaviour (Pettersson *et al.*, 1995). In our previous studies (Tjallingii & Mayoral, 1992), *R. padi* showed a distinct delay period between the two landmarks mentioned.

The aim of the study presented here is to investigate the effects of previous infestation of a plant on the 'fine structure' of probing behaviour by *A. fabae* on *Vicia faba* and *R. padi* on *Triticum aestivum*. Interest is especially focused on sieve element acceptance using the EPG technique as a main tool.

MATERIALS AND METHODS

Aphids. The aphid *Aphis fabae* used in these experiments was cultured on broad beans (*Vicia faba* L.) cv "3 x wit" under greenhouse conditions, at 20 ± 2 °C and L16:D8 photoperiod. This aphid has been kept in Wageningen since 1986 but originally stems from Hannover, Germany (Institut für Planzenkrankheiten, M. Poehling). *Rhopalosiphum padi* was reared on wheat (*Triticum aestivum* L.), cv Okapi at the same conditions mentioned above. The stock culture of this aphid was started with specimens collected in Spain (CSIC, Dept. of Biological Research, Madrid). For electrical penetration graphs (EPG) recording, apterous virginoparous adults, 2-4 days after the last moult, were

retrieved from the rather dense culture colonies on the respective rearing plants between 09:00 and 10:00 am.

Colonies of *A. fabae* were produced by placing two apterous adults inside a clip-on cage (2 cm diameter) during 4-5 days on the abaxial part of a new full-grown leaf of a broad bean plant. These plants were approximately 3 weeks old with 4-5 mature leaves. Thus, colonies of 15 to 40 larvae of different ages, usually concentrated in a compact group, developed on a leaf to be used in the colony treatments (see below). A similar procedure was used with *R. padi* on the second leaf of a wheat plant (plants 12-14 days old), obtaining groups of 10 to 20 larvae.

EPGs and experimental design. Before EPG recording, the collected aphids were starved in a Petri dish for about one hour. During this time they were wired with a 2 cm long gold wire, 20 µm in diameter, which allowed free movement of the aphid within the wire radius when placed on top of the leaf. Depending on the treatment (see below), this leaf was fixed in its normal position or reversed, the leaf side with the wired aphid up. The EPG technique (DC method), EPG set-up (Faraday cage, etc.) and the waveforms obtained have been extensively described elsewhere (van Helden & Tjallingii, 1993; Prado & Tjallingii, 1994; Tjallingii, 1978, 1985 & 1988). Acquisition of most of the recordings was directly done on a PC hard disc using STYLET 2.2 software (Tjallingii & Mayoral, 1992) but some EPGs were first stored on tape (Racal, type 7 DS recorder). All recording lasted 8 h and about 25 replicates were made per treatment.

Analysis. The same STYLET 2.2 software was used for EPG analysis. Waveforms A, B, C were considered as pathway phase, whereas waveforms E1 and E2 were regarded as phloem phase but kept separate. Waveforms G and F and other parameters were analyzed separately but not included in the results because they did not give much specific information.

Using the beginning of the first phloem phase (always starting with E1) and the first sustained phloem ingestion (E2 longer than 10 min) as landmarks, we divided probing behaviour for each aphid and used parameter definitions linked to that, as schematized by figure 1 (open arrows).

Some parameters needed calculation by a defined procedure in order to prevent ambiguous values for individuals which did not show a certain event. For example, when no phloem phase was shown by an aphid, the time to the first phloem phase or to the first sustained phloem ingestion in the experiment was considered to equal the total observation time. Thus, 8 h was taken as the time to reach the first phloem phase, or

sustained phloem ingestion respectively, since these aphids needed at least 8 h or more to show them. Although this calculation underestimated the values (means) of these parameters, we preferred to follow this calculation rather than to discard these aphids or to use zeros for their values. On the other hand, in some other parameters this procedure would provide a rather artificial situation and not considering these individuals in calculations was better (for example, the time between the first phloem phase and sustained phloem ingestion, parameter 6; Fig. 1 and Table 3). In such cases the number of individuals contributing to the mean was smaller than the total of individuals studied in a certain treatment, as indicated by "n" at the top of the tables (Tables 1 to 6). In the tables it is easy to recognise which method was used. When an additional, smaller number is given below a mean value (n= ...) it indicates that only aphids showing the phenomenon concerned were used for its calculation. When no additional number is given then all aphids were used, some of them with extrapolated values.

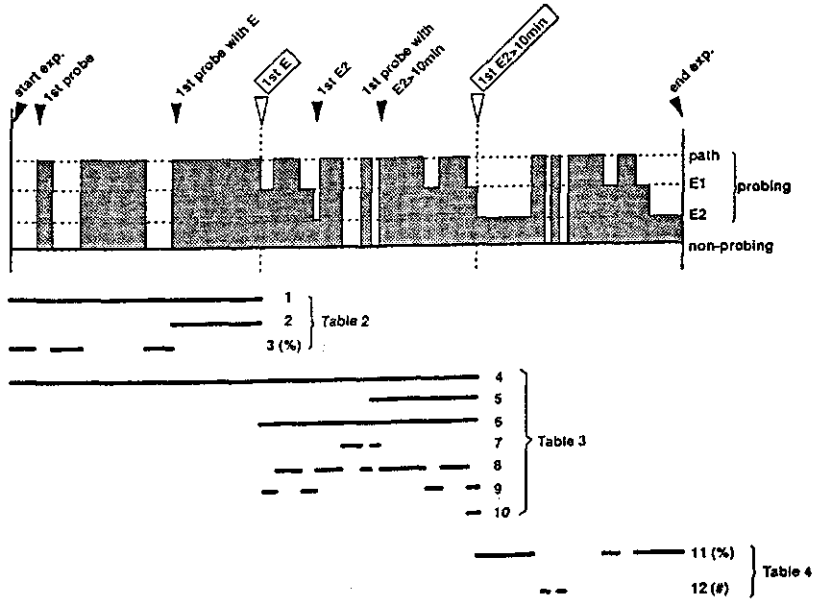

Figure 1. Diagram showing EPG parameters. Horizontal bars reflect for each parameter (number 1-12) the duration or summated duration. In two cases (%) relative values were used, i.e. for 3, as a % of 1; for 11, as a % of the time after the start of first sustained phloem ingestion; for 12 the number (#) of events is used. Open arrows indicate natural landmarks, solid arrows indicate other remarkable points in the sequence. Not all of them are used in the parameter's definitions.

Treatments: Individuals of *A. fabae* were monitored on the following feeding sites:
(1) Clean leaf, abaxial (= uncolonised leaf). A single aphid was monitored on the abaxial side of the leaf on the area where an empty clip-on cage was attached during the four previous days, as done with the colonised leaves.
(2) Clean leaf, adaxial. Similar to the previous treatment but now the aphid was placed on the adaxial side of the leaf.
(3) In colony, abaxial. The leaf with the colony on the underside of the leaf was reversed and the wired aphid was placed on the edge of the group, between other individuals.
(4) Opposite colony, adaxial. The aphid was put on the upper side, opposite the colony on the abaxial side. Initially, the infestation was tried on the adaxial side but survival and growth were very poor there.
(5) Removed colony site, abaxial. The clustered aphids were gently brushed from the abaxial side of the leaf some minutes before starting the experiment. The test aphid was placed on the site of the removed colony.
(6) Stem. The aphid to be monitored was placed in the last internodal space, close to the growing shoot tip. *A. fabae* has some feeding preference for this site (Lowe, 1967).

Only treatments 1, 3, 5, and 6 were used with *R. padi* on wheat plants. "Stems" (or pseudostems) were used as a feeding site as this species prefers the lower part of the plant (Wiktelius, 1987).

Statistical analysis. The different parameters and treatments were compared by the Kruskal-Wallis test, followed by a multiple comparison (α= 0.05) (Conover, 1980). When the significant level was close to the 5% using this test, a separate pair-wise comparison (Mann-Whitney U-test; α= 0.05) was also carried out.

Honeydew production of A. fabae. Honeydew excretion by individuals of *A. fabae* on previously colonised leaves (ca. 30 aphids) and on uncolonised or clean (control) leaves was monitored for 24 hours. EPG recording was performed simultaneously, wiring the dorsum of the aphid from the side of the body in order not to interfere with the deposition of honeydew droplets on the paper. Recording started one hour after the aphids were placed on the leaf in order to assure settlement and to avoid the aphids walking out of the suitable area (above the strip) for honeydew recording. The honeydew production was measured on a 24 hour honeydew clock, using a 2 cm wide strip of aluminium TLC plate (silica gel; Merck, type 60F254) impregnated with ninhydrin (in butanol and acetic acid solution) which stains the amino acids in the honeydew. The number of droplets was

counted and as an estimator for the amount of honeydew the diameter of deposited droplets was taken, using an eye-piece micrometer in a stereo microscope. Differences between treatments in respect to honeydew excretion, frequency and size of the droplets, were tested by the Mann-Whitney U-test (α=0.05).

RESULTS

Aphis fabae *on* Vicia faba. In comparison to the clean abaxial site, one treatment clearly showed the strongest effects of previous colonisation, i.e. the removed colony site. We will discuss first these effects and then the effects in the other treatments. Altogether we worked out approximately 70 different parameters from the EPGs but only the most relevant will be presented here.

Effects on the removed colony site. In respect to total and general parameters (Table 1) aphids on the removed colony site showed significantly more phloem ingestion (waveform E2) and less (but not significantly) phloem salivation (waveform E1). Non-probing and pathway phase were reduced, and more aphids reached sustained phloem ingestion (E2 > 10 min) during the 8 h experiment (Fig. 2) on the removed colony site as compared to the clean control leaf and also reached it sooner. Other parameters will be considered now in order of their occurrence in three successive periods of probing behaviour. They are shown in figure 1 and listed in table 2, 3, and 4 respectively. No clear differences were shown in durations of separate probes in the first period (data not shown in tables): i.e. the period until the first phloem phase (= first E1 waveform). In the treatment as well as in the control, equal numbers of probes shorter than 2 min, probes between 2 and 10 min, and probes longer than 10 min occurred. This may indicate that probably no important factors played a role, in the epidermis, mesophyll, and/or mesophyll-vascular parenchyma, respectively. Although aphids on the removed colony site showed a shorter time until the first phloem phase, both, from start of the experiment and from start of the probe concerned (parameters 1 and 2), this was not statistically significant. Nor was this the case for the somewhat lower percentage of non-probing (parameter 3, Fig. 1 & Table 2) during this period.

Table 1. Probing behaviour of *A. fabae* on *Vicia faba*. Total durations (h, min), numbers (#) or percentages of total times (%)

Probing site		Clean leaf abaxial n=25			Clean leaf adaxial n=25			In colony, abaxial n=25			Opposite colony, adaxial n=26			Removed colony, abaxial n=26			Stem n=25			Kruskal - Wallis
Parameter	(Unit)	Means	SE		Means	SE		Means	SE		Means	SE		Means	SE		Means	SE		p-value
Non-probing	(%)	11.1	1.4	a	14.0	1.7	a	11.8	1.6	a	12.5	1.9	a	5.9	0.9	b	12.0	1.6	a	**0.004**
Probes	(#)	14.6	1.7	bc	22.4	2.7	a	19.0	2.1	ab	14.7	1.8	bc	10.6	1.6	cd	8.6	1.1	d	**<0.001**
Pathway phase	(h)	3.4	0.3	a	3.5	0.3	a	2.9	0.3	a	2.9	0.3	ab	2.2	0.3	b	3.4	0.3	a	**0.015**
Phloem phase	(h)	2.1	0.4	b	2.2	0.4	b	2.7	0.5	ab	2.8	0.4	ab	3.7	0.3	a	2.5	0.4	b	**0.038**
Phloem phases	(#)	2.2	0.5		1.8	0.4		2.4	0.5		2.4	0.3		2.0	0.3		2.3	0.4		0.683
Phloem salivation	(min)	28.8	7.5		19.9	3.4		25.0	9.8		19.0	4.2		12.6	4.0		17.0	4.1		0.309
Phloem ingestion	(h)	1.6	0.4	b	2.0	0.4	b	2.3	0.5	b	2.5	0.4	b	3.6	0.4	a	2.2	0.4	b	**0.012**
Aphids with phloem phase	(#)	19			18			21			22			25			23			
Aphids with phloem ingestion	(#)	14			17			17			19			25			18			

Means followed by different letters within lines are significantly different according to the Kruskal-Wallis test followed by multiple comparisons (α= 0.05). SE= Standard error; n= number of replicates

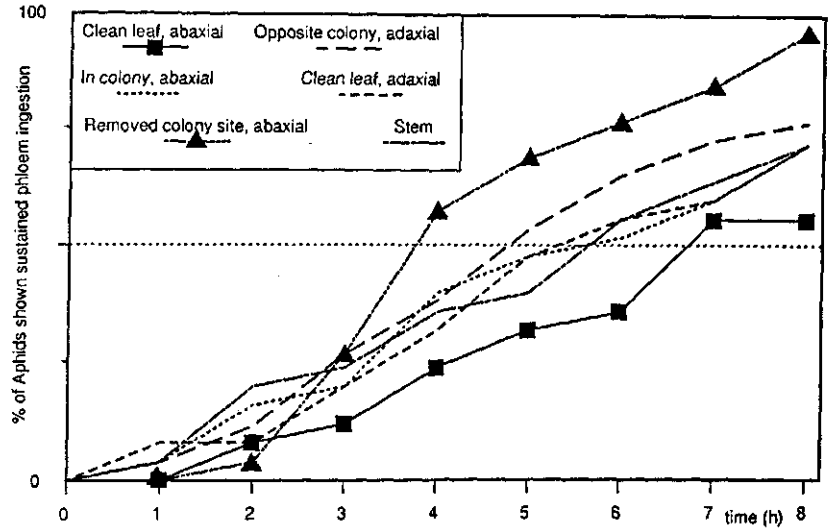

Figure 2. *Aphis fabae*. Percentage of aphids reaching a sustained phloem sap ingestion (E2>10 min). The two treatments which differed considerably in results are emphasised by symbols.

The period until the first sustained phloem ingestion (Fig. 1, Table 3), i.e. waveform E2 longer than 10 min, was shorter for the aphids on the removed colony site from the start the experiment as well as the probe concerned (parameters 4 and 5, Table 3). Since the comparable parameters in respect to the first phloem phase (parameters 1 and 2, Table 2) differed much less, it is of interest what occurred between the two landmarks. For aphids on the removed colony site the period itself (parameter 6, Table 3) was significantly shorter (1.1 h less), indeed, and non-probing and pathway during this period (parameters 7 and 8, respectively) were clearly reduced. However, most interesting was that far more aphids on removed colony sites achieved sustained ingestion during the first phloem phase (last parameter in Table 3). This resulted in less total sieve element salivation (waveform E1) and some reduction, but not significantly, of the period of sieve element salivation directly preceding sustained ingestion (parameters 9 and 10, respectively). Thus, sieve element factors again, seem to be very important here.

Table 2. Probing behaviour of *A. fabae* on *Vicia faba*. Period before the first phloem phase. Parameters numbers (N°) correspond to Figure 1

	Probing site		Clean leaf abaxial n=25		Clean leaf adaxial n=25		In colony, abaxial n=25		Opposite colony, adaxial n=26		Removed colony, abaxial n=26		Stem n=25		Kruskal - Wallis
N°	Parameter	Unit	Means	SE	Means	SE	Means	SE	Means	SE	Means	SE	Means	SE	p-value
1.	Time to first phloem phase in experiment	(h)	4.8	0.5	5.0	0.5	4.6	0.5	4.1	0.5	3.9	0.3	4.2	0.5	0.387
2.	Time to first phloem phase in probe	(h)	1.2 (n= 19)	0.3	1.1 (n= 18)	0.2	0.7 (n= 21)	0.1	1.0 (n= 22)	0.2	1.0 (n= 25)	0.1	0.9 (n= 23)	0.1	0.857
3.	% non-probing before first phloem phase	(%)	15.3 (n= 19)	2.2 ab	17.2 (n= 18)	2.1 a	17.1 (n= 21)	1.9 a	18.4 (n= 22)	1.9 a	11.5 (n= 25)	1.5 b	24.8 (n= 23)	5.3 a	**0.027**

Means followed by different letters within lines are significantly different according to the Kruskal-Wallis test followed by multiple comparisons (α= 0.05). SE= Standard error; n= number of replicates or, bellow individual means, numbers of aphids contributing to the parameters.

Table 3. Probing behaviour of *A. fabae* on *Vicia faba*. Period until the first sustained phloem ingestion. Parameters numbers (N°) correspond to Figure 1

	Probing site		Clean leaf abaxial n=25		Clean leaf adaxial n=25		In colony, abaxial n=25		Opposite colony, adaxial n=26		Removed colony, abaxial n=26		Stem n=25		Kruskal-Wallis
N°	Parameter	Unit	Means	SE	Means	SE	Means	SE	Means	SE	Means	SE	Means	SE	p-value
4.	Time to first sustained phloem ingestion in experiment	(h)	6.0	0.5 (a)	5.4	0.5 (ab)	5.2	0.5 (ab)	4.9	0.5 (ab)	4.3	0.4 (b)	5.1	0.5 (ab)	0.108 (pw)
5.	Time to first sustained phloem ingestion in probe	(h)	4.2	0.7 (a)	3.4	0.7 (ab)	2.9	0.7 (ab)	2.9	0.6 (ab)	1.8	0.3 (b)	3.1	0.6 (ab)	0.200 (pw)
6.	Time between first phloem phase and first sustained phloem ingestion	(h)	1.5 (n= 19)	0.4 a	0.5 (n= 17)	1.2 b	0.7 (n= 21)	0.3 b	0.9 (n= 22)	0.3 ab	0.4 (n= 25)	0.1 b	1.6 (n= 20)	0.5 a	**0.004**
7.	Non-probing between first phloem phase and first sustained phloem ingestion	(min)	3.1 (n= 14)	1.8 (a)	3.3 (n= 17)	2.5 (ab)	1.3 (n= 18)	1.2 (ab)	3.9 (n= 20)	2.2 (ab)	0.6 (n= 25)	0.6 (b)	5.2 (n= 18)	5.2 (ab)	0.344 (pw)
8.	Pathway phase between first phloem phase and first sustained phloem ingestion	(min)	33.4 (n= 14)	15.5 (a)	17.7 (n= 17)	9.1 (ab)	11.0 (n= 18)	3.1 (ab)	19.2 (n= 20)	10.3 (ab)	8.4 (n= 25)	3.4 (b)	19.6 (n= 18)	10.7 (ab)	0.139 (pw)
9.	Total phloem salivation before first sustained phloem ingestion	(min)	22.0 (n= 14)	6.8 a	7.3 (n= 17)	3.1 bc	9.4 (n= 18)	4.6 c	14.1 (n= 20)	3.6 ab	10.7 (n= 25)	3.9 c	14.5 (n= 18)	3.2 ab	**0.055**
10	Period of phloem salivation just before first sustained phloem ingestion	(min)	15.7 (n= 14)	5.7 (ab)	5.9 (n= 17)	2.8 (ab)	5.3 (n= 17)	2.0 (b)	5.6 (n= 20)	1.8 (ab)	7.4 (n= 25)	3.2 (b)	12.0 (n= 18)	3.0 (a)	0.150 (pw)
	Percentage of aphids with first sustained phloem ingestion during first phloem phase	(%)	21.1		66.7		57.1		40.9		70.8		43.5		

Means followed by different letters within lines are significantly different according to the Kruskal-Wallis test followed by multiple comparisons ($\alpha = 0.05$).
SE= Standard error; n= number of replicates or, bellow individual means, numbers of aphids contributing to the parameters; pw= pairwise comparisons tests and different letters below lines indicate significantly different means.

The involvement of phloem factors was strongly supported by probing activities in the period after the first sustained phloem ingestion (Table 4): i.e., the percentage of time spent in phloem phase (parameter 11), which was high, and the low number of interruptions (early termination) of phloem ingestion, reflected as separate probes (or non-probing periods, parameter 12).

Effects on other sites or treatments. Unexpectedly, aphids placed inside the colony (not removed) showed fewer differences in respect to the control than those on the removed colony site did (Table 1- 4). Most parameters showed intermediate values between the control and the removed colony site and were not significantly different from either of them. Because we suspected intraspecific interactions (pheromones, crowding, etc.) which probably caused these effects, we decided the 'opposite colony' treatment. However, colonies developed rather badly on adaxial leaf sides. Using the abaxial side for the colony urged us to use the adaxial site for the test aphids and to add a blank adaxial control treatment as well. Comparing results of the opposite colony site with its clean adaxial control, often showed an improvement in a similar sense as we found for the removed colony site and its respective control but the differences were smaller and mostly below the significance level. Apparently, the infestation effects were masked in the other treatments.

Finally, aphids on stems showed somewhat better overall reactions than the clean leaf (abaxial) (Tables 1- 4) but no clear distinction can be made between stems and leaf sites from our data, neither between leaf sites.

Honeydew excretion. The total number of excreted honeydew droplets was significantly higher on aphids feeding on the removed colony site, in accordance with the longer sap ingestion recorded by EPGs. Total honeydew excretion times could not be derived from TLC strips since some E2 periods had only a single droplet. Longer series of droplets during sustained ingestion showed no significant differences between aphids on the removed colony site and on clean control leaves (abaxial) in respect to the frequency of excreted honeydew droplets (per hour of phloem sap ingestion, waveform E2), neither in the droplet size (Table 5).

Table 4. Probing behaviour of *A. fabae* on *Vicia faba* after reaching the first sustained phloem ingestion. Parameters numbers (N°) correspond to Figure 1

	Probing site		Clean leaf abaxial n=25		Clean leaf adaxial n=25		In colony, abaxial n=25		Opposite colony, adaxial n=26		Removed colony abaxial n=26		Stem n=25		Kruskal-Wallis
N°	Parameter	Unit	Means	SE	Means	SE	Means	SE	Means	SE	Means	SE	Means	SE	p-value
11.	Percentage of phloem phase after first sustained phloem ingestion	(%)	84.5 (n= 14)	6.0 b	83.9 (n= 17)	6.7 b	87.4 (n= 17)	4.2 b	82.9 (n= 20)	5.6 b	97.0 (n= 25)	1.6 a	90.4 (n= 18)	4.2 ab	0.066
12.	Number of probes after first sustained phloem ingestion	(#)	0.9 (n= 14)	0.9 (ab)	3.0 (n= 17)	1.7 (a)	1.2 (n= 18)	0.8 (a)	2.5 (n= 20)	1.3 (a)	0 (n= 25)	0 (b)	0.5 (n= 18)	0.3 (a)	0.091 (pw)

Means followed by different letters within lines are significantly different according to the Kruskal-Wallis test followed by multiple comparisons ($\alpha = 0.05$). SE= Standard error; n= number of replicates or, bellow individual means, numbers of aphids contributing to the parameters; pw= pairwise comparisons tests and different letters below lines indicate significantly different means.

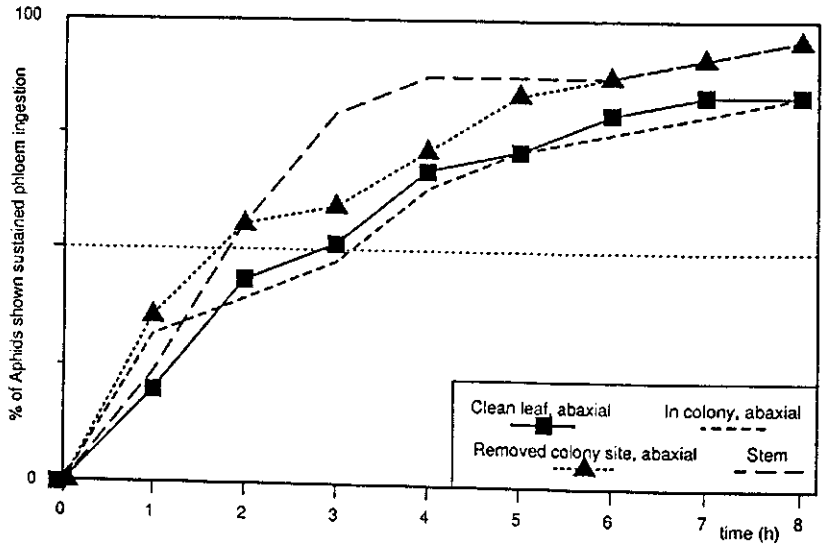

Figure 3. *Rhopalosiphum padi.* Percentage of aphids reaching a sustained phloem sap ingestion (E2>10 min). Same treatments emphasised by symbols as in figure 2.

Rhopalosiphum padi *on* Triticum aestivum. No significant differences related to previous colonisation of the plant were found in this aphid-plant combination. Although all the parameters retrieved were the same as in *A. fabae*, we restricted presentation here to the main ones in Table 6. Differences were found only between some plant parts (stem versus clean leaf). Aphids on stems spent significantly less time in total phloem salivation (E1 waveforms) and the E1 period just before phloem ingestion (E2) was significantly shorter (Table 6). Also, there was more phloem phase (mostly E2) after the first sustained phloem ingestion and this was less interrupted on stems than on leaves.

Table 5. Honeydew excretion of *A. fabae* on *V. faba*. Comparisons between aphids on clean (control) leaves versus previously colonised leaves

	Honeydew droplets/aphid				Droplet size	
Treatment	in 8 h experiment		per hour of E2		diameter (mm)	
	Mean	SE	Mean	SE	Mean	SE
Clean leaf (abaxial)	0.25 a	0.13	0.381 a	0.09	0.869 a	0.09
On site removed colony (abaxial)	2.38 b	0.77	0.553 a	0.08	1.001 a	0.03
(p-value)	0.001		0.121		0.182	

Differences according to the Mann-Whitney U-test. SE, standard error.

DISCUSSION

Aphis fabae, *EPGs*. The experiments showed most clearly the effects of previous infestation on leaves where the colony had been removed. Especially, phloem factors enhancing sieve element acceptance appeared to be involved, as reflected by more aphids reaching sustained phloem ingestion within the 8 h experiment, more phloem phase after reaching sustained phloem ingestion (parameter 11), and some more parameters. On the other hand, factors from mesophyll and non-vascular phloem tissue cannot be excluded, as reflected by less non-probing before the first phloem phase and more aphids showing phloem phase. This suggests that *A. fabae* apparently detect some changes already during the stylet route towards the phloem, and that these changes can be considered as an increased host plant acceptance.

Table 6. Probing behaviour of *Rhopalosiphum padi* on wheat. Parameters (N°) correspond to Figure 1

	Probing site		Clean leaf abaxial n=25			In colony, abaxial n=25			Removed colony, abaxial n=26			Stem n=25			Kruskal-Wallis
N°	Parameter	Unit	Means	SE		Means	SE		Means	SE		Means	SE		p-value
	Total phloem salivation	(min)	19.1	3.0	(a)	12.1	1.7	(ab)	15.0	3.6	(a)	9.2	2.0	(b)	0.056 (pw)
2.	Time to first phloem phase in probe	(h)	0.4 (n= 25)	0.1		0.3 (n= 24)	0.1		0.3 (n= 25)	0.1		0.3 (n= 24)	0.0		0.696
4.	Time to first sustained phloem ingestion in experiment	(h)	3.4	0.5		3.5	0.6		2.7	0.5		2.1	0.4		0.300
9.	Total phloem salivation before first sustained phloem ingestion	(min)	15.0 (n= 21)	7.5		6.1 (n= 21)	1.6		10.2 (n= 25)	3.7		5.1 (n= 24)	1.2		0.495
10	Period of phloem salivation just before first sustained phloem ingestion	(min)	2.7 (n= 21)	1.2	(a)	2.0 (n= 21)	0.7	(ab)	4.5 (n= 25)	2.2	(ab)	2.4 (n= 24)	0.9	(b)	0.204 (pw)
11.	Percentage of phloem phase after first sustained phloem ingestion	(%)	71.8 (n= 21)	6.7	ab	64.1 (n= 21)	6.4	a	75.8 (n= 24)	5.7	ab	88.5 (n= 24)	3.9	b	**0.042**

Means followed by different letters within lines are significantly different according to the Kruskal-Wallis test followed by multiple comparisons (α= 0.05). SE= Standard error; n= number of replicates or, bellow individual means, numbers of aphids contributing to the parameters; pw= pairwise comparisons tests and different letters below lines indicate significantly different means.

The main differences found in *A. fabae* are related to the two phloem phase activities: separate sieve element salivation, as reflected by the E1 waveform, and sieve element ingestion with concurrent salivation (Prado & Tjallingii, 1994), as reflected by waveform E2. Salivation into sieve elements might be required to prevent sieve element reactions like P-protein gelation or the formation of callose, blocking cell to cell transport in the phloem and the stylet food canal (Evert, 1990; Tjallingii & Hogen Esch, 1993; Cole, 1994; Tjallingii, 1994). However, we do not know the composition of the saliva secreted during the E1 waveform, nor do we know the biochemical or physiological effects in sieve elements. When a plant has been infested, an individual aphid may benefit from earlier salivation by others into the same leaf or vessels (systemic effect). Thus, less sieve element salivation (shorter E1) might be expected. Our results indicate that on the removed colony site the total sieve element salivation before sustained phloem sap ingestion (parameter 9) was reduced, although the reduction of the phloem salivation period just before sustained phloem sap ingestion (parameter 10) is not statistically significant (Table 3). This makes conclusions on this point uncertain. The increased sieve element ingestion, on the other hand, may have been caused by an improved quality of the phloem sap, or at least an increased content of feeding stimulants, as a result of the salivary components injected by *A. fabae* during the previous infestation on the removed colony site. We have no performance data of insects in the respective treatments. However, the situation on the feeding sites, i.e. treatments, may change rapidly. The colony effects may fade away after removing the colony. The blank leaf will become infested more heavily by the offspring produced, so that performance parameters, which generally take much longer than the 8 h used in our experiment, will lose more and more their relationship with the original treatment. Nevertheless, additional performance would have been interesting.

By comparison, the infestation effects on aphids inside a colony (not removed) were not significant. This might be due to interferences by the other aphids in the colony. Others (Ibbotson & Kennedy, 1951, 1959; Kay, 1976) found that the presence of other aphids or a colony might have an effect on the initial probing and settlement. We did not study the nature of these disturbances especially, so that olfactory interactions suggested by Pettersson *et al.* (1995) cannot be excluded. In fact, possible olfactory interactions were our main reason to introduce the 'opposite colony' and the 'removed colony' sites as a treatment. However, the acceptance of the two leaf sides apparently differed, which interfered with the infestation effects in the 'opposite colony' site. Similarly, the stems as a probing site showed differences with the abaxial leaf, even larger than the adaxial leaf did as a probing site. The longer non-probing time before the first phloem phase (parameter

3) seems in agreement with earlier reports (Ibbotson & Kennedy, 1959; Binns, 1977), suggesting that the stem ridges would stimulate walking before settling. Differences at phloem level were not detected on stems. In general, however, the differences between the clean (uncolonised) probing sites (plant parts) are difficult to explain on basis of our experiments and actually, remained outside the scope of our study. They are certainly worth being investigated further.

We think that the main effects we measured on the removed colony site were due to aphid induced changes in the plant material, especially, with respect to the chemistry. However, we realize that the removed colony site is a 'contaminated' area which probably contains volatiles and surface chemicals from plant and aphid origin that do not occur on the clean leaf. Nevertheless, it is likely that these contaminations affected the aphids less than the changed plant properties, since probing behaviour seems to be controlled mainly by internal plant factors: i.e. probing seems crucial in host plant selection and the gustatory sensory system in aphids seems evolved accordingly since sensilla are located internally (Tjallingii, 1976, 1978).

Honeydew excretion. Honeydew excretion (honeydew clock) recordings showed that total honeydew production was significantly higher on the removed colony site than on the clean control leaf, which is in accordance with the longer ingestion periods in the EPGs from these aphids and from the EPG results above (Table 1). However, no (significant) differences were shown in respect to the secretion rate (droplet intervals and sizes). The latter seems in agreement with suggestions that phloem sap feeding by aphids is regulated by the durations of ingestion periods rather than by changes in the ingestion rate during these periods (Tjallingii, 1995a).

Rhopalosiphum padi. For *R. padi*, in contrast to *A. fabae*, there appeared no beneficial infestation effects. Intraspecific competition and crowding has been reported to result in reduced longevity and fecundity (Chongrattanameteekul *et al.*, 1991a, b; De Barro, 1992), possibly, through a "deteriorated sap quality" (De Barro, 1992) but this suggestion is not supported by our data. Also, a spacing pheromone has been reported to act in such conditions (Pettersson *et al.*, 1995). Probing on stems, a known preferential feeding site, showed longer phloem phase after starting sustained sap ingestion (Table 6). This is an indication that the sieve elements were readily accepted on stems. *R. padi* needed a shorter time to accept its host (time to sustained sap ingestion) than *A. fabae* didi with its respective host (Figs. 2 and 3). This seems to be an intrinsic property of each aphid-plant combination (Tjallingii & Mayoral, 1992).

Our results show that the infestation effects are clearly aphid-plant combination dependent. We have no indications whether factors from the plant, (mono- versus di-cotyledon, sensitivity and/or way to be elicited by salivary components, etc.) or from the aphids (saliva composition, sensitivity to plant changes) play a major role. Nor do we have data on long term effects, like performance parameters. In general, we may conclude that changed aphid probing and feeding as a result of variations in plant tissue contents (in space and time) is a vast area in which insect-plant interactions are involved in a complex way. Our study is only an initial investigation in this field. More research is absolutely needed to better understand the observed effects.

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4. *Effects of experimental stress factors on insect behaviour in aphid-plant studies* [1]

ABSTRACT

Probing behaviour of *Aphis fabae* Scopoli and *Rhopalosiphum padi* (L.) was tested in different stress situations normally occurring in aphid-plant studies like feeding interruption or starvation, transfer to a new plant and wire attachment. The DC electrical penetration graph (EPG) technique and a "honeydew clock" were used to collect data on behavioural effects of these stress conditions. Increased interruption times (deprivation from the host-plant) stimulated the aphids to insert their stylets earlier. The time before showing the first phloem contact was hardly affected by the applied conditions. When contact with its host-plant was interrupted for 1 min *A. fabae* started phloem feeding earlier than with longer interruption times, but only when replaced on the same plant on which it fed before. It is concluded that this is partly an effect of previous experience, which vanished with longer interruption, and partly caused by changed plant properties due to earlier feeding. The observation, when tested on the previous feeding site, that less "phloem salivation" (E1) occurred before starting sustained sap ingestion supports this conclusion. Behaviour of both aphid species were somewhat affected by wiring, showing stylet penetration earlier, longer pathway phase (including F waveform, stylet penetration difficulties), phloem feeding occurred later and honeydew excretion was reduced. However, generally, no significant changes appeared in the sequence and time course of probing events resulting from aphid transfer, feeding interruption, and movement restraints.

[1] This chapter has been submitted to Entomologia Experimentalis et Applicata

INTRODUCTION

Probing behaviour by aphids, i.e. stylet penetration, plays a key role in aphid-plant interactions during host-plant selection (Pollard, 1973; Montllor, 1991; Pickett *et al.*, 1992) and virus-vector relationships (Sylvester, 1980; Martin *et al.*, 1997). Electrical monitoring has been shown to be a suitable technique to study probing behaviour and aphid host- plant relationships (McLean & Kinsey, 1964; Tjallingii, 1988; van Helden & Tjallingii, 1997). The events occurring during stylet penetration, which are recorded as waveforms, reflect aphid activities as well as stylet positions in the plant tissue (Tjallingii, 1988). The occurrence, sequence and duration of events during stylet penetration can be interpreted by parameters, such as time till the first probe, time till start of phloem phase, duration of phloem phase and sustained phloem ingestion, etc., as described earlier (Prado & Tjallingii, 1997). In most aphid-plant studies, as in electrical penetration graph (EPG) experiments, aphids are put on a new plant. Aphids are removed from their host plants before recording, thereby interrupting their feeding and stylet insertion and often causing cornicle excretion, an alarm reaction. Moreover, the aphids are deprived of food for some time and their locomotion is restricted as a wire is glued to their dorsum to make them part of an electrical circuit. So far no indication has been found of any effect of the electrical current or of substantial movement of charged particles on the probing behaviour (Tjallingii, 1985), at least not at currents actually used. Also, when glue components were carefully selected, no intoxication effects occurred. However, effects on probing behaviour due to locomotion restraints by the wire tether have been found, especially at the beginning of experiments (Tjallingii, 1986). Large aphids seem to be less affected than smaller insects, like whiteflies (Lei *et al.*, 1997a). Some wired aphids, e.g. *Acyrthosiphon pisum* (Tjallingii, 1978) and *Rhopalosiphum padi* (unpubl.) survived for up to ten days while feeding and reproducing and without any apparent physiological effect. On the other hand, wired *Nasonovia ribisnigri* aphids were more reluctant to start probing than free ones (Montllor & Tjallingii, 1989; van Helden, 1995). All these interferences differ from the aphid's normal stay on a host plant. Therefore, the question arises whether the aphids still behave normally, i.e. whether their observed behaviour is still reflecting normal host-plant selection and acceptance. Hence, every species to be monitored needs to be tested in order to detect behavioural modifications, especially when less suitable plants or resistant cultivars are involved (Caillaud *et al.*, 1995).

Host-plant selection and acceptance behaviour typically show a sequence of probes, interrupted by non-probing intervals. These probes are displayed in EPGs as a sequence of activities with increasing duration. The first probes mainly show waveforms reflecting

an intercellular penetration pathway phase (Tjallingii & Hogen Esch, 1993). Gradually, periods of phloem phase become more abundant and 10 min or longer of phloem ingestion has been used as a criterion for sieve element acceptance (Tjallingii, 1990; Prado & Tjallingii, 1997). Tjallingii (1986) demonstrated that the time course of this sequence is not very different between wired and free, non-wired aphids, which seems to indicate that tethering does not completely disturb this sequence. However, the "free" aphids used as controls in these experiments were also exposed to a number of treatments that may affect this time course of activities. Especially their removal from the original feeding plant (and site) and the period of food deprivation may be important, but their effects remain undetected in such experiments.

Previous feeding experience on the original rearing plant can induce some "memory template" in insects (Chapman & Bernays, 1989) leading perhaps to an early acceptance of the plant also in aphids. At the same time, aphid probing can modify the physiology of the feeding site or the whole plant which can show an induced resistance to aphids (Pettersson, 1996; Wool & Hales, 1996), or the opposite, i.e. to show an induced higher susceptibility (Prado & Tjallingii, 1997). Thus, in every case of a transfer to a new plant the aphid will be confronted with a situation that differs from its previous feeding plant or feeding site, even if the plant is identical with respect to species, cultivar and age. Klingauf (1972) already showed that the duration of the first walk and first probe on a new plant can be influenced by the time lapse since removal from the rearing plant, although his data showed an effect up to only 2 hours. Actually, we also found that these two parameters are rather unreliable (Prado & Tjallingii, 1997) and seem to depend more on the feeding status and, possibly the amount of stress before access to the new plants. Moreover, 2 hours without food seems a rather short time in relation to the time normally spent by aphids to achieve sustained phloem ingestion, which was found to be as long as 3 to 7 hours (Tjallingii & Mayoral, 1992).

The objective of this work was to study the effects of transfer and manipulation as well as the effects of previous plant feeding on probing behaviour, especially for those parameters used in host-plant selection studies. Two aphid host-plant combinations and three transfer period intervals from the original plant to a new plant were used. Also, the plants to which the aphids were transferred were varied and wired as well as free, i.e. non-wired aphids were tested.

MATERIALS AND METHODS

Aphids. Two aphid species, *Aphis fabae* Scopoli and *Rhopalosiphum padi* (L.) were tested for their feeding behaviour on broad beans (*Vicia faba* L.) cv "3 x wit" and on wheat (*Triticum aestivum* L.) cv Okapi, respectively. They were reared under greenhouse conditions at 20±2°C and L16:D8 photoperiod on the same plants they were tested.

Plants. The broad bean plants used in the experiments were approximately three weeks old with 4-5 mature leaves. The wheat plants were 12-14 days old with a fully expanded second leaf.

EPGs. The Electrical Penetration Graph (EPG, DC system) was recorded from apterous virginoparous adults (2-4 days after moulting) placed on the abaxial side of the youngest full grown leaf of broad bean plant or on the second leaf of a wheat plant. Before starting the EPG aphids were wired with a 20 mm long, 20 µm diameter gold wire, using water based (non toxic solvent) silver paint. For a full description of the EPG technique, experimental set-up and pattern descriptions see Tjallingii (1978, 1985, 1988 and 1997).

Effects of feeding interruption and transfer. Tested aphids were taken from their rearing plant and wired. Subsequently, the aphids were placed on a fresh host plant for 48 h. Their probing behaviour was recorded during the first three hours and the last hour of this period in order to be sure that the aphids behaved/probed normally, i.e. without periods of non-probing longer than half an hour. Only aphids showing pathway or phloem phase activities during the last hour prior to the removal were used. After this 48 h period, probing of these aphids was interrupted for a period (i) of 1, 10 or 100 min by gently lifting the aphid from the plant. After the interruption, supposed to be a stressful situation, the aphid was either placed back to the same plant and probing site, or transferred to a new plant of the same species. Then, EPG recording was resumed for 8 hours. Considering one aphid as a replicate, 19 to 23 replicates were used. The offspring produced during the 48 h pre-treatment period was removed when aphids were placed back on the same plant.

Wire effects. Probing behaviour of *A. fabae* and *R. padi* with a wire attached to the dorsum and permanently connected to the amplifier, as is normally done in the EPG technique, was compared with aphids having no wire but only a small drop of silver paint on the dorsum. The latter aphids could freely move and their EPG was recorded at

intervals of 0.25, 0.50, 1.0, 1.50, 2.50, 4.0, 6.0 and 8.0 h after access to the plant. Recording was done only for a few seconds by gently touching the silver paint drop with an electrode by means of a micromanipulator. The aphids seemed not to be disturbed by the wire and no immobilisation reflexes were observed either. The numbers of aphids showing a certain waveform at each interval were compared between permanently wired and free aphids. Twenty-five to twenty-seven aphids per treatment were recorded.

Honeydew excretion. The number of honeydew droplets excreted during 24 h was compared between wired, as done for EPG recording, and free aphids. Aphids were starved for one hour prior to the recording. The frequency of honeydew production was measured on a 24 h honeydew clock using a TLC aluminium silica gel chromatography paper impregnated with ninhydrin (in butanol and acetic acid solution) which stains the amino acid content of the honeydew (Mittler, 1958). Results from free aphids that moved out of the area of the paper for honeydew detection were discarded. Aphids were placed on the abaxial side of the leaf and the honeydew clock placed at about 2 mm under the aphids. Sixteen and twelve *A. fabae* and ten and nine *R. padi* were recorded for free and wired aphids respectively.

Parameters and some terminology. Only the parameters obtained during the 8-h EPG following the interruption interval were compared between the treatments. Patterns A, B and C were lumped, and considered as a pathway phase (intercellular path with cell punctures). A probe is the period between the start of any stylet penetration and the stylet withdrawal. Phloem phase is the period with the stylet inserted into the sieve element (EPG shows waveforms E1 or E2). Phloem salivation (E1) is the first period in a phloem phase showing saliva injection into a sieve element. Phloem ingestion (E2) is any period of phloem ingestion, sustained or not. Sustained (sap) ingestion (E2>10 min) is phloem sap ingestion lasting longer than 10 min. For schematic representation of some parameters see Chapter 3 or Prado & Tjallingii (1997).

Statistical analysis. The different parameters and treatments were compared by the Kruskal-Wallis test, followed by a multiple comparison ($a=0.05$) (Conover, 1980). No transformation was found to match data with normal distribution and therefore, differences between transfers (to the same or to the new plant) and interruption intervals were compared by the Mann-Whitney Unpaired Test. Nevertheless, interactions and the effect of each variable were checked by an analysis of variance. Honeydew results were also analysed by the Mann-Whitney U Test.

RESULTS

STARVATION AND TRANSFER EFFECTS.

Initial probing. The time until the first probe decreased significantly with increased time of interruption in *A. fabae* (Table 1), irrespective of whether the aphids were placed on the same plant or transferred to a new plant. The duration of the first probe (in spite of some numerical differences), the total number of probes, and the total probing duration, were not significantly different between any of the treatments. *R. padi* (Table 2) also showed a decreased in time until the first probe with increased interruption time, but only when transferred to a new plant. Other significant differences were found, only on a new plant as well, with respect to the duration of the first probe and the total probing duration. Comparing the effects of transfer revealed significant differences for the interruption periods of 1 and 100 min for the first period of non-probing, but in an opposite sense, i.e. a higher value was found when transferred to a new plant after 1 min interruption and a lower value after 100 min interruption (Table 2, underlined values).

In general, interruption time showed a rather clear negative correlation with the first non-probing time, except for *R. padi* on the same plant. No other consistent effects were shown by increasing interruption periods or transfer types.

Reaching the phloem. Parameters 1 to 5 in Table 3 and 4 refer to a period "within experiment", when taking the time from the start of the first probe in the experiment until the event concerned (waveform). Similarly, "within probe" is used when the time was taken from the start of the probe in which the event concerned occurred until the start of the event itself.

A. fabae on its previous feeding plant (Table 3A), showed no significant differences with increased interruption time in time to reach first phloem phase within the experiment, as well as within a probe (probe showing a phloem phase, parameters 2 and 3). However, a tendency was found for an increased delay of phloem ingestion (sustained or not; parameters 4 - 6). This trend was not found in aphids transferred to a new plant, and at the 1 min interruption the difference between transfer plants was significant for two parameters (4 and 6; underlined values). The two remaining parameters (1 and 7) do not indicate any clear consequences or causes for the tendency shown by the former parameters and no other effects either. *R. padi* (Table 4B) showed an increased non-probing period before reaching the first phloem phase (parameter 1) when its probing had been interrupted for 100 min.

Table 1. General parameters and initial probing behaviour of *A. fabae* after a period of feeding interruption (i).

A. Aphids returned on the same plant	(unit)	i= 1 min n= 20 Means (SE)	i= 10 min n= 19 Means (SE)	i= 100 min n= 23 Means (SE)	Kruskal-Wallis p-value
1. Time to 1st probe	(min)	6.4 (1.3) a	4.2 (1.6) b	1.4 (0.5) c	**< 0.001**
2. Duration of 1st probe	(min)	7.4 (2.6)	14.2 (7.3)	8.6 (5.2)	0.306
3. Total number of probes	(#)	11.8 (2,0)	11.2 (1.9)	12.7 (2.4)	0.888
4. Total probing duration	(h)	7.5 (0.1)	7.5 (0.1)	7.6 (0.1)	0.440
B. Aphids transferred to a new plant	**(unit)**	**i= 1 min n= 20 Means (SE)**	**i= 10 min n= 21 Means (SE)**	**i= 100 min n= 20 Means (SE)**	
1. Time to 1st probe	(min)	7.6 (1.3) a	6.7 (3.1) b	1.6 (0.4) b	**<0.001**
2. Duration of 1st probe	(min)	8.6 (4.1)	37.9 (23.0)	80.0 (37.7)	0.564
3. Total number of probes	(#)	9.5 (1.6)	9.1 (1.8)	8.8 (0.1)	0.803
4. Total probing duration	(h)	7.4 (0.1)	7.5 (0.1)	7.6 (0.1)	0.153

Means followed by different letters within rows are significantly different according to the Kruskal-Wallis test followed by multiple comparisons (α =0.05). (SE = Standard error). Comparisons of each interruption time between transfers were tested by the Mann Whitney U test. Significant differences (α=0.05) are underlined.

Table 2. General parameters and initial probing behaviour of *R. padi* after a period of feeding interruption (i).

A. Aphids returned on the same plant	(unit)	i= 1 min n= 22 Means (SE)	i= 10 min n= 21 Means (SE)	i= 100 min n= 21 Means (SE)	Kruskal-Wallis p-value
1. Time to 1st probe	(min)	2.7 (0.4)	4.0 (1.0)	3.4 (1.1)	0.862
2. Duration of 1st probe	(min)	83.2 (35.2)	39.4 (22.3)	39.2 (22.9)	0.774
3. Total number of probes	(#)	9.7 (1.7)	10.2 (1.9)	10.6 (1.5)	0.784
4. Total probing duration	(h)	7.5 (0.1)	7.3 (0.2)	7.2 (0.2)	0.434
B. Aphids transferred to a new plant		**i= 1 min n= 20 Means (SE)**	**i= 10 min n= 20 Means (SE)**	**i= 100 min n= 22 Means (SE)**	
1. Time to 1st probe	(min)	4.2 (0.5) a	2.8 (0.5) b	1.7 (0.5) c	**<0.001**
2. Duration of 1st probe	(min)	41.5 (14.7) ab	77.5 (34.0) a	16.6 (8.4) b	**0.039**
3. Total number of probes	(#)	10.5 (1.7)	8.3 (1.5)	14.0 (1.9)	0.055
4. Total probing duration	(h)	7.6 (0.1) b	7.7 (0.1) a	7.4 (0.1) b	**0.027**

Means followed by different letters within rows are significantly different according to the Kruskal-Wallis test followed by multiple comparisons (α =0.05). (SE = Standard error).Comparisons of each interruption time between transfers were tested by the Mann Whitney U test. Significant differences (α =0.05) are underlined.

However, this initial effect did not delay the first phloem phase or phloem ingestion and neither did it affect the number (or %) of aphids showing any sustained phloem ingestion (parameter 7). The few differences between transfer treatments (underlined values) were irregularly scattered among treatments.

Phloem parameters. After 1 min interruption on the same plant, *A. fabae* showed a significant shorter phloem salivation (E1) just before starting a sustained feeding (Table 5, parameter 1) either compared with other intervals or with aphids transferred to a new plant. On the same plant this salivation period gradually increased with interruption time, whereas on a new plant no such effect was shown. No further effects of plants or intervals were found. *R. padi* (Table 6) here again, showed some differences irregularly scattered among treatments. No correlation with interval duration or consistent differences between plants (underlined values) was found for these parameters. Phloem salivation just before ingestion (parameter 1), on the same plant and at 1 min interval was also smaller than on a new plant, as in *A. fabae*. However, no gradual change with intervals occurs on the same plant, and on a new plant the figures are less clear than those observed in *A. fabae*.

Differences between aphid-plant combinations (Table 7). *A. fabae* reached the phloem in about 3.2 h (intervals lumped) after the start of the first probe, and started sustained sap ingestion after 4.5 h, whereas *R. padi* needed 0.8 and 2.2 h, respectively. These values are significantly different between both species (Mann-Whitney U test, $p<0.01$). *A. fabae* had fewer periods of phloem salivation (E1) than *R. padi*, but they lasted longer, resulting in longer duration of individual phloem salivation periods (E1). However, once the phloem phase was reached for the first time both aphids spent about 71% of the time with their stylets in the sieve elements.

WIRE EFFECT (Figs. 1 and 2). The main wire effects detected in both aphid species were fewer individuals showing non-probing behaviour in the beginning (first 90 min in *A. fabae* and first 30 min in *R. padi*), and more insects exhibiting F waveforms (stylet penetration difficulties) in wired aphids. Especially later on, a lower number of aphids showed phloem sap ingestion. Xylem ingestion (waveform G) and phloem salivation (E1), on the other hand, were hardly affected by wiring.

EFFECTS OF WIRING ON HONEYDEW EXCRETION. Wiring significantly reduced honeydew excretion only in *R. padi* (Table 8). This is in agreement with the observed

Table 3. Probing behaviour of *A. fabae* before reaching the phloem and after a period of feeding interruption (i).

A. Aphids returned on the same plant	(unit)	**i= 1 min n= 20** Means (SE)	**i= 10 min n= 19** Means (SE)	**i=100 min n= 23** Means (SE)	**Kruskal-Wallis p-value**
1. Non-probing to 1st phloem phase within experiment	(%)	12.1 (1.8) n=20	7.4 (1.3) n=17	11.6 (1.7) n=22	0.119
2. Time to 1st phloem phase within experiment	(h)	2.6 (0.4)	3.4 (0.5)	3.1 (0.4)	0.473
3. Time to 1st phloem phase within probe	(h)	0.8 (0.2)	1.9 (0.6)	1.3 (0.4)	0.221
4. Time to phloem ingestion within probe	(h)	1.4 (0.5) b	2.4 (0.6) ab	2.8 (0.6) a	**0.047**
5. Time to sustained ingestion within experiment	(h)	3.9 (0.6)	4.4 (0.6)	4.5 (0.5)	0.806
6. Time to sustained ingestion within probe, (only aphids showing the event)	(h)	0.8 (0.1) n=17	1.2 (0.2) n=16	1.7 (0.3) n=19	0.055
7. Aphids showing sustained ingestion	(#) (%)	17 85 %	16 84.2 %	19 82.6 %	
B. Aphids transferred to a new plant		**i= 1 min n= 20** Means (SE)	**i= 10 min n= 21** Means (SE)	**i= 100 min n= 20** Means (SE)	
1. Non-probing to 1st phloem phase within experiment	(%)	12.4 (2.9) n=19	9.3 (1.9) n=17	9.1 (2.4) n=19	0.451
2. Time to 1st phloem phase within experiment	(h)	3.0 (0.5)	3.8 (0.5)	3.0 (0.5)	0.390
3. Time to 1st phloem phase within probe	(h)	1.1 (0.4)	2.3 (0.6)	1.4 (0.4)	0.550
4. Time to phloem ingestion within probe	(h)	2.1 (0.5)	3.0 (0.7)	3.3 (0.7)	0.530
5. Time to sustained ingestion within experiment	(h)	4.9 (0.5)	4.7 (0.5)	4.8 (0.6)	0.992
6. Time to sustained ingestion within probe, (only aphids showing the event)	(h)	1.6 (0.2) n=17	1.5 (0.3) n=16	1.9 (0.3) n=14	0.341
7. Aphids showing sustained ingestion	(#) (%)	17 85%	16 76.2 %	14 70 %	

Means followed by different letters within rows are significantly different according to the Kruskal-Wallis test followed by multiple comparisons (α =0.05). (SE = Standard error).
Comparisons of each interruption time between transfers were tested by the Mann Whitney U test. Significant differences (α =0.05) are underlined.

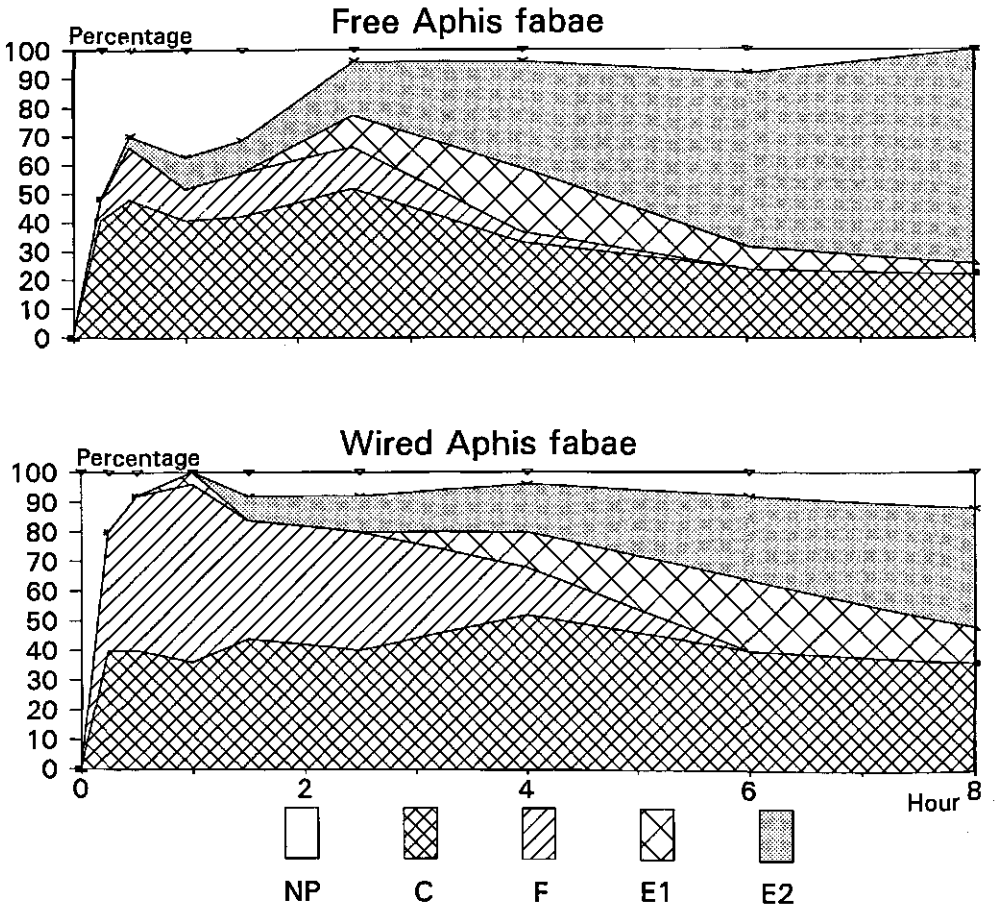

Figure 1. Probing behaviour waveforms comparison between free and wired *A. fabae*. NP: non-probing; C: extracellular pathway (excluding F); F: stylet penetration difficulties in extracellular position; E1: salivation into sieve element; E2: phloem sap ingestion

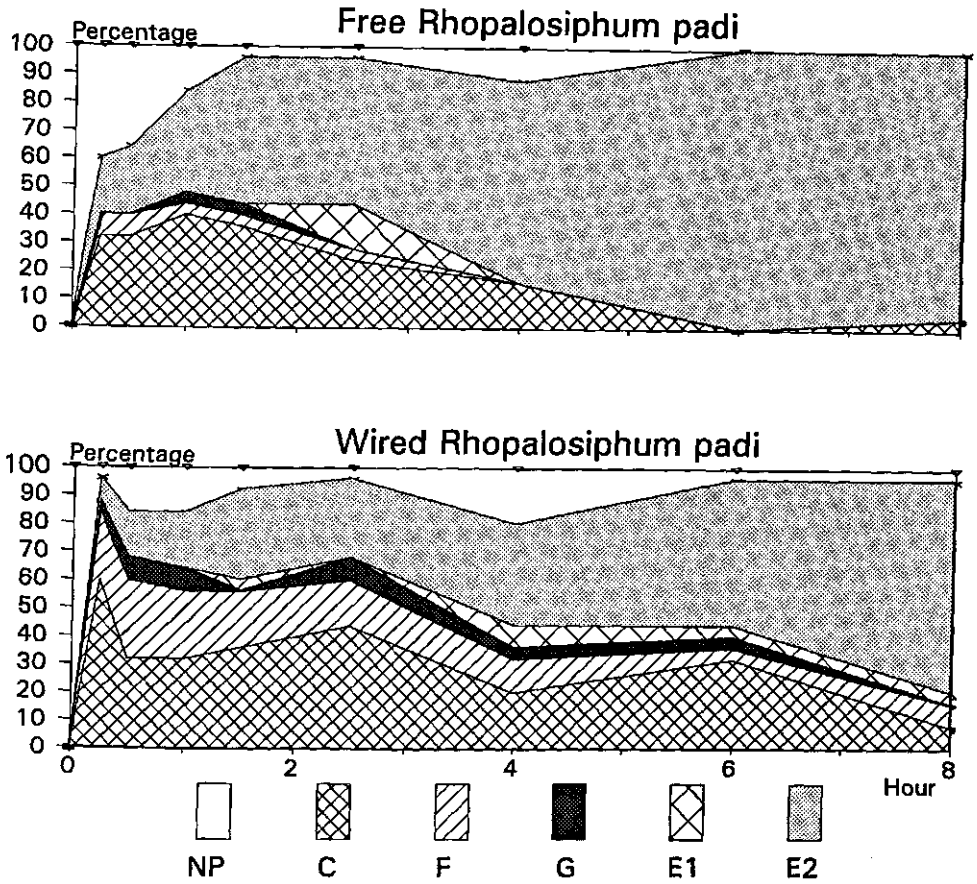

Figure 2. Probing behaviour waveforms comparison between free and wired *R. padi*. NP: non-probing; C: extracellular pathway (excluding F); F: stylet penetration difficulties in extracellular position; G: xylem ingestion; E1: salivation into sieve element; E2: phloem sap ingestion

reduction of phloem sap ingestion (E2) by wired aphids as shown in Fig. 1 and 2. Though a similar reduction in E2 was observed for *A. fabae* this was not reflected in the honeydew excretion. *R. padi* excreted 4 to 8 fold more honeydew droplets in 24 h than *A. fabce*, for wired and free aphids, respectively.

DISCUSSION

In this study almost 60 parameters were calculated using numbers and duration of events or periods between certain events. To some parameters all aphids contributed, to others only those that showed the event concerned to avoid biased results (Tjallingii, 1996). Not all differences that were statistically significant could be interpreted. Differences with respect to some parameters were either isolated, in contradiction with other parameters, or not well correlated among treatments. In such cases we preferred to avoid drawing conclusions.

An increased interruption or starvation period (aphids deprived from their food plant) stimulated both aphid species to insert their stylets earlier, which is in agreement with some earlier studies (Klingauf, 1987). Whereas, it has widely been reported that starved aphids transmit non-persistent plant viruses more efficiently due to early and more frequently probes (Sylvester, 1989; Powell, 1993), it seems unlikely that starting probing a few minutes earlier, as such, has any causal relation to this. The duration of the first probe and some other (not shown) initial probing parameters appeared to be unaffected by the interval time under our experimental conditions, but one should interpret these probing parameters cautiously because they often show a huge variability. As shown for *R. padi*, the interruption effect on the time to the first probe was absent when the aphids were returned to their original feeding plant (Table 2A, parameter 1), although in some cases significant differences appeared of which the biological relevance remains unclear. Total probing time and the total number of probes were not affected by increasing the interruption time, neither was the period of total non-probing between first probe and first phloem phase. Numbers of probes (visually detected) has been reported to increase with starvation time for *Myzus persicae* and *Acyrthosiphon pisum* (Sediles, 1992) in studies with unwired aphids during an observation period as short as 60 min, which is not comparable to our conditions.

For *A. fabae*, some earlier phloem phase activity was shown after short interruption intervals (significant with respect to the time to phloem ingestion within a probe). This also appeared in values of other parameters when aphids were returned to their original

Table 4. Probing behaviour of *R. padi* before reaching the phloem and after a period of feeding interruption (i).

A Aphids returned on the same plant	(unit)	**i= 1 min n= 22 Means (SE)**	**i= 10 min n= 21 Means (SE)**	**i= 100 min n= 21 Means (SE)**	**Kruskal-Wallis p-value**
1. Non-probing to 1st phloem phase within experiment	(%)	8.0 (2.5)	8.7 (2.8)	16.5 (4.1)	0.280
2. Time to 1st phloem phase within experiment	(h)	0.9 (0.1)	0.6 (0.1)	1.0 (0.3)	0.367
3. Time to 1st phloem phase within probe	(h)	0.4 (0.1)	0.2 (0.0)	0.4 (0.1)	0.123
4. Time to phloem ingestion within probe	(h)	0.5 (0.1)	0.3 (0.0)	0.5 (0.1)	0.704
5. Time to sustained ingestion within experiment	(h)	2.2 (0.3)	2.6 (0.5)	2.0 (0.4)	0.855
6. Time to sustained ingestion within probe, (only aphids showing the event)	(h)	0.5 (0.0) n=22	0.4 (0.1) n=20	0.6 (0.1) n=20	0.331
7. Aphids showing sustained ingestion	(#)	22	20	20	
	(%)	100 %	95.2 %	95.2 %	
B. Aphids transferred to a new plant	(unit)	**i= 1 min n= 20 Means (SE)**	**i= 10 min n= 20 Means (SE)**	**i= 100 min n= 22 Means (SE)**	
1. Non-probing to 1st phloem phase within experiment	(%)	6.7 (1.6) b	4.3 (1.1) b	14.9 (2.3) a	**0.002**
2. Time to 1st phloem phase within experiment	(h)	0.9 (0.2)	0.5 (0.1)	0.7 (0.1)	0.525
3. Time to 1st phloem phase within probe	(h)	0.5 (0.1)	0.3 (0.0)	0.3 (0.1)	0.677
4. Time to phloem ingestion within probe	(h)	0.6 (0.1)	0.4 (0.1)	0.5 (0.1)	0.447
5. Time to sustained ingestion within experiment	(h)	2.0 (0.4)	1.4 (0.4)	3.0 (0.5)	0.055
6. Time to sustained ingestion within probe, (only aphids showing the event)	(h)	0.5 (0.1) n=20	0.4 (0.1) n=19	0.7 (0.1) n=21	0.359
7. Aphids showing sustained ingestion	(#)	20	19	21	
	(%)	100 %	95 %	95.5 %	

Means followed by different letters within rows are significantly different according to the Kruskal-Wallis test followed by multiple comparisons (α =0.05). (SE = Standard error).
Comparisons of each interruption time between transfers were tested by the Mann Whitney U test. Significant differences (α =0.05) are underlined.

feeding plant and site (Table 3A) though not statistically significant. Also, the salivation time just before the first sustained ingestion period seems to support that (Table 5A, parameter 1). These effects were not found when *A. fabae* was transferred to a new plant, neither for *R. padi*, irrespective of the transfer plant. Facilitation did not result unambiguously in increased phloem feeding (Table 5 and 6, parameters 3 and 4). The foregoing seems in accordance with what we found on leaves previously colonised by these aphid species (Prado & Tjallingii, 1997). Thus, possibly the 48 hours of probing activity (some offspring had been produced as well) by *A. fabae* may have changed the *Vicia* leaf locally and this result can be explained as a small colony effect. On the other hand, the facilitating effects on initial phloem phase activities appeared especially at the 1 min interruption, either statistically significant or only numerically. Therefore, some short-term memory effect of previous experience cannot be excluded. In general, however, the exploratory behaviour of the aphid seems to be 'reset' by the interruption, which is an important conclusion for the interpretation of results in food plant selection experiments.

Some differences between both aphid species were evident. *R. padi* reached the phloem and started sustained phloem sap ingestion earlier than *A. fabae,* and exhibited shorter periods of salivation into the sieve elements (E1) as well. A difference has also been found between *A. fabae* and *Sitobion avenae* with respect to the former parameter (Tjallingii & Mayoral, 1992). Nevertheless, after the first phloem phase both aphids, *A. fabae* and *R. padi*, showed a similar proportion of time (about 71%) with their stylets in the phloem. The observed differences are probably dependent on the aphid species, the plant species, or the specific aphid-plant combination. It is still unknown why *A. fabae* needs so long to explore the plant tissue (pathway waveforms) without any phloem phase and why this period, as well as the phloem salivation period just before sap ingestion is much longer than in *R. padi.* Plant species characteristics may be responsible. However, the absolute distance between epidermis and phloem in bean plants and wheat, although possibly playing some role, could not fully explain the differences found in some cases (Lei *et al.*, 1997b; Gabrys *et al.*, 1997). Earlier suggestions that sieve element salivation might prevent possible coagulation of phloem proteins (Evert, 1990; Tjallingii & Hogen Esch, 1993) might explain the longer salivation before ingestion in *Vicia* as phloem proteins are absent in wheat (Eleftheriou, 1990). However, aphids probing on earlier colonised plants, which might be expected to profit from anti-coagulant effects accomplish by the colony, did not show a convincing reduction of phloem salivation before sap feeding in previous experiments (Prado & Tjallingii, 1997).

Table 5. Probing behaviour of *A. fabae* after reaching the phloem and after a period of feeding interruption (i).

A. Aphids returned on the same plant	(unit)	**i= 1 min n= 20** Means (SE)	**i= 10 min n= 19** Means (SE)	**i= 100 min n= 23** Means (SE)	**Kruskal-Wallis p-value**
1. Period of phloem salivation just before 1st sustained ingestion (aphids + event)	(min)	7.4 (4.4) b n=17	15.2 (6.7) ab n=16	18.0 (5.8) a n= 9	0.040
2. Total phloem salivation	(min)	28.8 (8.6)	31.7 (11.7)	37.2 (7.4)	0.352
3. Total phloem ingestion	(h)	3.5 (0.6)	3.0 (0.5)	3.2 (0.4)	0.775
4. Time phloem phase after starting 1st phloem phase	(%)	73.4 (7.1)	69.2 (8.2)	73.1 (6.7)	0.993
B. Aphids transferred to a new plant	(unit)	**i= 1 min n= 20** Means (SE)	**i= 10 min n= 21** Means (SE)	**i= 100 min n= 20** Means (SE)	
1. Period of phloem salivation just before 1st sustained ingestion (aphids + event)	(min)	24.9 (8.0) n=17	20.8 (8.5) n=16	34.7 (11.1) n=14	0.344
2. Total phloem salivation	(min)	39.1 (8.2)	34.2 (8.1)	39.0 (8.9)	0.632
3. Total phloem ingestion	(h)	3.0 (0.5)	3.0 (0.5)	3.0 (0.6)	0.957
4. Time phloem phase after starting 1st phloem phase	(%)	73.1 (7.0)	70.4 (8.3)	66.9 (8.4)	0.825

Means followed by different letters within rows are significantly different according to the Kruskal-Wallis test followed by multiple comparisons (α =0.05). (SE = Standard error). Comparisons of each interruption time between transfers were tested by the Mann Whitney U test. Significant differences (α =0.05) are underlined.

Free aphids initially showed higher numbers of non-probing insects than wired aphids, which can be explained by their unrestrained possibilities to walk and change probing sites. Remarkably, higher numbers of aphids show waveform F when wired. This waveform has been correlated with "mechanical difficulties" during stylet penetration, i.e. their mandibles and maxilla were dislocated (Tjallingii, 1987). Waveform F could be caused by stress or by movement restrictions, for example, no or fewer possibilities to rotate the stylets inside the stylet sheath, thereby limiting the angle over which the tips can be changed. This should be investigated further. The occurrence of F waveforms during early probing may have delayed phloem sap ingestion, hence reducing the total duration of phloem sap ingestion (E2) as well, in the 8-h recordings. In other studies on wired and free aphids no increase of F waveform has been reported (Tjallingii, 1986; van Helden, 1995).

Table 6. Probing behaviour of *R. padi* after reaching the phloem and after a period of feeding interruption (i).

A. Aphids returned on the same plant	(unit)	i= 1 min n= 22 Means (SE)	i= 10 min n= 21 Means (SE)	i= 100 min n= 21 Means (SE)	Kruskal-Wallis p-value
1. Period of phloem salivation just before 1st sustained ingestion (aphids + event)	(min)	3.2 (0.7) n=22	5.7 (1.3) n=20	4.3 (1.1) n=20	0.205
2. Total phloem salivation	(min)	18.8 (2.8)	18.4 (2.5)	18.4 (3.2)	0.976
3. Total phloem ingestion	(h)	4.8 (0.5)	5.2 (0.5)	4.3 (0.4)	0.338
4. Time phloem phase after starting 1st phloem phase	(%)	71.4 (5.7)	74.1 (6.1)	64.3 (5.5)	0.422
B. Aphids transferred to a new plant	**(unit)**	**i= 1 min n= 20 Means (SE)**	**i= 10 min n= 20 Means (SE)**	**i= 100 min n= 22 Means (SE)**	
1. Period of phloem salivation just before 1st sustained ingestion (aphids + event)	(min)	6.1 (2.3) a n=20	1.9 (0.7) b n=19	5.1 (1.8) a n=21	**0.048**
2. Total phloem salivation	(min)	19.4 (3.8)	18.1 (4.1)	28.1 (3.6)	0.065
3. Total phloem ingestion	(h)	4.9 (0.4) ab	5.6 (0.4) a	4.1 (0.4) b	**0.021**
4. Time phloem phase after starting 1st phloem phase	(%)	74.0 (5.0)	79.9 (5.0)	63.4 (5.1)	0.054

Means followed by different letters within rows are significantly different according to the Kruskal-Wallis test followed by multiple comparisons (α =0.05). (SE = Standard error).Comparisons of each interruption time between transfers were tested by the Mann Whitney U test. Significant differences (α =0.05) are underlined.

Wiring seems to cause decreased honeydew excretion (i.e. number of droplets), reflecting less sap ingestion in *R. padi*. However, honeydew excretion by free aphids may have been overestimated in both species as only aphids remaining at their original site, probably those that started early with sustained phloem ingestion, were recorded. Aphids that walked out of the area of honeydew detection on paper were discarded. Wired aphids could not walk out of the honeydew recording site. To consider the excretion rate as a direct measure of ingested food is partly true. Droplet size is an indirect way to estimate the volume of a droplet but inaccuracy is also present as viscosity cannot be measured. Comparisons can be done only within an aphid species so far droplets size varies little in individuals (c.a. 5.6% in *A. fabae* and 6.1% in *R. padi*, unpubl.). Any comparison

between species is useless because of the difference in aphid size, food canal, host plant (sieve element pressure), etc.

Summarising, we may conclude that transferring *A. fabae* and *R. padi* to their own host plant affected the subsequent behaviour in different ways. The interruption time affected mainly the first non-probing period, negatively correlated, whereas some effects may be due to previous plant experience when interrupted for a very short period (1 min). This memory effect by previous experience disappeared very fast. In general, however, interruption of feeding seems to cause a reset of the behaviour, i.e. same delay, sequence and duration of probing activities, independent of interruption time differences between 10 and 100 min, the most common time used in insect-plant experiments. Thus, the sequence of events observed when aphids are removed from their original rearing plant seems unaffected by the transfer to a new plant, at least with respect to interruption time. We did not perform transfers to non-host plants or resistant cultivars. Therefore, it is impossible to estimate the role of previous plant experience in such test situations. Wiring, which limits the aphid movements and restricts the feeding area, seems initially to enhance probing, but later to reduce sap feeding somewhat and, as a consequence, reduce honeydew excretion. Thus, the number of probes, probing time, and other parameters will be overestimated in wired aphids kept on unsuitable or resistant host plants. Wired aphids on non-host plants are forced to stay and appear to show probing, while in normal situations they would move to other plants (Tjallingii, 1986; Caillaud *et al.*, 1995). Apart from aphid effects, our results showed some effects on probing behaviour which seem to be caused by the plant as a result of earlier probing by aphids. These effects were weak and specific for *A. fabae-Vicia* as it has been demonstrated for heavier infestations (Prado & Tjallingii, 1997).

Table 7. Behavioural differences between *A. fabae* and *R. padi*.

	(unit)	*A. fabae*	*R. padi*
1. Time to 1st phloem phase from 1st probe	(h)	3.2 (0.2) a	0.8 (0.2) b
2. Time to 1st sustained sap ingestion from 1st probe	(h)	4.5 (0.2) a	2.2 (0.2) b
3. Number of phloem salivation periods	(#)	3.0 (0.2) b	8.4 (0.6) a
4. Average duration of phloem salivation	(min)	15.0 (2.5) a	2.9 (0.3) b
5. Percentage of phloem phase after starting 1st phloem phase	(%)	71.1 (3.0) a	71.0 (2.2) a
6. Period of phloem salivation just before 1st sustained ingestion	(min)	19.7 (3.1) a	4.4 (0.6) b

Treatments followed by different letters into rows are significantly different according to the Mann-Whitney U-test (α =0.05).

Table 8. Honeydew excretion on free and wired aphids. Number of honeydew droplets within 24 h (1 h on plant pre-recording)

	A. fabae	*R. padi*
Free aphids	9.8 (1.6) a n = 16	80.2 (6.1) a n = 10
Wired aphids	7.4 (2.0) a n = 12	31.0 (8.9) b n = 9

Treatments followed by different letters into columns are significantly different according to the Mann-Whitney U-test (α =0.05).

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5. Backgrounds of phloem salivation and sap ingestion by aphids

ABSTRACT

Probing behaviour of the aphid *Sitobion avenae* (F.) on resistant and susceptible wheat, *Triticum* spp., was monitored for 8 h using electrical penetration graphs (EPGs). Adults were placed on resistant *T. monococcum* line TM 44, on moderate resistant TM 47, and on susceptible *T. aestivum* cv Millaleu. It was confirmed that resistance factors are located in the phloem as most aphids failed to ingest phloem sap on resistant plants. When parameters related to phloem salivation were critically evaluated longer phloem salivation periods (E1) before sap ingestion (E2) on the resistant line TM 44 could not be found. Therefore, this aphid-plant combination, *S. avenae* on line TM 44, appeared not to be more suitable than any other, for further research into the function of E1 in plant penetration behaviour. No honeydew excretion was recorded during a few long phloem salivation periods (E1 waveforms) by *S. avenae* and *Rhopalosiphum padi*, which confirm the idea that no significant ingestion takes place during this phase. Variation in droplet size during E2 waveforms was small, but excretion intervals varied considerably. Regular and irregular honeydew deposition were compared with waveforms features (frequencies and amplitudes) during phloem ingestion (E2), but no correlation could be demonstrated between these features and droplet intervals, nor with droplet sizes. The long droplet intervals and the time lag between ingestion and excretion form a problem in these correlations.

INTRODUCTION

Earlier experiments (previous chapters) have indicated the importance of phloem phase activities in aphid-plant interactions and the impact of biotic factors on them, as studied using electrical penetration graph (EPG).

Phloem salivation, occurring during E1 waveforms (Chapter 2), is supposed to play some specific role before phloem ingestion starts. The results of the pre-infestation experiments (Chapter 3) did not completely clarify that role and therefore another

approach was chosen. Caillaud *et al.* (1995) investigated the resistant *Triticum monococcum* line TM 44 and suggested that some phloem factor was responsible for resistance as the phloem salivation (E1 waveforms in EPGs) was found to last longer and subsequently hardly any phloem ingestion (E2 waveforms) occurred. Therefore, S. avenae on line TM 44 has been used here to investigate the involvement of sieve element salivation and the acceptance of a sieve element for feeding in the resistance of this line.

Sieve element ingestion is supposedly mainly a passive process due to the hydrostatic pressure of the phloem sap in the plant. The ingestion rate has been found to be very stable over long periods of time as reflected by honeydew clock recording (Banks & Macauley, 1964) and it seems that volume intake is controlled by the length of the feeding period rather than by changing the intake rate (Tjallingii, 1995). Notwithstanding the very stable excretion rate of honeydew during long periods of EPG recorded phloem ingestion (waveform E2), a considerable variation has been observed during the ingestion waveforms (Tjallingii, 1990). EPG waveform correlation with honeydew recording were also studied by Lei *et al.* (1996) in whitefly larvae, producing smaller honeydew droplets at a much higher frequency than aphids.

The aim of this study is to investigate the role of phloem salivation activity (E1) in a case of host-plant resistance, and the relation of E1 and E2 waveform activities with ingestion and honeydew excretion rates by aphids.

MATERIALS AND METHODS

Plants. Seeds of *T. monococcum* TM 44 and TM 47 were obtained from INRA-Rennes, France, while those of *T. aestivum* cv Millaleu came from INIA-La Platina, Chile. Plants were grown under greenhouse conditions, at 20±2°C and L16:D8 photoperiod, and used for experiments at about 12-14 days old at the stage of two leaves.

Aphids. S. avenae originated from a stock culture kept at the Chile University, Santiago. *Rhopalosiphum padi* came from a colony started with a clone collected in Spain (CSIC, Dept. of Biological Research, Madrid). Aphids were reared on barley and wheat, respectively, in an environmental chamber at 22 ± 2 °C and L16:D8 photoperiod.

EPGs. The electrical penetration graph (EPG) was recorded from apterous virginoparous adults 2-4 days after moulting. The EPG technique (DC system), has been described elsewhere (van Helden & Tjallingii, 1993; Prado & Tjallingii, 1994; Tjallingii 1988). Recordings lasted 8 h and started in the morning about 1 h after collecting aphids from their rearing plants and after wiring them, and signals were directly stored on a computer harddisk and analysed later. Each treatment was repeated 23 or 25 times.

Waveform analysis. The same software used for acquisition, Stylet 2.2, was employed for analysis. Waveforms A, B and C were together considered to represent the pathway phase (Tjallingii, 1990; Prado & Tjallingii, 1994). Phloem salivation (E1) and phloem sap ingestion (E2), the two waveforms referred to as phloem phase, could clearly be recognised from each other but sometimes they shared a transition period during which both waveforms were mixed, making it difficult to decide when phloem salivation ended and phloem sap ingestion started. It is relevant to point out this difficulty, because the biological background of this intermixed area is still unknown. An E2 waveform longer than 10 min was considered as sustained sap ingestion and interpreted as phloem acceptance. More than 70 parameters were calculated, but only the most relevant are presented. Certain parameters were calculated using all aphids and using the maximum possible value for aphids that had shown the event concerned (Prado & Tjallingii, 1997), but at the same time, these parameters were also calculated excluding those aphids which never showed the event (i.e. presence or absence of phloem related parameters) (van Helden *et al.*, 1997).

Honeydew production. Honeydew excretion and EPGs were recorded simultaneously. EPGs of adult apterae of *R. padi* were recorded on wheat cv Okapi and *S. avenae* on wheat cv Millaleu and line TM 44. Electrode wires were attached to the dorsum of the aphid from the lateral side in order not to obstruct falling honeydew droplets. Honeydew recording started one hour after the aphids were placed on the leaf to assure settlement and to avoid that aphids walked out of the area above the strip for honeydew recording. Honeydew production was measured on a 24 h honeydew clock, using a 2 cm wide strip of aluminium TLC plate (silica gel; Merck, type 60F254) impregnated with ninhydrin (in butanol and acetic acid solution) which stains the amino acids in the honeydew. The number and diameter of deposited droplets was measured to estimate the amount of honeydew produced, by using an eyepiece micrometer in a stereo microscope.

Statistical analysis. The treatments were compared by the Kruskal-Wallis test, followed by a multiple comparison (α=0.05) (Conover, 1980). In case of significance close to 10% the Mann-Whitney U test was applied to compare pairs. Proportional differences were analysed with the chi square test (α=0.05).

Honeydew excretion intervals and droplet sizes were tested by analysis of variance. A correlation analysis was done between intervals and wave frequency, and wave amplitude.

RESULTS

The period of time between placing the aphids on the plant and their first probe was not significantly different between the lines or cultivar (Table 1, parameter 1). Likewise, no other parameter related to the time before the first phloem phase showed significant differences between treatments (Table 1, parameters 2 to 5). These results indicate that no constraints were present during stylet penetration on the route to the phloem, which leads to the conclusion that no resistance factors are located at the epidermal or the mesophyll level.

During phloem phase the insects exhibited differences in behaviour on different lines or cultivar. Similar numbers of aphids showed phloem phase (Table 2, parameter 6) on each plant according to the chi-square test (p=0.31). However, only a small proportion of the aphids placed on the resistant TM 44, about half of those placed on the moderately resistant TM 47, and about 80% on the susceptible cultivar Millaleu showed sustained ingestion of phloem sap (Table 2, parameter 7). On TM 44 about 65% of the aphids presented only phloem salivation (E1 waveforms) without sap ingestion, whereas on line TM 47 and Millaleu this was only a low percentage (Table 2, parameter 8).

The duration of the first phloem phase (1st E) was significantly shorter in TM 44, indicating earlier stylet withdrawal from the sieve element (Table 3, parameter 9). In most cases only E1 was observed, followed by a return to the pathway phase without sap feeding (E2). Duration of the first period of E1 on TM 44 was similar to those on TM 47 and Millaleu (Table 3, parameter 10). On these plants there was longer sap ingestion (Table 3, parameter 11) resulting in a longer phloem phase on these more susceptible plants (Table 3, parameters 10 and 11).

Even if the time to reach the phloem was similar, a longer time until first E2 (phloem ingestion) within the experiment and within the probe was shown on TM 44, as less

phloem phases switched to ingestion, and considerably less total phloem ingestion was shown as compared to the more susceptible plants (Table 3, parameters 12, 13 and 14).

Table 1. Probing behaviour of *S. avenae* on *Triticum spp.* until first phloem phase

Treatment	TM 44 n=23	TM 47 n=23	Millaleu n=25	Kruskal-Wallis
(unit)	Means (SE)	Means (SE)	Means (SE)	p-value
1. Time to 1st probe (min)	6.6 (1.2)	17.4 (9.4)	11.4 (3.6)	0.955
2. Non-probing duration before 1st phloem phase (*) (min)	71.2 (15.4) n=22	64.5 (14.4) n=19	65.5 (10.0) n=23	0.866
3. % of non-probing before 1st phloem phase (*) (%)	32.2 (3.9) n=22	30.5 (3.4) n=19	36.1 (4.0) n=23	0.558
4. Time to 1st phloem phase from 1st probe (h)	3.5 (0.5)	3.6 (0.5)	3.2 (0.5)	0.930
5. Time to 1st phloem phase within probe (*) (h)	0.9 (0.2) n=22	0.6 (0.1) n=19	0.7 (0.1) n=23	0.878

(*) Only aphids showing the event were considered. Differences between treatments were not significant according to the Kruskal-Wallis test. Se= standard error; n= number of replicates

Table 2. Numbers of aphids showing phloem phase activities.

Treatment (unit)	TM44 Resistant line	TM 47 Moderate resistant line	Millaleu Susceptible cultivar	Chi-square test
6. Aphids showing phloem phase (E) (# and %)	22 95.7 %	19 82.6 %	23 92.0 %	Chi-square: 2.35 p=0.308
7. Aphids showing sustained ingestion (# and %)	7 a 30.4 %	12 a 52.2 %	20 b 80.0 %	Chi-square: 11.99 **p=<0.001**
8. Aphids showing phloem salivation only (# and %)	15 a 65.2 %	4 b 17.4 %	2 b 8.0 %	Chi-square: 11.25 **p=<0.001**

Different letters within a row indicate significant differences according to the chi-square test

Other parameters related to phloem salivation (E1) (Table 4). The number of phloem phases was not different (parameter 15) among treatments indicating similar attempts to pierce the phloem. The following parameters did not show significant differences between treatments: total phloem salivation (E1 duration) (parameter 16), the number of E1 periods (parameter 17), the mean duration of individual periods of phloem salivation (parameter 18), total phloem salivation before first sustained ingestion (parameter 20), salivation before the first sustained ingestion within the probe (parameter 21), salivation just before the first sustained ingestion (parameter 22) and, the mean duration of E1 before the first ingestion (parameter 24). TM 44 showed a higher contribution of phloem salivation to the phloem phase (parameter 19). The duration of phloem salivation (E1) just before any ingestion (E2) was not different when tested by the Kruskal-Wallis test, but when compared by pairs (Mann-Whitney U test) TM 44 showed more E1 than the most susceptible cultivar Millaleu (p= 0.043, parameter 23).

Table 3. Parameters related to the first phloem phase

Treatment	TM 44 n=23		TM 47 n=23		Millaleu n=25		Kruskal-Wallis
(unit)	Means	(SE)	Means	(SE)	Means	(SE)	p-value
9. Duration of 1st phloem phase (min)	16.2 b	(5.9)	81.6 ab	(25.5)	84.6 a	(18.3)	**0.016**
10. Duration of 1st phloem salivation (min)	7.8	(3.4)	11.7	(3.7)	8.2	(1.5)	0.417
11. Duration of 1st phloem ingestion (h)	0.3 b	(0.1)	1.3 a	(0.4)	1.6 a	(0.3)	**0.001**
12. Time to 1st ingestion within the experiment (h)	6.6 a	(0.5)	5.4 ab	(0.6)	3.9 b	(0.5)	**0.003**
13. Time to 1st ingestion within a probe (min)	6.0 a	(0.6)	3.5 b	(0.7)	2.1 b	(0.5)	**0.001**
14. Total sap ingestion (h)	0.6 a	(0.2)	1.5 ab	(0.4)	2.1 b	(0.3)	**0.001**

Means followed by different letters within lines are significantly different according to the Kruskal-Wallis test followed by multiple comparisons ($\alpha = 0.05$). SE= standard error; n= number of replicates

Honeydew recording and E1. No honeydew excretion could be demonstrated during long periods of E1 waveforms on *S. avenae* or *R. padi.* Concurrent EPG and honeydew recordings of continuous E1 periods, lasting maximally 3 h, are exceptional but did occurr in both aphids. Periods of alternating E1-E2 did not produce honeydew.

Honeydew excretion and phloem waveforms. The excretion rate recorded by a honeydew clock appeared not as continuous as expected during continuous E2 as recorded by the concurrent EPG (Fig. 1). Aphid 1 interrupted excretion (between 450 and 600 min) without any change in the simultaneously recorded waveform E2 (phloem ingestion). In aphid 2 the intervals between droplets, in general about twice a long as in aphid 1, showed a much greater variation. Aphid 3, recorded for 30 h, showed that excretion rate became more regular during the long sustained ingestion period (Fig. 1). The first series of 7 and the subsequent series of 7 droplets of aphid 1, forming two rather uniform but different series of droplets, could be used for comparison within one individual and even, within one phloem phase and sieve element. The intervals between droplets in these two series appeared to differ significantly, whereas their diameters did not (Table 5). Droplets

Table 4. Parameters related to phloem salivation (*)

Treatment	TM 44 n=23		TM 47 n=23		Millaleu n=25		Kruskal-Wallis
(unit)	Means	(SE)	Means	(SE)	Means	(SE)	p-value
15. Number of phloem phase (#)	3.3	(0.5)	2.1	(0.4)	2.7	(0.4)	0.134
16. Total phloem salivation (E1) (min)	48.2	(12.7)	27.4	(5.8)	32.6	(7.5)	0.731
17. Number of E1periods (#)	3.9	(0.7)	2.8	(0.5)	3.3	(0.6)	0.354
18. Mean duration of individual E1 periods (min)	12.2 n=22	(3.5)	10.7 n=19	(2.1)	10.1 n=23	(1.5)	0.942
19. Phloem salivation contribution to the phloem phase (**) (%)	73.2 a	(7.9)	40.4 b	(8.6)	28.0 b	(6.1)	**<0.001**
20. Total E1 before 1st sustained ingestion (***) (min)	42.2 n=22	(12.4)	28.7 n=19	(5.8)	23.7 n=23	(7.1)	0.517
21. Total E1 before 1st sustained ingestion within probe (min)	23.9 n=7	(6.2)	15.5 n=12	(3.5)	20.7 n=20	(6.5)	0.418
22. E1 duration just before 1st sustained ingestion (min)	14.7 n=7	(2.5)	13.4 n=12	(3.1)	18.6 n=20	(6.5)	0.802
23. E1 duration just before any ingestion (min)	21.1 n=13	(7.9) (a)	16.2 n=18	(4.0) (ab)	9.8 n=38	(1.4) (c)	0.111 (p.w.)
24. Mean duration of E1 before 1st ingestion (***) (min)	13.9 n=22	(3.6)	14.9 n=19	(3.5)	10.1 n=23	(1.4)	0.831

(*) E1: salivation into sieve elements; (**) (E1/E*100); (***) Excluding aphids without phloem phase and using the value of E1 in absence of E2. Means followed by different letters within lines are significantly different according to the Kruskal-Wallis test followed by multiple comparisons (α= 0.05). SE= standard error; n= number of replicates

diameters showed no significant correlation with their preceding intervals.

Phloem ingestion waveforms of aphids 2 and 3 showed the typical E2 "peaks" and "waves" (Fig. 2A), suggested earlier (Tjallingii, 1990) to reflect saliva pump activity and cibarial valve activity of the "food pump", respectively (see also Table 3, Chapter 1).

Aphid 1, however, showed a rather curious difference with respect to this general appearance of the waveform. During the first period of the honeydew excretion (droplet series < 1 >, Table 5) clear peaks were shown (Fig. 2B), whereas during the second period, after about 600 min (Fig. 1), the E2 waves became dominating, masking the peaks, which gave the signal an appearance of waveform G, i.e. active ingestion from the xylem. The presence of the E2 peaks (not present in G), though completely masked, could be demonstrated (Fig. 2E) by filtering out the predominant E2 waves, using inverse FFT filtering (c.f. Fig. 2D, "waves" frequencies were removed so that the "peak" frequency remained and the signal was regenerated: Fig. 2E). As no other waveforms had been observed other than the gradual changes within E2, the phloem location of this activity was clear. Its similarity with waveforms showed during xylem ingestion could suggest an active (or "real") sucking with a strong cibarial pump activity. Therefore, the relation with excretion intervals was further studied.

Two aspects of the E2 "wave" contribution were tested in aphids 1 and 2. From the Fourrier spectrum (FFT derived auto power spectra; c.f. Fig. 2D) the wave frequency could be derived (Table 5, w. freq.) and also, for aphid 1 only, the amplitude of the E2 waves was measured (amp(mV)). These wave aspects were measured in 10 seconds waveform samples at the beginning of each droplet interval period (paired observations, Table 5). No correlation between interval duration and E2 wave frequencies could be demonstrated and no correlation with the droplet size (diameter) either. For the amplitude (amp(mV)) a slight negative correlation with intervals was found but this was not significant.

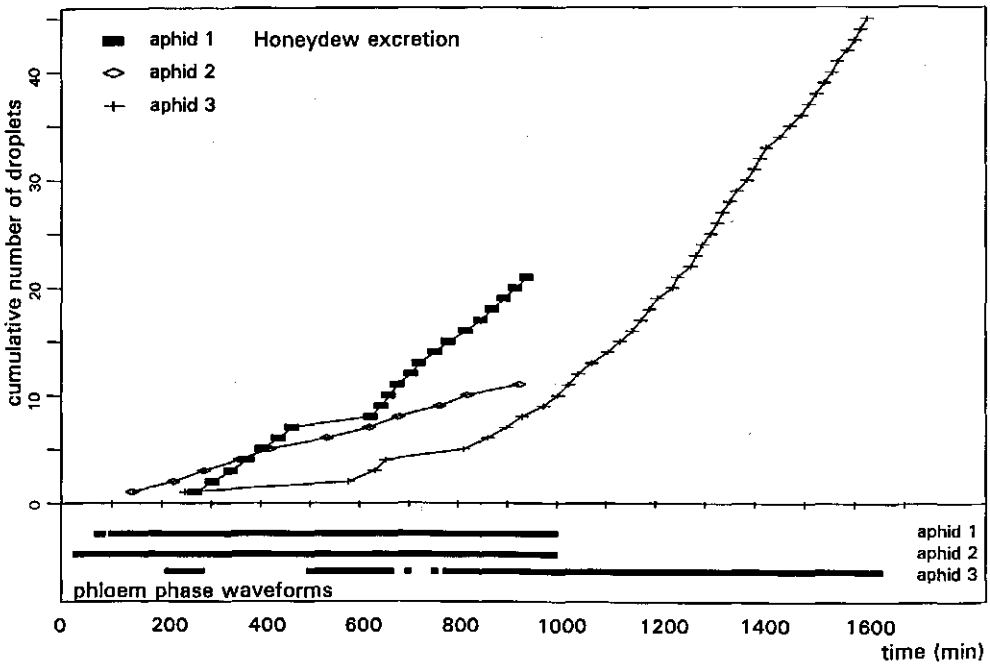

Figure 1. Honeydew excretion (clock) and EPG recording of three aphids. Droplets cumulatively, upper part, and phloem ingestion periods (E2 waveforms), lower part (horizontal bars).

DISCUSSION.

Sieve element salivation (E1). As demonstrated by Caillaud *et al.* (1995b) *S. avenae* on the resistant *T. monococcum* line TM 44 showed phloem phases, as rapidly as on susceptible plants but it took aphids three times as long until any E2 occurred. Aphids switched less frequently from waveform E1 to E2, but more often from E1 back to C (pathway) on the resistant TM 44, which was explained as a failure to start feeding. The time until E2 within the experiment and the mean duration of E1 just before E2 started were longer on TM 44, and total E2 was shorter in their study, whereas the time until the first E1 within experiment and probe, the mean E1 and E2 periods, as well as the total E1 were similar to the susceptible plants. Those observations were confirmed in this study, although the shorter E1 before any ingestion (parameter 23), which presumably is the same as Caillaud' *et al.* "mean duration of E1 before E2", is is not completely convincing (pairwise different, but Kruskal-Wallis not). Further differentiating E1 before E2 (mainly parameters 20-24, Table 4) demonstrated that the duration of E1 before E2 was not longer on TM 44, especially not, when considering E1 just before sustained E2 (i.e. > 10 min; parameter 22, Table 4). Although the statistical reliability of the similarity shown by parameter 22 is possibly less firm, as only 30% of aphids on TM 44 showed any sustained E2 (parameter 7), it is more critical than parameter 23. Restricting the calculations to the cases where E1 preceded E2 periods longer than 10 min, implies that E2 ingestion has been really established and presumably sieve element restrictions have been overcome on those aphids shifting to E2. Thus, our data do not demonstrate a substantial increase of E1 before E2 to overcome any supposed sieve element constraints in this cultivar as compared to the susceptible.

As a consequence, we may conclude that, for the underlying mechanism of the inability to start feeding on TM 44, there is no reason to suggest that difficulties to suppress a sieve element reaction are more likely than for example the presence of an anti-feedant, for example. Also, this aphid-plant combination does not seem to be more suitable to study the function of sieve element salivation (E1) than any other. It may be useful to investigate resistance mechanisms of other plants in which avoidance of phloem feeding rather than toxic effects or low nutritional quality has been suggested as the causal factor (van Helden *et al.*, 1993; Cole, 1994; Therasa *et al.*, 1996).

Table 5. Intervals and diameters of honeydew droplet recorded from aphid 1 and 2 and concurrently recorded "wave" frequencies and amplitudes (amp(mV);aphid 1 only). In aphid 1 three series (col 3) of rather uniform intervals were compared to one another but EPG parameters were only derived for the first 2 series. Averages (avg) and standard errors (se) and statistical differences (different letters $p < 0.05$) relate to droplet series.

Aphid 1								
	Droplet		Interval (*)		Diameter		EPG	
#	min	series	min	avg se sign	mm	avg se sign	w. freq	amp (mV)
1	270	----	35		0.85		6.05	1.12
2	305	|	37	32.5	0.94	0.925	6.84	1.37
3	342	<1>	33	1.5	0.95	0.016	6.74	1.81
4	375	|	30	a	0.95	a	7.32	1.90
5	405	|	33		0.94		7.52	2.73
6	438	----	27		0.93		7.32	2.88
7	465		160		0.90			
8	625	----	22		0.86		7.23	2.39
9	647	|	16	19.8	0.88	0.889	7.32	2.44
10	663	<2>	17	2.3	0.88	0.028	7.23	2.54
11	680	|	28	b	0.90	a	7.23	2.25
12	708	|	15		0.89		6.93	2.49
13	723	----	32		0.94		7.13	2.73
14	755		27		0.88			
15	782		36		0.91			
16	818	----	32		0.88			
17	850	|	22	26.3	0.88	0.904		
18	872	<3>	23	2.5	0.89	0.011		
19	895	|	23	a	0.94	a		
20	918	|	22		0.93			
21	940	----			0.95			

Aphid 2 Droplets		Interval (*)	Diameter	w. freq.
#	min	min	mm	Hz
1	140	84	1.05	7.91
2	224	62	0.98	8.11
3	286	72	0.91	7.71
4	358	61	0.89	8.20
5	419	115	0.86	8.01
6	534	86	0.80	7.91
7	620	60	0.78	6.35
8	680	83	0.88	7.52
9	763	57	0.91	6.25
10	820	105	0.84	6.64
11	925		0.85	6.54

* interval following on droplet time

Honeydew recording and E1. The lack of honeydew excretion during periods of E1 waveforms in *S. avenae* as well as in *R. padi* supports the conclusions reported in Chapter 2, that not substantial sap ingestion occurs during sieve element salivation. On the basis of these results the ingestion of small amounts of fluid, possibly used to taste the plant sap as a part of the host-plant selection process, cannot be excluded, however. Honeydew excretion took only place during continuous feeding, usually after some hours of food intake. During alternating periods of E1-E2 no honeydew excretion was shown.

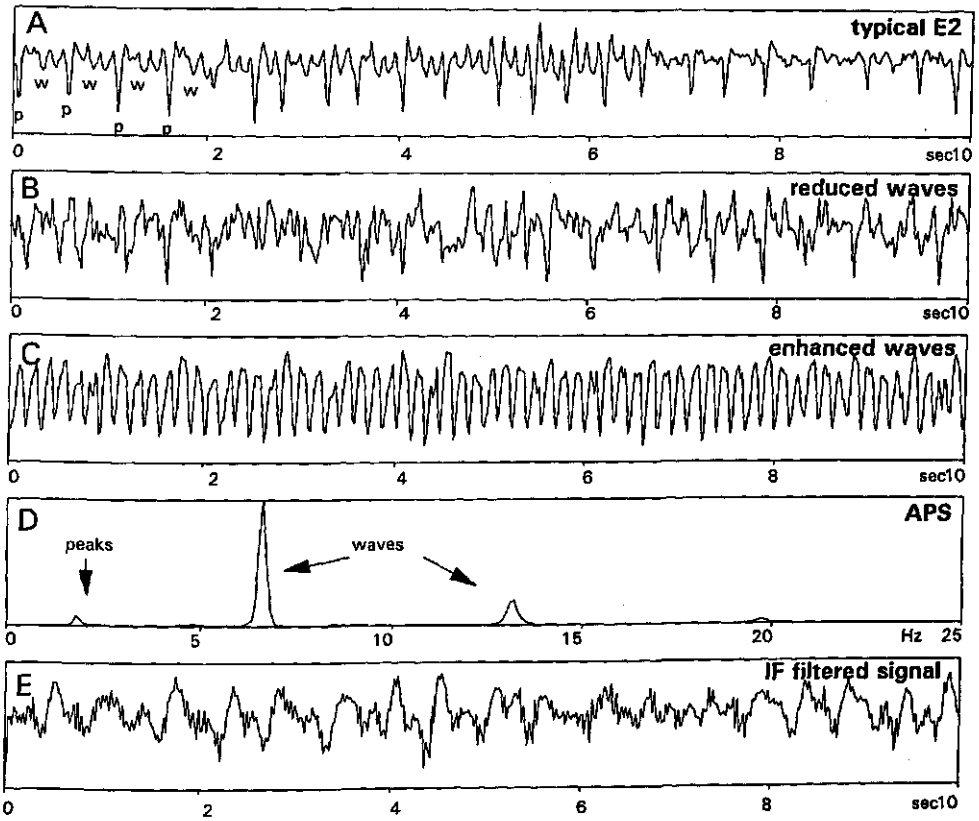

Figure 2. Phloem ingestion (E2) waveforms and frequency spectrum. A, clear recording of typical E2 waveform with peaks (p) and waves (w), aphid 2. B, E2 waveform with mainly peaks, aphid 1. C, dominating waves in E2, aphid 1. D. Auto power spectrum of signal in C, frequency contributions of peaks and waves indicated. E, same signal as in C but frequencies 5-15 Hz filtered out (inverse Fourrier), making the masked E2 peak features visible.

Honeydew excretion and phloem waveforms. Spots of honeydew droplets collected on the TLC paper showed a very constant diameter for individual aphids, irrespective of the preceding interval, which is in agreement with earlier reports (Tjallingii, 1995). The diameter closely reflects the droplet volume, but the relation is presumably affected by the viscosity of the fluid and the relative humidity during recording, given the same silica layer (TLC plate material). The diameter seems to be strongly determined by the size of the aphid, and juveniles on the same leaf produced much smaller droplets but at higher frequencies. Within individual insects the excretion rate, therefore, seems to be rather well reflected by the number of droplets per unit of time. Interruption of honeydew excretion, as shown by aphid 1 between 450 and 600 min, may be due to either reduced ingestion or reduced excretion. The unaltered continuation of the E2 waveform during this interval suggests that ingestion continued, but that the fluid was retained for some time in the gut. When excretion resumed thereafter, its rate seemed to be increased as reduced droplet intervals were shown for some time (7 droplets series 2, Table 5 and Fig. 1). Thus, the excretion rate is changed by the interval, not by the droplet size. Apart from the 2.5 h interruption by aphid 1, which is rather exceptional, the droplet intervals appeared to become more or less constant, at least in long recordings. This supports earlier findings (Tjallingii, 1995).

Ingestion during E2 has been suggested as passive intake, mainly due to the hydrostatic pressure of the phloem sap (Tjallingii, 1995). The ingestion rate, if fluctuating with the E2 "wave" frequency, seems to be smoothened by a buffer capacity formed by the gut between ingestion and excretion.

The droplet frequency of aphid honeydew is so low that the delay between ingestion activity and excretion difficults the establishment of a good correlation between both activities and, very likely, the excretion rate largely depends on the amount of sap in the gut. The fuller the gut the more direct ingestion will presumably affect excretion. The interval between the start of feeding and the first honeydew droplet will be larger than the interval between droplets after reaching a continuous ingestion and at the end of a feeding period. In the context of the foregoing considerations the use of the honeydew excretion intervals and the droplet size for correlation with ingestion signals is rather arbitrary. Another point is the honeydew clock. Although this is a good technique for measuring ingestion differences on different plant cultivars, for example, it seems inadequate to accurately correlate the ingestion activities, so that in further studies on the relationships between EPG waveforms and ingestion rate we will need other and more accurate methods.

It may be clear from this study that phloem phase activities consist of a number of components that need further investigations in order to better understand their biological functions.

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6. Summary and conclusions

Aphid-plant interactions occurring during plant penetration, or probing, have determined the evolution of these insects as well as the abilities of plants to survive huge densities of these insects. For aphids, as phloem sap feeders, the interactions at sieve element level must be of special importance. During the phloem phase of probing, there are two aphid activities, categorised as waveforms E1 and E2 in electrical penetration graphs (EPGs), which have been studied here. The initial E1 activity appeared to be responsible for the inoculation of a persistent plant virus (BYDV), thus reflecting saliva injection into the sieve element (Chapter 2). No apparent sap ingestion was found during E1. The relation of the subsequent E2 activity with phloem sap ingestion was confirmed in this study, as the E2 duration appeared to be positively correlated with the acquisition success of the virus. Moreover, the results supported the idea that concurrent salivation during E2 does not reach the sieve element. Instead, it is presumably mixed with the ingested sap in the fused apical part of the two stylet canals.

The "obligatory" occurrence of sieve element salivation (E1) before ingestion (E2) starts suggested a function in suppressing wound reactions of the sieve elements, such as phloem protein gelation or callose formation, or some other activity in preparation of phloem feeding. Therefore, the extensive saliva injection of an aphid colony might influence the probing behaviour of the individuals and explains the sometimes observed better performance. Hence, the occurrence and duration of phloem salivation (E1) was subsequently studied under this condition (Chapter 3). The aphid *Aphis fabae* showed reduced phloem salivation when probing on previously colonised bean leaves (*Vicia faba*), which seems in agreement with this hypothesis. However, no shortened E1 was found in the combination *Rhopalosiphum padi-Triticum aestivum*. Thus, no general conclusion can be drawn. Nevertheless, a beneficial effect of plant changes at phloem level could be inferred from the observed shorter phloem salivation and the more continuous phloem sap ingestion by *A. fabae* on previously infested plants. The ingestion rate, as reflected by recorded honeydew excretion, remained unaltered but the ingestion periods were prolonged.

The effects of aphid manipulation during the experiments, including interruption of feeding, moving to a new plant, depriving from food, etc., on the experimental results were investigated (Chapter 4). Also, the impact of attaching

them to a wire for EPG recording was examined. Increasing the interval between removal from the plant and giving access to the test plant led to a reduction of time until the first probe on test plants. A shorter time until the first phloem phase was found in *A. fabae* when deprived of food for 1 min and replaced on the same plant, suggesting a short "aphid memory" and/or a better plant acceptability of the already probed plant. Also, a short "memory" effect was observed in the 1 minute intervals, slightly reducing the time until the first phloem phase. A general effect, however, was that an interruption of feeding resulted in a "reset" of probing behaviour, appearing as a similar sequence, duration and pattern of probing activities, irrespective of the length of the interval between 1 and 100 minutes. Confinement of an aphid by a wire slightly increased pathway activities, but once phloem phase was reached it did not affect phloem feeding, at least in tests with suitable host plants as were used here.

The final chapter describes some more detailed aspects of the phloem phase activities. Phloem salivation was supposed to be prolonged when compared to a susceptible cultivar, during aphid probing on a line of *Triticum monococcum* with resistance to the aphid *Sitobion avenae.* This plant-aphid combination was used as a model system in order to investigate further the biological function of phloem salivation. However, it appeared that there was no prolonged phloem salivation when the parameters were selected critically. Additional honeydew excretion experiments with *S. avenae* and *R. padi* showed that E1 activities were not related to honeydew excretion, i.e. no honeydew secretion occurred during this waveform. Also, no relation was found between variations in the phloem ingestion waveforms (E2), frequencies and amplitudes of certain details, and excretion rate. If these waveform variations reflect fluctuations in ingestion rate, the excretion rate seems not adequate to measure them.

Samenvatting en conclusies

De bladluis-plant interacties die optreden tijdens de stiletpenetratie zijn bepalend geweest voor de evolutie van bladluizen en voor het vermogen van planten om hoge dichtheden van deze insecten te verdragen. Vooral de interacties die plaatsvinden op het niveau van de floëem- of zeefvaten zijn van groot belang omdat bladluizen zich met het floëemsap voeden. Met Elektrische Penetratie Grammen (EPGs) werden de twee golfpatronen (E1 en E2) onderzocht die in de floëemfase van de penetratie (met de punt van de stiletten in een zeefvat) kunnen worden onderscheiden. Gedurende E1, aan het begin van een zeefvatpunctie, werd inoculatie van een persistent virus (BYDV) gevonden, waaruit geconcludeerd werd dat de bladluis in deze fase speeksel in het zeefvat (hoofdstuk 2) injecteert. Opname van zeefvatsap gedurende E1 werd niet aangetoond. Gedurende het hierop volgende E2 patroon kon wel opname van zeefvatsap worden aangetoond omdat de duur van E2 positief gecorreleerd bleek met de kans op acquisitie van het virus. De resultaten van deze proeven bevestigden bovendien de hypothese dat de speekselsecretie die gedurende E2 optreedt niet in de plant terechtkomt. Aangenomen word dat dit speeksel in het apicale deel van de stiletten, waar het speekselkanaal en het voedselkanaal fuseren, wordt gemengd met het opgenomen zeefvatsap.

Het "noodzakelijke" optreden van speekselsecretie in het zeefvat (E1) voordat de eigenlijke voedselopname (E2) begint, suggereert dat E1 een functie heeft ter voorbereiding op de voedselopname of voor het onderdrukken van afweerreacties in de zeefvaten, zoals het tegengaan van de gelering van de zogenaamde P-proteïnen of de vorming van callose. De omvangrijke speekselinjectie van een bladluiskolonie kan het penetratie-gedrag van een individu vergemakkelijken en zo de soms geobserveerde betere groei en ontwikkeling verklaren. Daarom werd het optreden en de duur van de speekselsecretie (E1) met en zonder eerdere bladluisinfectie bestudeerd (hoofdstuk 3). De bladluis *Aphis fabae* (Zwarte bonenluis) vertoonde op bonenbladeren (*Vicia faba*) die eerder met bladluizen waren geïnfecteerd een korter E1 patroon. In de combinatie *Rhopalosiphum padi* - *Triticum aestivum* werd echter geen korter E1 patroon gevonden. Er werd dus geen algemeen geldende verklaring gevonden. Wel kan geconcludeerd worden dat een eerdere bladluisinfectie van de plant voordelig is voor *A. fabae* omdat in dat geval de E1 patronen korter waren en er meer voedselopname (E2) waargenomen werd. De opnamesnelheid zoals die via de uitscheiding van honingdauw werd bepaald, was onveranderd, maar de E2 periodes waren langer.

De effecten van de manipulatie van de insecten voor en gedurende de proeven zoals de onderbreking van voedselopname, overzetten op een nieuwe plant, hongeren etc. werden onderzocht (hoofdstuk 4). Ook de invloed van een beperking van de bewegingsvrijheid door het 'aanlijnen' voor de EPG-opnames werd bestudeerd. Een toename van het interval tussen het verwijderen van een plant en plaatsing op een nieuwe plant leidde tot een verkorting van de tijd voor de eerste penetratie. De kortere tijd tot aan het eerste floëemcontact zoals die werd gevonden bij *A. fabae* bij een korte onderbreking (1 minuut) en terugplaatsing op dezelfde plant suggereerde een soort "herinnerings"-effect bij de bladluis of een betere geschiktheid van de plant. In het algemeen leidde de onderbreking van de voedselopname tot een soort 'herstart' van het penetratiegedrag: het EPG vertoonde een vergelijkbare opeenvolging en duur van patronen, onafhankelijk van de duur van de onderbreking (tussen 1 en 100 minuten). In onze proeven, waarbij alleen waardplanten gebruikt werden, bleek dat de beperking van de bewegingsvrijheid door de gouddraad-elektrode resulteerde in een kleine toename van de penetratietijd voor het eerste floëemcontact, de voedselopname zelf bleek niet beïnvloed te worden.

Voor het laatste hoofdstuk werden de activiteiten gedurende de floëemfase meer gedetailleerd bestudeerd. Daartoe werd de bladluis-plant combinatie *Sitobion avenae* en een resistente lijn van *Triticum monococcum* gebruikt omdat daarbij een verlenging van de duur van de speekseluitscheiding (E1) op zou treden. Dit modelsysteem zou daarom geschikt zijn om de biologische functie van E1 te bestuderen. Na een kritische analyse van de juiste parameters bleek er echter geen sprake te zijn van langere E1 periodes. Honingdauwregistraties van *S. avenae* en *R. padi* lieten zien dat gedurende het E1 patroon geen uitscheiding van honingdauw plaatsvindt. Ook werd er geen verband gevonden tussen variaties in frequentie en amplitude van onderdelen van het voedselopnamepatroon E2 en de snelheid (frequentie en druppelgrootte) waarmee honingdauw werd uitgescheiden. Als deze variaties binnen het E2 patroon al een relatie hebben met de opnamesnelheid, dan lijken honingdauwregistraties geen goed instrument om dit te meten.

Resumen y conclusiones

La interacción áfido-planta que ocurre durante la inserción del estilete en el tejido de la planta ha determinado la evolución de estos insectos como también las habilidades de la planta para sobrevivir altas densidades de estos insectos. En áfidos, por alimentarse de floema, las interacciones a este nivel son especialmente importantes. Existen dos actividades durante la fase floemática de la alimentación, catalogadas como ondas E1 y E2 en el sistema de monitoreo electrónico ("electrical penetration graphs", EPG), las cuales fueron estudiadas en este trabajo. La primera actividad del áfido al llegar a un tubo criboso del floema fue responsable de la inoculación de un virus persistente (BYDV, virus del enanismo amarillo de la cebada) y fue relacionada a la inyección de saliva (Capítulo 2). No se encontró indicios de ingestión durante el período correspondiente a la onda E1. El vínculo de la siguiente onda durante la fase floemática (E2) con ingestión de savia floemática fue confirmada en este estudio, debido a la correlación positiva encontrada con la adquisición de virus persistente. Además, los resultados obtenidos confirman la idea que la saliva producida por el pulgón durante la ingestión no llega a los tubos cribosos. Esta se mezcla con la savia ingerida en el extremo del estilete, donde el canal alimenticio y salivar están fusionados.

El hecho que la salivación en los tubos cribosos (E1) siempre ocurre previo a la ingestión (E2), sugiere que esta actúa como un supresor o inhibidor de la reacción del tubo criboso al daño producido por el estilete (coagulación de la proteína del floema, formación de callosa, etc.) o representa alguna otra actividad indispensable para comenzar la alimentación. Las agregaciones de pulgones en plantas son comunes y mejoran el desarrollo de los individuos de algunas especies. Esta gran inyección de saliva producida por la colonia y cambios en la planta misma podrían reflejarse en el comportamiento alimentario del insecto. Por lo tanto se estudió el efecto de una colonia de áfidos, en la presencia y duración de la salivación en el floema (E1) (Capítulo 3). El áfido *Aphis fabae* mostró una reducción de la salivación cuando se alimentó en una hoja previamente colonizada por individuos de la misma especie (y eliminada previo al monitoreo del comportamiento), lo que parece confirmar la hipótesis planteada. Sin embargo, en la combinación *Rhopalosiphum padi* - *Triticum aestivum*, no existió tal relación. Por lo tanto, la conclusión no puede ser generalizada. No obstante, los efectos benéficos de la colonia se reflejó en posibles cambios en la planta a nivel del floema, deducido de la reducción de la salivación en el floema y de la prolongada ingestión encontrada en *A. fabae* en plantas previamente infestadas. La taza de ingestión reflejada

en la secreción de mielecilla, permaneció inalterada, en cambio, se produjo un período de ingestión mas prolongado.

Fueron investigados los efectos de la manipulación de los insectos, interrupción de la alimentación, traslado a una nueva planta, etc., sobre los resultados experimentales (Capítulo 4). También se estudió el impacto del electrodo atado el dorso del insecto en el comportamiento alimentario. Una mayor demora en colocar el áfido en una nueva planta implicó una inserción mas temprana del estilete. La primera fase floemática se observó mas temprano luego de un período de interrupción de la alimentación de 1 minuto en *A. fabae* al ser repuesto el pulgón en su antigua planta. Es probable la existencia de una breve "memoria" para aceptar la planta anticipadamente y/o un efecto de la planta que predispone una mejor aceptabilidad. Sin embargo, el efecto general es que la interrupción de la alimentación produce un "reinicio" total en el comportamiento alimentario con una secuencia, duración y patrón similar de comportamiento, independiente del tiempo de interrupción de la alimentación (1 a 100 minutos). La restricción impuesta al áfido por la atadura al electrodo aumenta levemente la fase de "ruta" (fase de penetración intercelular del estilete con breves inserciones en las células), pero una vez en el floema la alimentación no es afectada, al menos en áfidos alimentándose de sus plantas hospederas como las estudiadas aquí.

El último capítulo describe con mas detalles algunos aspectos de las actividades del pulgón durante la fase floemática. Se hipotetizó una prolongada salivación del áfido *Sitobion avenae* en el floema de una línea resistente de *Triticum monococcum*. Se usó esta combinación como modelo para investigar en más detalles la función biológica de la salivación. Sin embargo, no se encontró una clara prolongación de la salivación al analizar detalladamente los parámetros relacionados a esta actividad. La secreción de mielecilla en *S. avenae* y *R. padi* no estuvo relacionada a la presencia de la onda E1 (no existió secreción de mielecilla durante esta actividad, E1). Tampoco se encontró una relación entre las variaciones encontradas durante la ingestión (onda E2), frecuencias y amplitudes, y la secreción de mielecilla. Si las variaciones en las ondas E2 son consecuencia de fluctuaciones en la taza de ingestión, la secreción de mielecilla no parece reflejar estos cambios.

Publications

Chapters of this thesis has been published or submitted as:

Prado, E. & W. F. Tjallingii, 1994. Aphid activities during sieve element punctures. Entomologia Experimentalis et Applicata 72: 157-165.

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Curriculum Vitae

Ernesto Javier Prado Cordero was born on 25 May 1951, in Santiago, Chile. He attended the primary and secondary school at the Instituto Nacional. He started studies in Agronomy at the Catholic University in Santiago in 1969. Immediately after graduating he began his specialisation in Entomology with staying at the Musée d'Histoire Naturelle de Paris and at the INRA-France (1975-1976), being involved in taxonomy and biological control studies. He spent several years in Sweden (1977-1979) at the Institution för växtskydd, Department of Plant Protection (Stockholm). Back to Chile in 1980, he worked for the Agricultural Ministry at the quarantine laboratory and fruit fly monitoring and eradication program. Thereafter, he moved to the Agricultural Research Institute (INIA), his actual employer, conducting research in IPM on different crops and fruit orchards. At the end of 1991, he started his PhD studies at Wageningen Agricultural University in the subject of aphid-plant interactions.