Probing behaviour of thrips

Behavioural study on the feeding of Western flower thrips related to *Tomato spotted wilt virus* transmission and host plant susceptibility

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Frodo Kindt

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Contents

Abstract

Western flower thrips, *Frankliniella occidentalis*, feeds in a piercing sucking manner and is a worldwide pest on many ornamental and vegetable crops. Thrips can feed on different parts of the plant; leaves, stems, flowers and fruits. On leaves thrips can ingest the cell contents of epidermal, mesophyll, and parenchymal cells, but their mouthparts cannot reach the vascular tissue. Western flower thrips can also transmit *Tomato spotted wilt virus* (TSWV) during probing. My research has shown that individual probes can be divided in several phases using an electrical monitoring system, electrical penetration graph (EPG) technique. Six different phases have been distinguished: P, Q, R, S, T, and U. These phases were shown to reflect different behavioural activities and are represented by different waveforms. Waveform P represents mandibular stylet insertion through the surface of the leaf, during waveform Q the maxillary stylets are inserted and salivation occurs and waveform R represents ingestion of the cell contents. Of waveform S and U it is only known that they reflect mandibular action, and during waveform T probably the stylets are retracted. Not all these waveforms occur in every individual probe and waveform transients are often gradual therefore I suggest for practical reasons to distinguish only phases for: the 'puncture phase, PQ', also including S, and the 'feeding phase, R', also including T and U. Transmission of TSWV occurs with salivation during waveform Q in the puncture phase. TSWV can be transmitted in a single probe without ingestion, but only in one of 125 probes transmission was found to be successful. Therefore, more probes made by a viruliferous thrips result in a higher chance of virus transmission. The probability of TSWV transmission is not the same in every probe; the duration of the salivation phase has a positive effect whereas the duration of the ingestion phase has a negative effect. Host plant resistance against TSVW works well in pepper, but combined with thrips resistance it can be more durable. This can be concluded because this study found that thrips resistance only results in small changes in the probing behaviour and these changes hardly influence TSWV transmission.

Voorwoord

Allereerst wil ik Nina (Joosten) bedanken zonder jou had ik lang niet zoveel kunnen doen. Je hebt voor mij bergen werk verzet en je bent regelmatig voor mij in het weekend op het lab geweest. Natuurlijk was Freddy (Tjallingii) ook onmisbaar met zijn ongelooflijke kennis over EPGs en alles wat daarbij komt kijken. Regelmatig ben ik bij je gekomen met een probleem of vraag en samen kwamen we er altijd wel uit. Ook Dick (Peters) hoort bij de mensen die voor mij onmisbaar waren, want zonder jou was het project er sowieso niet geweest en als ik een vraag had op het virologische vlak dan kon ik altijd bij je terecht. Daarnaast heb je het geduld gehad om mij te helpen beter te leren schrijven. Ook was jij degene die me het meest aanspoorde om op te schieten. Paul (Maris) heeft mij vooral in het begin erg veel geholpen met de meer praktische problemen en we hebben ook onze resultaten regelmatig bediscussieerd. Helaas hebben we nooit echt samengewerkt aan experimenten. Ik vond het fijn om in een zo'n klein team te werken en heb erg veel aan jullie gehad.

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CHAPTER 1

General introduction

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1. Thrips

Together with whiteflies (Aleurodidae), scale insects and mealy bugs (Coccidea), thrips belong to the most damaging pest insects in agriculture and horticulture and, possibly, they are as damaging as aphids (Aphididae) (Lewis, 1997a). Thrips cause direct damage by probing or feeding on plants, and indirectly, by transmitting *Tospoviruses*. Solely the mere presence of thrips in a crop may already lead to economic losses since some countries do not allow import or export of such crop products. Thrips belong to the order of the Thysanoptera, which consist of two suborders, Terebrantia and Tubulifera, together including nine families. Approximately 5% out of 5000 identified species (Gaston and Mound, 1993) are considered as a pest (Appendix I in Lewis (1997b). Some of the pest species occur worldwide and cause problems in many different crops because they are polyphagous, while other species form only a local pest or are only a problem in a single crop. All pest species belong to two families, Thripidae and Phlaoethripidae each in one of the different suborders. Only eight thrips species are known as vectors of *Tospoviruses*. They all belong to the genera *Thrips* and *Frankliniella* within the family Thripidae (Nagata and Peters, 2001). Formerly *Thrips tabaci* was considered to be the most important vector in Europe, but nowadays *Frankliniella occidentalis*, originating from North America, which has been unintentionally spread to other continents, has become more important and the main vector worldwide (Ullman et al., 1997; Nagata and Peters, 2001).

1.2. Economic importance

The damage caused by thrips worldwide or per country is difficult to measure. The damage caused by *F. occidentalis* in the Netherlands was approximately 33 million Euro's in 1998, not including the damage caused by *Tospoviruses*. This is based on a model, developed to estimate damage caused by different pests per year (Roosjen et al., 1998). The additional loss caused by *Tomato spotted wilt virus* (TSWV) is presumably about 20 million Euro's (Roosjen et al., 1998).

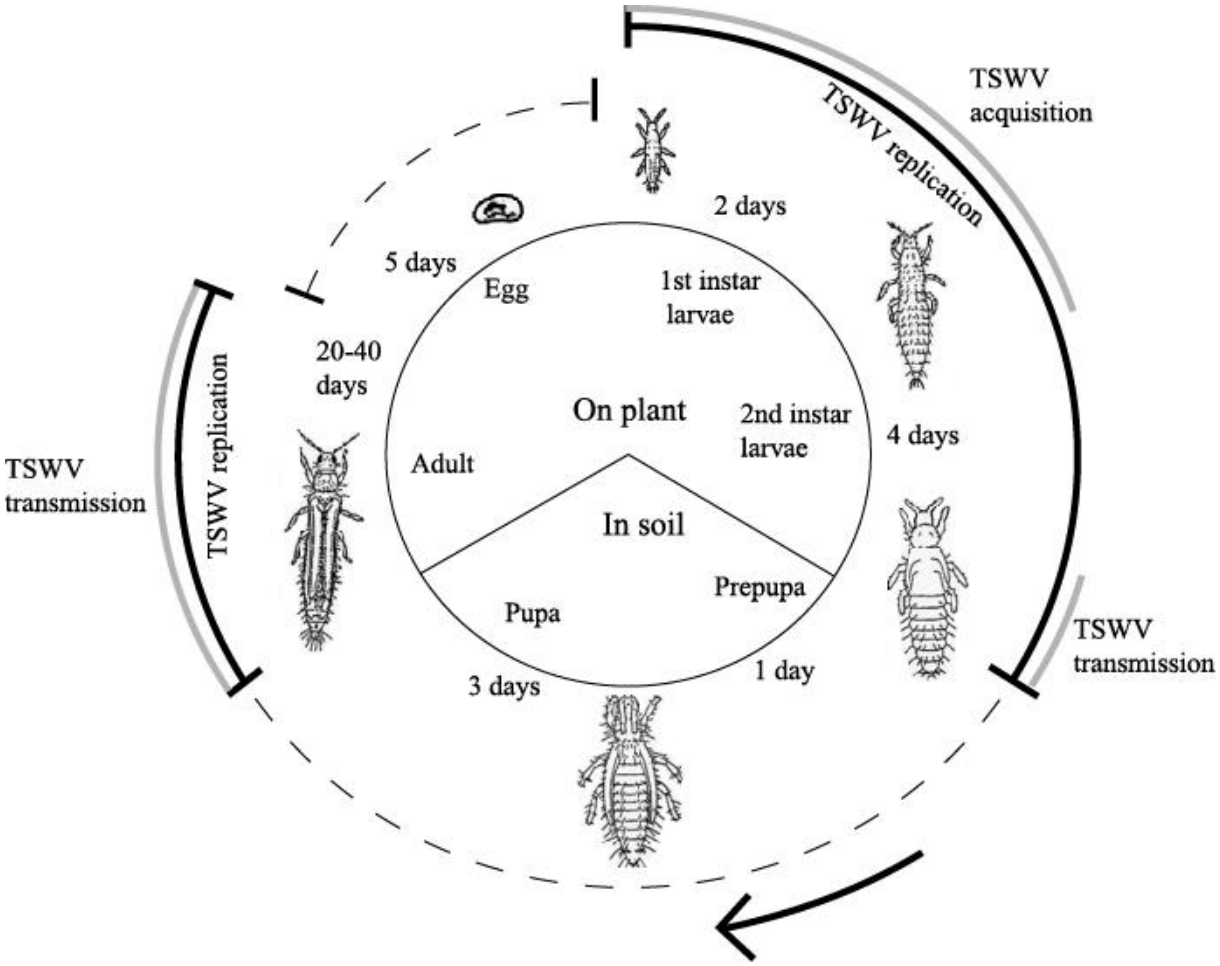

Fig. 1. Life-cycle of *F. occidentalis* (inner circle) and the infection-cycle of *Tomato spotted wilt virus* (TSWV, outer circle).

1.3. *Frankliniella occidentalis*

The common name of *F. occidentalis* is Western flower thrips since it was found first in the western USA and then described by Pergande in 1895. Why *F. occidentalis* is such an important pest and vector species can partly be understood on basis of its life history parameters and behaviour. The life cycle of *F. occidentalis* consists of two larval stages, two pupal stages and an adult stage (Fig. 1). During their entire adult lifespan females can lay eggs in plant tissues, in which they are inserted by their ovipositor. Fertilised eggs develop into females and unfertilised eggs will become males so that females are diploid and the males are haploid. On *Capsicum annuum* the generation time from egg to fertile adults is about 17 days at 25º C (Maris, 2004).

For several reasons both chemical and biological control of *F. occidentalis* is difficult. Firstly, due to intensive spraying, thrips populations have developed resistance to a range of common insecticides (Jensen, 2000). Secondly, thrips are minute insects, only 1.2-1.9 mm

long, slender, and very mobile, properties which are disadvantageous for control with natural enemies. Also the adults can easily escape by flying away, with their double-fringed wings. They also have a cryptic lifestyle, by living inside flowers, which protects them from sprayed contact insecticides and natural enemies. Also the pupal stages of *F. occidentalis* do not feed and life in the soil and are therefore very difficult to control with pesticides. Sufficient control can be obtained with natural enemies, but it is almost impossible to wipe out a whole population, just a few thrips are often enough to infect a whole field or greenhouse compartment with for example *Tomato spotted wilt virus* (TSWV) (Tommasini, 2003). Another reason why *F. occidentalis* has become such an important pest species is that it has been distributed rapidly over the world due to increased global distribution of all sort of crops and crop products (Goldbach and Peters, 1994). Also, the spread of *F. occidentalis* is enhanced by its opportunistic feeding habits, and its broad host range including many of crop and wild plant species (Peters, 1998). Moreover, this thrips species can survive on many living plant parts and they can feed on pollen, nectar and in dense populations even cannibalism has been shown to occur (Kirk, 1997).

1.4. Probing behaviour

Thrips are equipped with a piercing-sucking type of mouthparts (Heming, 1978; Chisholm and Lewis, 1984; Hunter and Ullman, 1989). Insertion of these mouthparts can most adequately be described as 'probing'. In fact this means the insertion of a 'sensor', to test the quality of the plant's surface and interior, in terms of structure (mechano-reception) and chemistry of the cell contents (chemo-reception). Behaviourally, probing includes mouthpart 'piercing or penetration' into the plant tissue, 'salivation' and 'feeding', all coinciding with a constant monitoring by the insect's sensory system (Hunter and Ullman, 1994). With terms as 'feeding', 'ingestion', or 'penetration' only a single of these activities will be indicated, neglecting the others.

Frankliniella occidentalis causes direct damage by probing on all kinds of plant parts; leaves, stems, fruits and flowers. Especially, probing on young buds can lead to growth distortions of the leaves, flowers or fruits (Tommasini and Maini, 1995). On leaves, probing causes the well-recognisable silver scars, which have been used to quantify feeding damage (van Dijken et al., 1994; Maris et al., 2003b). Most direct damage also implies cosmetic damage causing additional economic losses in case of ornamental flowers and fruits. Quantitative yield losses are particularly huge when population densities are very high and/or

plants are infested in an early stage (van Dijken et al., 1994). The damage on leaves can vary from a few ruptured cells in the epidermis to total necrosis of considerable parts of the leaf (Wardle and Simpson, 1927; Chisholm and Lewis, 1984; Childers and Achor, 1991). First instar larvae cause less damage than second instar larvae. Also, the host plant suitability can affect the amount of damage. All these factors do influence the probing behaviour and therefore the damage. Thrips mouthparts (stylets) are not long enough to reach the vascular bundle. Therefore, they must rely on feeding from cells of the epidermis, mesophyll, and palisade parenchyma from which they suck the cell contents after stylet penetration.

1.5. Morphology of the mouthparts

The morphology of thrips mouthparts is unique and they consist of an enveloping mouth cone, including a single left mandibular stylet without any functional canal and a pair of maxillary stylets, which form together a tube (Hunter and Ullman, 1992) (Fig 2). Before stylet penetration and feeding thrips place the tip of their mouth cone onto the plant surface. The paraglossal lobes of the mouth cone move apart and the labial pads are unfolded and pressed against the plant surface. By a head nod the mandible is thrust against and through the plant surface layers, the cuticle and the outer epidermal cell wall. Since the mandible is fixed to the exoskeleton it can only be protruded when the whole head capsule moves downward and backward and subsequently the more flexible mouth cone is shortened by muscular action (Chisholm and Lewis, 1984; Heming, 1993). The maxillary stylets are inserted into the hole made by the mandible and the thrips sucks up the contents of the punctured cells (Kirk, 1997). The feeding period may vary from less than a second to more than an hour (Heming, 1993; Harrewijn et al., 1996a; Harrewijn et al., 1996b) and ends when the thrips retracts its stylets and lifts its mouth cone. Then either a new probe on an adjacent spot is made or the insect walks away. Thrips feed on the contents of the damaged cells of the leaf tissue but not every stylet penetration activity is followed by a period of ingestion and therefore again, we prefer to indicate plant penetration by ‘probing’. Ingestion of the cell’s fluid contents and organelles can result in totally emptied cells (Chisholm and Lewis, 1984). Many cells are completely destroyed after intensive feeding and others may show extreme plasmolysis.

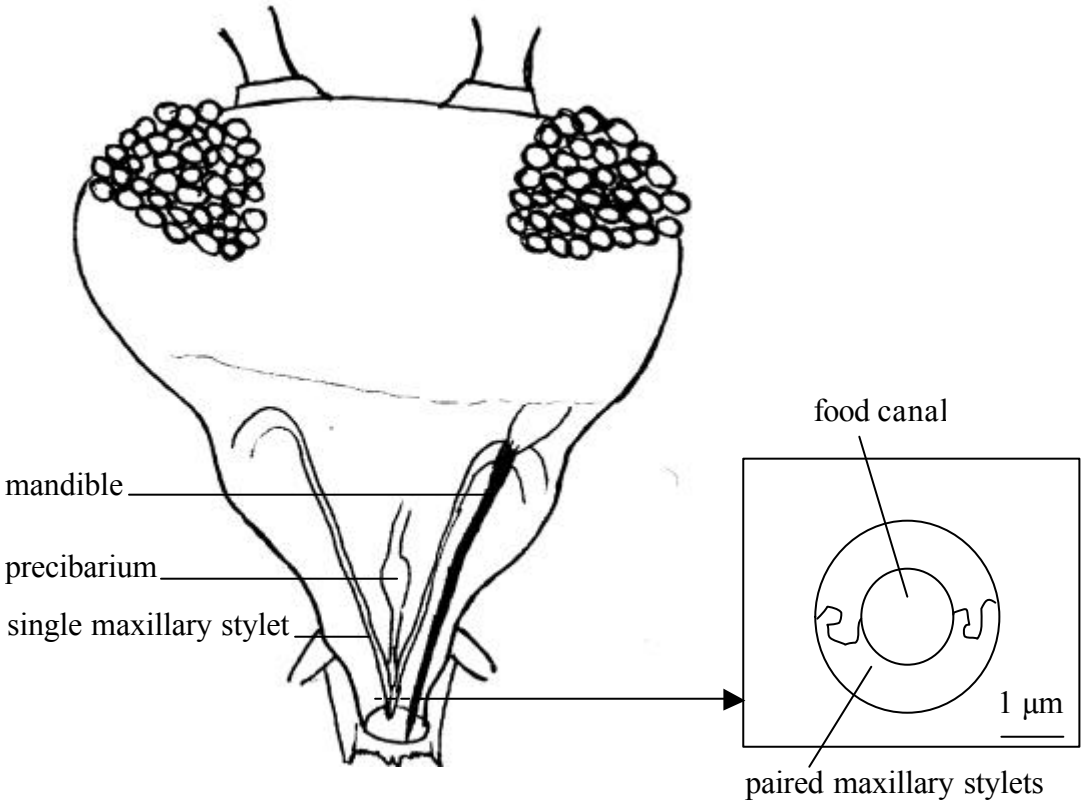

Fig. 2. Schematic representation of the mouthparts from a frontal view of the head of *Frankliniella occidentalis* and a cross-section of the paired maxillary stylets (free after Risler, 1957).

Whether these effects are only due to the stylet punctures and subsequent ingestion is not known. Necrosis of parts of the leaf could also be the result of subsequent desiccation or hypersensitive reactions of the plant to salivary components excreted by the thrips. Little is known about the salivation by the thrips. There is at least one pair of salivary glands (Ullman et al., 1992a; Kritzman et al., 2002). The salivary ducts of these lobed glands lead to the salivarium. From the salivarium a canal leads to the point where the hypopharynx ends. At this point the salivarium canal meets the precibarium (Fig. 3) (Hunter and Ullman, 1992) and the routes of the food and the saliva come together here. The maxillary stylets come together here as well (Fig. 3, inset ***I***), but they are not yet completely fused to form a closed canal, which occurs more distally (Fig. 3, inset ***II***). Therefore, the excreted saliva can follow a route either via the maxillary stylet canal or via the space surrounding the stylets to reach the plant puncture via the mouth cone opening (Fig. 3). Childers and Achor (1991) found salivary plugs after stylet penetration, which were suggested to seal the hole and acting together with the labial pads, to prevent any seepage of plant sap. These salivary plugs or flanges have also been found with other piercing-sucking insects (Miles, 1988). Saliva excretion by thrips has been observed after stylet penetration in Parafilm® membrane covered water (Chisholm and Lewis, 1984).

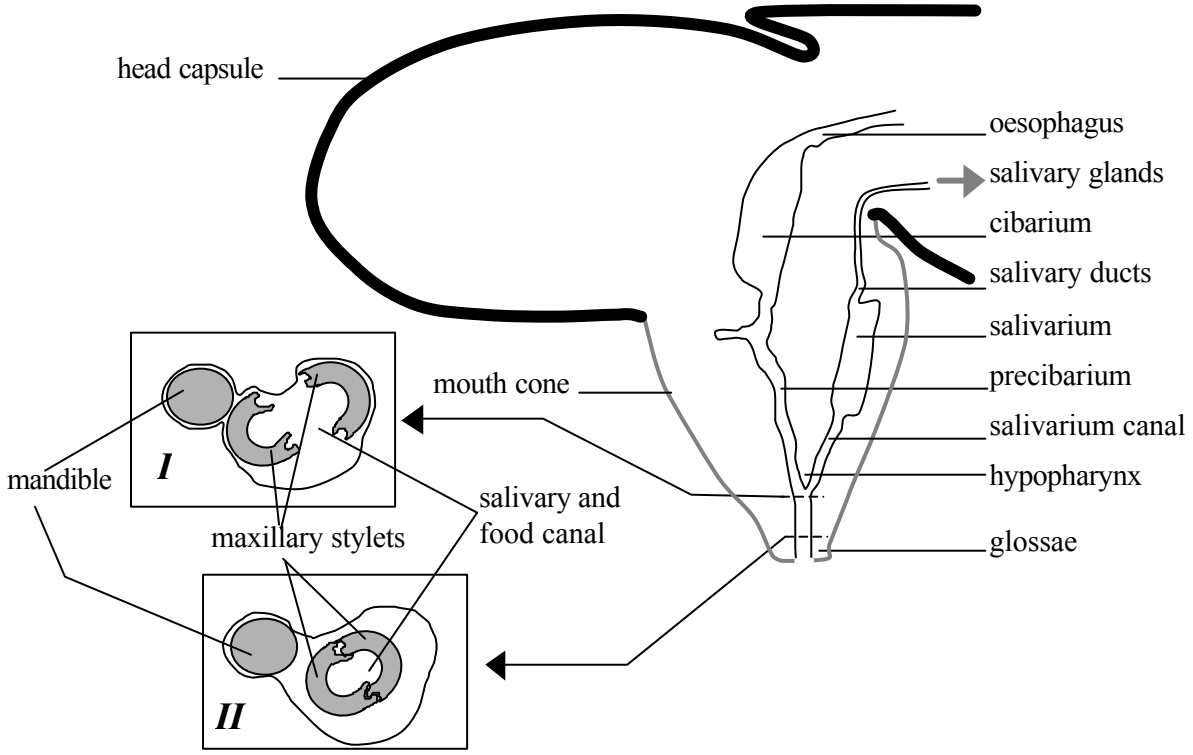

Fig. 3. Schematic representation of the median section of the head of *Frankliniella. occidentalis* with hard head capsule with more flexible mouth cone. Inserts represent cross sections at two levels of the mouth cone, the upper section (***I***) before and the lower (***II***) after the formation of a functional maxillary canal (free after Kirk, 1997 and Hunter and Ullman, 1992).

2. Monitoring of probing behaviour

Probing behaviour can be observed through a (stereo)microscope (Heming, 1978) with or without video recording (Chisholm and Lewis, 1984). Ingestion can be inferred from contractions of the cibarial muscles in the rather transparent head capsule. The frequency of these contractions is between 2-6 Hz as observed in *Limothrips cerealium* (Chisholm and Lewis, 1984). However a quantitative analysis of the probing activities by visual observation is difficult and very laborious, because only one thrips can be observed at a time. Analysis of ingested quantities has been undertaken by using radio-active tracers. The ingested volume has been measured in this way and the feeding rate was calculated (Day and Irzykiewicz, 1954; Chisholm and Lewis, 1984; Wiesenborn and Morse, 1985; Wiesenborn and Morse, 1986). However, the time of exposure to a labelled diet was used in these studies instead of the exact time of ingestion, which cannot be obtained from visual observation. For this purpose, electronic monitoring of probing behaviour is a suitable technique (Tjallingii, 1986a).

2.1. Electrical penetration graph (EPG) technique

Electronic monitoring of the probing behaviour of piercing-sucking insects has been introduced by McLean and Kinsey (1964) and further developed by Tjallingii (1988) known now as the electrical penetration graph (EPG) technique. Originally, it was used for aphids but later it was also applied to other piercing-sucking insects (Walker, 2000). Presently, EPG studies have been used in whiteflies (Janssen et al., 1989; Lei et al., 1996; Lei et al., 1998), leaf- and planthoppers (Backus and Hunter, 1989; Kimmins, 1989; Lett et al., 2001), mealy bugs (Calatayud et al., 1994) and also in thrips (Hunter et al., 1993; Harrewijn et al., 1996b).

In principle, EPG comprises a simple electric circuit in which the insect and the plant are incorporated (Fig. 4). Plant and insect are connected with electrodes in a circuit that also contains a voltage source and a resistor. When the insect's mouthparts penetrate into the plant, the circuit is completed and the fluctuating voltage across the resistor results in a EPG signal. The voltage changes during probing are due to two different electrical origins, fluctuations of electrical resistance and electromotive force (emf) (Tjallingii, 2000). In the EPG signals, recorded and visualised by computer, different waveforms can be distinguished, characterised by amplitude, frequency, and other features. These waveforms have a relationship to the behavioural activities and events of the probing insect. For aphids, many of such relations between insect activities and EPG waveforms have been established (Tjallingii, 1995). For thrips, correlating the behaviour with EPG waveforms has started more recently (Hunter et al., 1993; Tjallingii, 1995; Harrewijn et al., 1996b).

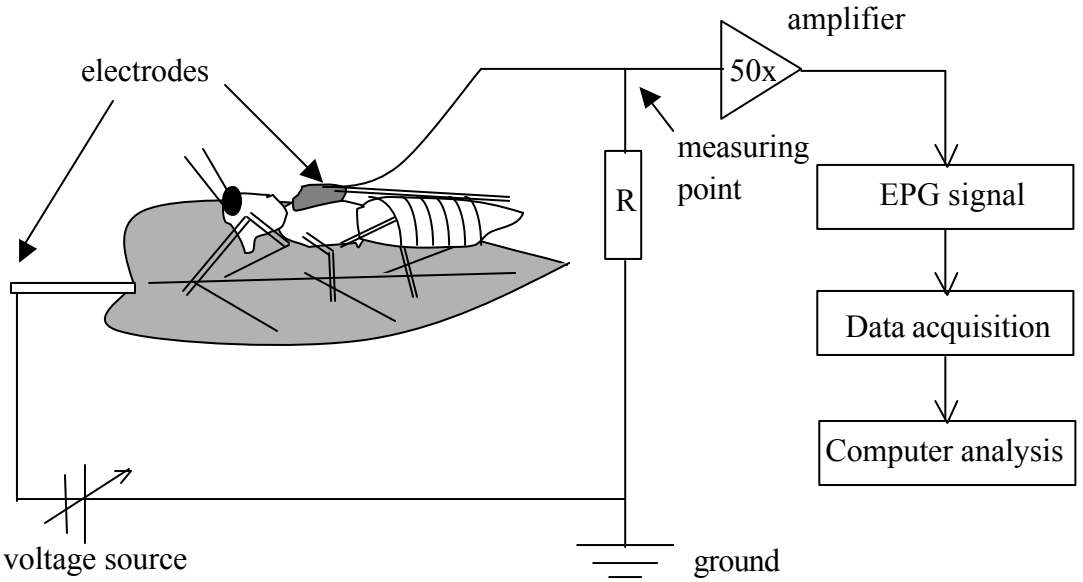

Fig. 4. Electro penetration graph (EPG) set-up. The principle electrical circuit of the DC-EPG system with a thrips and attached leaf incorporated.

2.2. EPG relations to natural probing behaviour

EPG recording can be used to quantify all activities related to probing and the effects of plant resistance on these activities. However, these recordings are made under artificial conditions, the most important being the tethering of the insects by the electrode wire that limits their movements. The electrical current is minute and no negative effects have been found so far (Tjallingii, 1985). The tether effect has been investigated for aphids (Tjallingii, 1986b; Hardie et al., 1992; Caillaud et al., 1995; Prado and Tjallingii, 1999) and whiteflies (Lei et al., 1997). The effects found are not negligible, but on the other hand, almost every technique used in behavioural studies does affect inevitably the insect's behaviour. In general, all EPG studies should actually be supported by control experiments of free moving insects. Although the tether effect is the same for insects on susceptible and resistant plants, it can lead to an underestimation of resistance in non-host plants or resistant cultivars (Tjallingii, 1986b; Caillaud et al., 1995). The effect of tethering on virus transmission has not been studied but will likely depend on the effect on the specific insect's activities that are important for transmission and on the virus-insect-plant combination as well.

3. *Tomato spotted wilt virus*

3.1. Properties

Tospoviruses are exclusively transmitted by a limited number of thrips species, of which *F. occidentalis* is currently regarded as the main vector worldwide (Ullman et al., 1997; Nagata and Peters, 2001). The *Tospoviruses* form a distinct genus of plant-pathogenic viruses within the large family of *Bunyaviridae* which is further restricted to mammals (van Regenmortel et al., 2000). The animal-infecting bunyaviruses include several important human and animal viruses, and these are mainly transmitted by blood-sucking arthropods such as mosquitoes, sandflies and ticks.

TSWV is the type species of the *Tospoviruses* and, like all bunyaviruses, has a tripartite single-stranded RNA genome (Goldbach and Peters, 1994). The TSWV particles are quasi-spherical in shape with a lipid envelope containing two kinds of glycoproteins (Fig. 5) and a diameter between 70 and 110 nm.

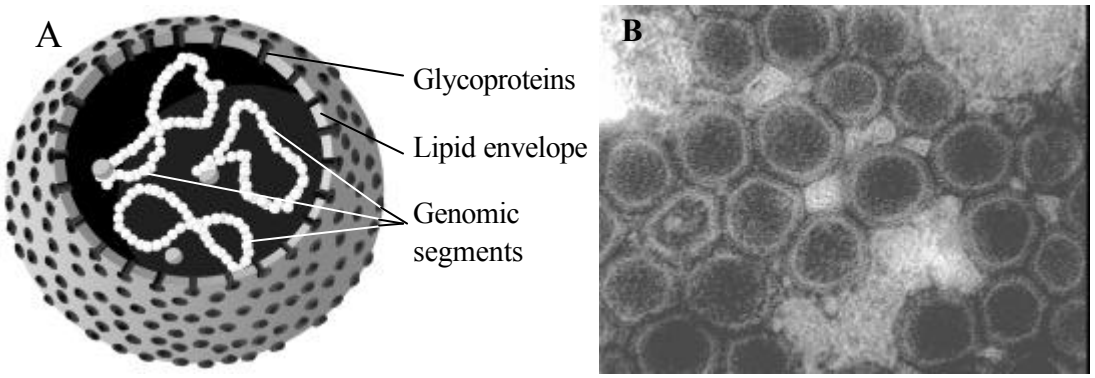

Fig. 5. *Tomato spotted wilt virus*, **A,** Schematic representation of the virus particle. Glycoproteins, lipid envelope and the three genomic segments are indicated; **B,** Electron micrograph of purified TSWV particles.

3.2. Thrips transmission of *Tospoviruses*

TSWV is circulatively and propagatively transmitted (Fig. 1). Circulatively, because it is ingested by its vector and transported in the insect from the gut to the salivary glands before it can be transmitted to a new host. Propagatively, because TSWV is replicated in the thrips but this has no known negative effects (Wijkamp et al., 1996a; Maris, 2004). The transport route through the vector is almost completely known (Ullman et al., 1992b; Wijkamp et al., 1993; van de Wetering et al., 1996; Nagata et al., 1999; Nagata and Peters, 2001; Chatzivassiliou et al., 2002; de Assis et al., 2002). Only first instar larvae can successfully acquire TSWV. After ingestion, the virus infects the epithelium of the midgut and subsequently the circular and longitudinal muscle tissues of the midgut. The virus particles must then be transported to the salivary glands. Most likely, this occurs via a transient contact between the midgut and the salivary glands during larval development (Moritz, 2002) and not via the haemolypmh or the suggested ligaments between the salivary glands and the midgut (Ullman et al., 1989). After replication in the salivary glands the virus can be transmitted by the late second larval and adult stages of the insect. Virus inoculation occurs with excretion of the saliva into the plant (Wijkamp and Peters, 1993; Nagata and Peters, 2001).

The minimum duration required for TSWV transmission is not exactly known, probably one probe is sufficient. It has been found that transmission occurred within 5 min and the median inoculation access period, the time that 50% of the thrips has transmitted TSWV, depends on the host and was 59 min on petunia leaf discs and 133 min on *Datura stramonium* (Wijkamp et al., 1996b). TSWV is acquired during feeding, the minimal time for acquisition found is 5 min, but this was also the shortest duration tested.

3.3. TSWV symptoms

When a susceptible plant is successfully infected, TSWV will spread systemically through the plant (Best, 1968). TSWV disease symptoms can greatly vary, depending not only on plant species or cultivar, but also on plant age and growing conditions. Among the symptoms encountered in systemically infected hosts are chlorosis, mottling, wilting, stunting, and necrosis. In several cases the infected plant will sooner or later die. In some plant species, including Petunia, TSWV infection induces local lesions, and such plants are used to determine thrips' capabilities to transmit TSWV or to quantify thrips transmission under laboratory conditions or semi-field situations (Maris et al., 2003a; Maris, 2004).

3.4. TSWV and thrips resistance in host plants

TSWV has a very broad host range, as have most of its vectors. More than 1100 species in 100 plant families are known to be susceptible, dicotyledons as well as monocotyledons (Peters pers. comm.). Many vegetable crops, like lettuce, tomato and pepper (*Capsicum* spp.) are frequently infected. In some host plants TSWV resistance traits have been identified and these have been used in virus resistance breeding programs. For instance, in chrysanthemum (Daughtrey et al., 1997), tomato (Stevens et al., 1992) and pepper single dominant genes for TSWV resistance have been identified and commercially used (Black et al., 1991; Boiteux and de Ávila, 1994). However, the risk of a single dominant resistance gene (vertical resistance) is a possible break down in field conditions (Boiteux et al., 1993; Kumar et al., 1995; Cho et al., 1996; Thompson and van Zijl, 1996). Combining such a virus resistance with vector resistance could potentially reduce virus spread and therefore, reduce selection pressure on the virus by the resistance, thus providing a more sustainable crop protection (Kumar et al., 1995). Vector resistance could be defined as a trait of a plant that decreases the performance of thrips as virus vector. However, the opposite may also occur if vector resistance would alter the feeding behaviour of thrips resulting in higher probing frequency and thus enhanced virus transmission (van de Wetering, 1999). What kind of effect the vector resistance has on virus transmission will depend on the influence of the plant's resistance on the probing behaviour of thrips.

4. Outline of this study

At the start of the studies compiled in this thesis, information on the effects of thrips resistance on the spread of TSWV was limited. Preliminary experiments performed with a thrips resistant chrysanthemum accession revealed that this accession became more readily infected with TSWV than a susceptible accession, possibly by an altered feeding behaviour of the vector (van de Wetering, 1999). Given this seemingly adverse effect, an STW-funded research project encompassing two PhD research lines was started aiming to determine potentially positive and adverse effects of thrips resistance on TSWV-spread (see thesis of Maris, 2004) and to relate these effects with possibly altered probing behaviour of the thrips (this thesis). Pepper (*Capsicum* spp) was chosen as model crop. A single dominant TSWV resistance gene had been introgressed in some pepper accessions, while other accessions had good levels of thrips resistance, allowing a comparative study of possible synergistic and/or antagonistic effects of thrips resistance and virus resistance.

To be able to determine the effects of thrips resistances on the probing behaviour it was first necessary to carefully describe the probing behaviour of *F. occidentalis*, especially the probing activities involved in TSWV transmission. A combination of the electrical penetration graphs (EPG) technique and simultaneous video monitoring was chosen as main experimental approach. Chapters 2 and 3 describe the thrips' probing activities and how these are reflected in EPG waveforms, including sap ingestion that is responsible for TSWV acquisition. In Chapter 4, the activities involved in the inoculation of TSWV in terms of EPG waveforms are identified. Subsequently, Chapter 5 presents comparative EPG analysis using four different pepper accessions, differing in virus and vector susceptibility. With the information of the role of probing activities in virus transmission (Chapter 4) the expected impact of the cultivars are evaluated. In Chapter 6 finally the results obtained are summarized and discussed.

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CHAPTER 2

Characterisation of the feeding behaviour of Western flower thrips in terms of electrical penetration graph (EPG) waveforms

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CHAPTER 2

Characterisation of the feeding behaviour of Western flower thrips in terms of electrical penetration graph (EPG) waveforms

Abstract

The Western flower thrips, *Frankliniella occidentalis* (Pergande) causes damage to plants when they are feeding. Also, this thrips species transmits *Tomato spotted wilt virus* (TSWV) during stylet penetration. We investigated the penetration behaviour (probing) of thrips on pepper leaves and on liquid diet by electrical penetration graph (EPG, DC-system) recording. In addition, we used high-magnification video observations to correlate EPG waveforms with the insect's posture, head movements, and muscle contractions. Also, EPGs were correlated with probing on liquid diets containing radioactive tracers to distinguish and quantify ingestion waveforms. The previously described waveforms P, Q, and R were distinguished and additionally, a new waveform 'S' was distinguished. Waveform P could be linked with mandibular leaf penetration, waveform Q with insertion of the maxillary stylets, and waveform R with ingestion of cell contents, whereas waveform S could not be correlated with any behavioural activity. Histology of the feeding damage in pepper leaves shows that *F. occidentalis* ingests from multiple cells per probe.

1. Introduction

The western flower thrips (WFT), *Frankliniella occidentalis* (Pergande) (Thysanoptera; Thripidae), is a world-wide pest in many ornamentals and vegetables. Direct WFT damage includes silver scars on leaves, flowers and/or fruits and growth distortions by feeding in a piercing-sucking manner (Chisholm and Lewis, 1984). In addition, vectoring of *Tomato spotted wilt virus* (TSWV) by WFT can also cause large economic losses. Feeding behaviour of thrips has been described by Chisholm and Lewis (1984), Heming (1978; 1993), Harrewijn, et al. (1996a,b) and Hunter and Ullman (1989). These studies showed that the mandible first pierces a hole in the leaf and that subsequently cell contents are ingested via the maxillary stylets (Chisholm and Lewis, 1984). Saliva is probably excreted in between the piercing and the sucking activities (Harrewijn et al., 1996a). In these previous studies different names have been used to describe the different phases of the feeding behaviour. We will define 'probing' to indicate all piercing-sucking activities during leaf contact by the mouthparts, which includes mandibular and maxillary movements, saliva excretion, and ingestion of the cell contents.

There is so far no evidence regarding which probing activities are involved in the acquisition and inoculation of TSWV. The virus is circulatively and propagatively transmitted (Nagata and Peters, 2001). First and early second instar WFT larvae acquire the virus, whereas inoculation is restricted to second instar larvae just before pupation as well as adults (Chatzivassiliou et al., 2002; Wijkamp and Peters, 1993). The virus can be acquired and inoculated in periods as short as five minutes, but the efficiency of acquisition and inoculation is known to increase with the length of the access period (Wijkamp and Peters, 1993). The virus has been detected in the salivary glands of viruliferous thrips (Wijkamp et al., 1993; Nagata et al., 1999) and is probably ejected with the saliva into a plant when a thrips is probing. During which phase of probing the saliva is secreted is not known.

Probing behaviour can be studied by electrical penetration graph (EPG) monitoring. Two different systems are currently used, the AC-system (McLean and Kinsey, 1964) and the DC-system (Tjallingii, 1988). In these systems a piercing-sucking insect and its host plant (or liquid diet) are made part of an electrical circuit, which is completed as soon as the insect's mouthparts penetrate the plant. Both, AC- and DC-systems record the changes in electrical resistance that are associated with probing. Only the DC-system records the voltages evoked (electromotive forces) within the insect-plant system (Tjallingii, 2000). The fluctuating

voltages cause different waveforms, which can be displayed on a computer monitor. Few studies have been made on the probing behaviour of thrips using any of these monitoring systems (Hunter et al., 1993; Harrewijn et al., 1996a,b; van de Wetering et al., 1998; Groves et al., 2001). Hunter et al (1993) distinguished four different waveforms with the AC-system and suggested that these waveforms represented probe initiation, salivation/stylet movement, ingestion, and probe termination, respectively. Later, Harrewijn et al. (1996a), using the DC-system, distinguished three waveforms and suggested that these reflect mandible penetration, salivation, and ingestion of the cell contents, respectively. However, none of these waveforms were correlated with behavioural observations.

The aim of the study described in this Chapter was to investigate the feeding behaviour of WFT in more detail and to describe the feeding activities in terms of electronic monitoring waveforms (EPG-DC system) by integrating the EPG recordings with head movements, muscle contractions, and food intake. Also, we studied the leaf histology in relation to probing damage.

2. Materials and Methods

2.1. Insects and plants

The western flower thrips population used was collected in the spring of 2000 on sweet pepper (*Capsicum annuum* cv. Mazurka) in a greenhouse and reared continuously on bean pods (*Phaseolus vulgaris* cv. Prelude) in a climate room at 16/8 h of light/dark and 25° C. The pepper accession "Pikante reuzen" was used in the present studies. This accession is susceptible to both thrips and TSWV (Maris et al., 2003). The plants were grown in a greenhouse at 16/8 h light/dark and 24 ± 2° C. In all experiments with plants 3-4 weeks old seedlings were used. Also two liquid diets were used: tap water enclosed by stretched Parafilm®, or 10% sucrose solution enclosed by stretched Parafilm®. All experiments were carried out at 21 ± 3° C.

2.2. Electrical Penetration Graph (EPG) technique

To record probing behaviour we used the DC-EPG system in a 1, 4, or 8 channel version (made at Wageningen University, WU), as developed by Tjallingii (1988, 2000) to record

probing behaviour of Homopteran. Specific modifications were made to be able to use thrips in this system. Gold wire of 10 µm in diameter was used, because of the small size of the thrips, especially of the L1 and L2 instars. Thrips were wired on a specially developed 'manipulation table' (Fig. 1). This table consisted of a small plastic ball (1.6 cm in diameter), from which a top section was removed (Fig. 1). This ball table was placed on a cylinder socket so that it could be rotated 360º and tilted 45º. A vacuum-channel was vertically drilled through the ball. The diameter of the vacuum channel in the ball was 0.5 mm wide for adult females and 0.2 mm for adult males and larvae. Individual thrips were anaesthetised by CO_2 and gently sucked onto the channel by vacuum, which was connected to the socket underneath the ball. To sustain anaesthetisation during wiring CO_2 gas was delivered to the insect on the manipulation table, which was surrounded by a Perspex cylinder (diameter 8 cm, height 6 cm). The CO_2 flow was stopped after proper positioning of the thrips. A little droplet of silver glue (water soluble glue mixed with fine silver powder) was put on the dorsal side of the mesothorax with a thin needle and the wire placed in the glue at an angle of 75-90° with the abdomen. Thrips recovered from the anaesthesia within a few minutes, were connected to the input of the EPG amplifier and lowered onto the substrate after the recording was started. The data were stored on a hard disk of a computer at a 100 Hz. sample rate and analysed with Stylet 3.7 Dos-software (developed at WU).

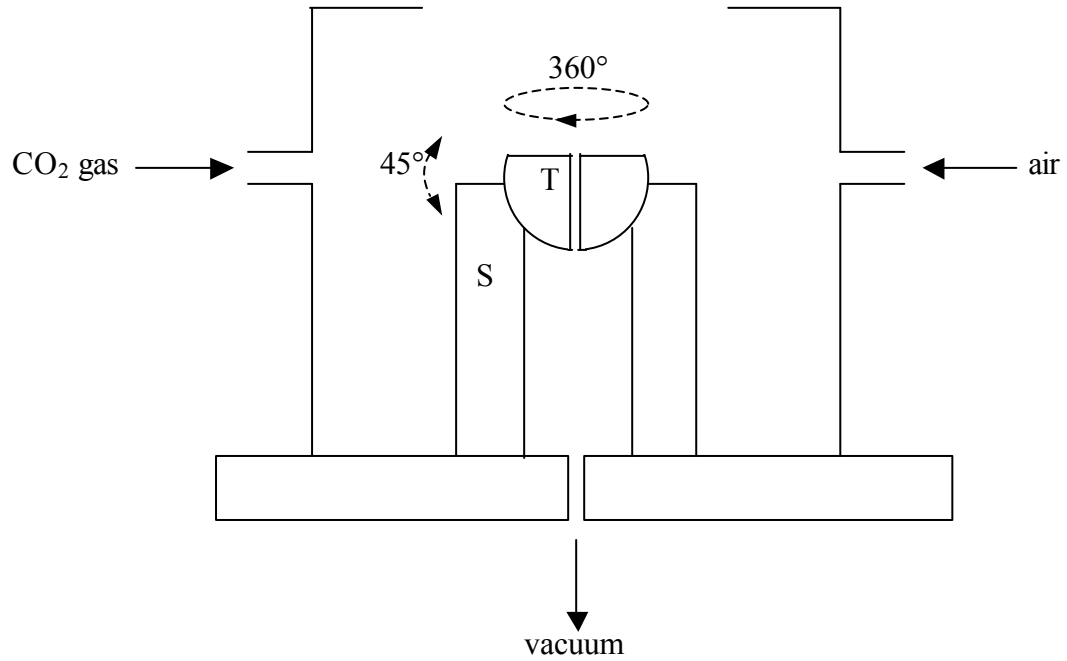

Fig. 1. Cross-section of the manipulation table for thrips wiring. A thrips is placed on the top of the ball table (T) and sucked by vacuum onto the hole (0.5 mm, females or 0.2 mm, males and larvae). The ball can be rotated 360º and tilted for 45º in its socket (S). The thrips is anaesthetised by an inlet of CO_2 and can be revived by an inlet of air.

2.3. High-magnification video

High-magnification video observations were made with a 3-colour CCD camera (Panasonic AW-300E, with studio card AW-PB305 and remote control WV-RC550) and recorded by a SVHS recorder (Panasonic AG-7330). The camera was mounted on a stereomicroscope (Zeiss Stemi SV 11, max. 264x). Two 'cold light' sources (Schott KL1500) were used with flexible light conductors. The light sensitivity of the camera could be adjusted with the gain control to prevent overheating of the thrips at high magnifications. The thrips were placed on the adaxial side of a leaf of a pepper plant or on a 10% sucrose solution in sachets of stretched Parafilm® membranes. The thrips were kept in focus by rotating and tilting the insect and the substrate with an apparatus as used by van Helden *et al.* (1994). The thrips were usually placed in a position on the substrate such that we could observe their lateral side and view their head and especially the mouth cone. Some additional video recordings were made with a ventral view from membrane-probing insects. The video was recorded through a microscope slide cover slip and liquid diet (10% sucrose or water) covered by stretched Parafilm® membrane.

2.4. Video observations in combination with EPG

For off-line analysis of the video and EPG recordings, the two signals were synchronised using the audio track of the SVHS tape for the EPG signal. In a modulator (made by the Electronics Department, WU) the voltage of the EPG signal (input) modulated the amplitude of a 1 kHz sine wave (output) making the signal suitable for analogous recording on the sound track of the videotape. Only the positive part of the sine wave was displayed in a sound track window of the video editing program Adobe Premiere 5.1®, whereas the digitised video (using a Miro DC30 plus card) images were displayed in a different window.

2.5. Estimation of ingestion using radioactive tracers

To quantify ingested volumes, thrips were allowed to feed on a Parafilm® covered solution of 10% γ ^{32}P D-ATP-labelled sucrose (30-40 MBq/ml, Amersham Pharmacia Biotech) while EPGs were recorded. Only those thrips were used which had fed during at least one hour before testing, but after wiring. Wired adult males and females, as well as first and second instar larvae were given access to the solution for 15-30 min. After this period each individual

was transferred into 2 ml of scintillation fluid (Lumasafe™ plus) to which 1 µl of 10% Triton was added to immerse the thrips. Two vials with scintillation fluid and Triton were used as background blanks and two other vials with 1 µl of the ^{32}P-labeled 10% sucrose solution were used as standards. The radioactive emission of each thrips was counted in a liquid scintillation counter (Beckman LS6000TA). Counts were corrected for background values and converted to volumes using a standard series. Only thrips with counts of at least twice the background value were considered to have ingested the solution. Corresponding waveform R durations were retrieved from the EPGs. The correlation between the duration of R and the volume ingested was calculated and analysed by Pearson Correlation test (α = 0.05, SPSS 11.0). The ingestion rate of the different developmental stages was determined by linear regression analysis (α = 0.05, SPSS 11.0).

2.6. Histology of leaf damage

Leaf damage caused by thrips probing was studied histologically. Pieces of about 3 x 3 mm were cut from undamaged and damaged leaves of four-week old pepper plants. After fixation and embedding in paraffin (Langdale, 1994), 8 µm cross sections were made and the paraffin was removed by 3 washes in xylene. For staining first a pre-stain was used, i.e. iron Hematoxylin (Merck). Safranin-O (Sigma) was used in combination with Fast Green (Sigma) to stain the cellulose walls and chloroplasts. After dehydration and clearing the sections were mounted in DPX (Fluka). The thickness of the upper and lower epidermis, the palisade parenchyma, and spongy mesophyll was measured by light microscopy (Leica Microsystems, maximal magnification 40x). Also, the dimensions of the palisade parenchyma cells and the shortest distance from the leaf cuticle to the vascular tissue were determined. Leaves with severe, moderate and minor thrips damage were used. For severe damage a four-week old pepper plant was infested with 12 adult thrips for 48 h. For moderate damage 4-5 adult thrips were allowed to infest a plant for 12- 24 h. For minor damage a probing site of an individual adult thrips was marked and sectioned.

3. Results

3.1. Electrical Penetration Graphs (EPGs) of thrips

To elucidate which thrips EPG waveform is related to ingestion or salivation and to establish during which process TSWV is acquired or inoculated, it is crucial to identify and define the separate probing activities. Four different waveforms, labelled P, Q, R and S were identified (Fig. 2A).

The P waveform was characterised by one or a few high peaks occurring at the beginning of a probe, i.e. the period of plant penetration (Fig. 2A, B). The average duration of P was usually less than one second (about 0.25 s), but ranged between 0.05 and 1.30 s. The maximum amplitude (voltage) of the P-peak was higher than that of any other waveform. The electrical origin of the P waveform was found to be based on electromotive force fluctuation (emf), i.e. voltage changes evoked by biological phenomena in the insect-plant system (c.f. Tjallingii, 2000) as described by Harrewijn *et al.* (1996a), but there may also be resistance changes. The Q waveform formed a transition phase between P or S and R, and was mainly characterised by a voltage dip. This dip was visible in most of the penetrations, but its appearance and duration was not very consistent. The electrical origin of this dip in the Q waveform was based on an emf component as shown by Harrewijn *et al.* (1996a). The R waveform was the most regular and dominant waveform in the thrips EPGs, characterised by regular waves with square-like peaks (i.e. without additional peaks or dips; Fig. 2A, B). The frequency of these waves was 3-8 Hz, which differed between individuals and often fluctuated during a probe. Additional peaks of waveform R (Fig. 2B) were superimposed on the main waveform. The function or activity of these additional peaks remains to be elucidated. The peak-to-peak amplitude of the main R waves varies between individuals, but also between and within probes made by a single thrips. In general, the amplitude is larger on liquid diets than on plants. The main electrical origin of the R waves is emf, but there is also a minor electrical resistance component.

The waveform S, newly distinguished in this study, was previously included as part of the P wave (Harrewijn *et al*., 1996a). It is characterised by a short sequence of regular waves at a 9-17 Hz frequency and a peak-to-peak amplitude somewhat bigger than in waveform R. Waveform S mostly occurred between waveforms P and Q, but sometimes interrupted an R period or occurred at the end of a probe. The electrical origin and behavioural correlations of

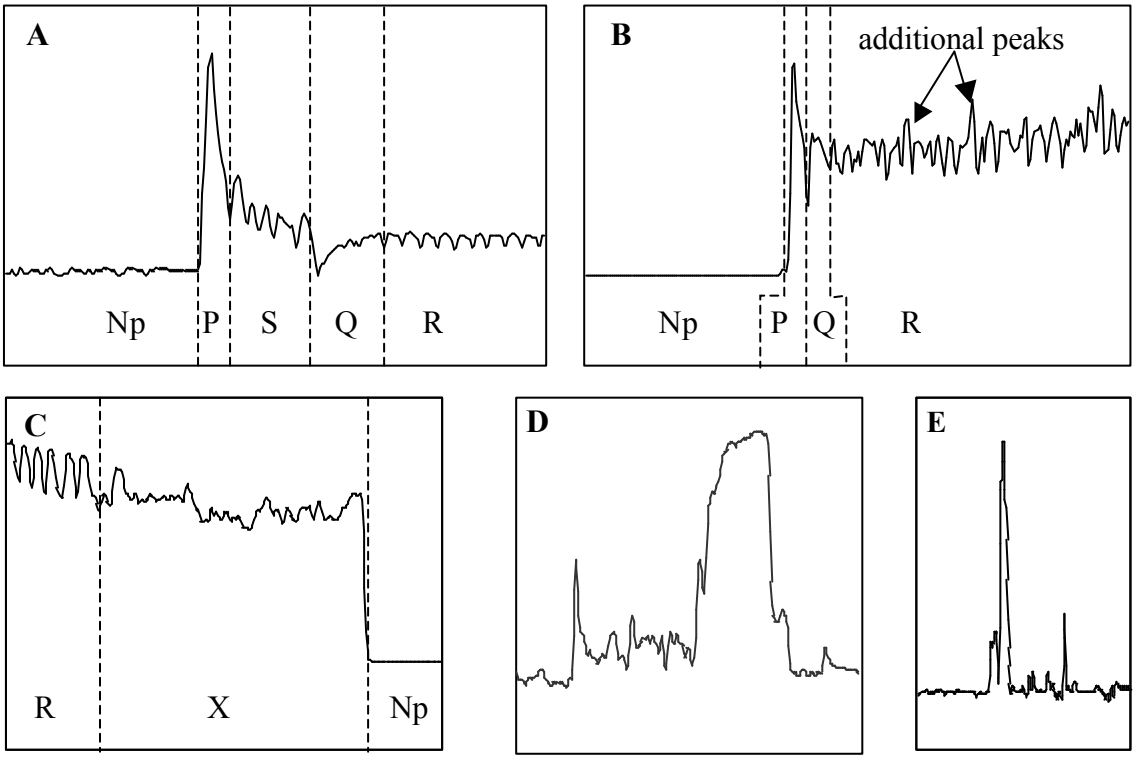

Fig. 2. EPG waveforms of the Western flower thrips. **A,** characteristic sample of 5 s with all possible waveforms on a pepper leaf. Np = non penetration, P = penetration with the mandible, S = repetitive head nodding, Q = insertion of maxillary stylets and R = ingestion of cell contents; **B**, characteristic sample of 5 s on a liquid diet, 10% sucrose solution enclosed with stretched Parafilm®; **C**, sample of 4 s with waveform X, a part in a probe which cannot be classified as one of the regular waveforms; **D**, sample of 3 s showing a pseudo-probe; **E**, sample of 2 s showing a pseudo-probe.

the S waveform are still unknown. Waveform S was often shown by thrips on bean pods but rarely on pepper leaves. Except for S, all EPG waveforms were shown on leaves, bean pods and liquid diets (Fig. 2B) but not all waveforms occurred in every probe. Short probes often consisted of P and Q only. The waveforms in most EPG recordings were not as well shaped as shown in Figure 2A. For example, the R waveforms often had additional peaks as in Figure 2B. The duration of probes was quite variable, from less than 1 s to more than 1.5 h.

Some probes occasionally contained rather noisy signals, not matching any known waveform. These have been designated X signals (Fig. 2C). The X-signals might be distorted waveforms, short circuited by some parallel electrical contact with the leaf. The X-signals were mostly observed at the end of a probe, though sometimes they interrupted R or occurred after Q. Electrical leaf contacts without probing as concluded from video recording seemed to occur as well now and then and were referred to as 'pseudoprobes' (Fig. 2D+E).

3.2. High-magnification video observations of the probing of thrips

Some aspects of the feeding behaviour of WFT were also studied visually, using a high-magnification video set-up. About 30 thrips (most females, but also males and both juvenile instars) were used for EPG recording for 1 h and ca. 30 thrips (both sexes and instars) between 5 and 20 min. The total duration of recordings was about 36 h. Reviewing video forward and backward and frame by frame showed that each probe started by placing the mouth cone on the leaf surface followed by a quick downward movement of the head, which was sometimes repeated a few times ('nodding'). During the downward movement of the head, the mouth cone was shortened between leaf and head (Fig. 3C) as was observed by Chisholm and Lewis (1984). When the head was raised again, the mouth cone remained in contact with the leaf. After head nodding, hardly any movements of the body were observed until the probe was ended. With proper illumination some muscle pulsations were seen inside the head and the mouth cone of the thrips, in larvae especially, which have a more transparent cuticle. The pulsations were visible from ventral, dorsal as well as the lateral angles. When ending a probe the head was lifted and the mouth cone subsequently lost contact with the leaf while the maxillary stylets were retracted between the glossae, which occasionally was visible. Some probes occurred directly after the previous one; in other cases the insect walked away. Thus, high-magnification observations allowed us to distinguish three phases in probing behaviour: (1) probe initiation with nodding, (2) probing without body movements but with internal pulsation in the head, and (3) probe termination with stylet withdrawal.

3.3. Correlation of video observations and the EPG waveforms

High-magnification video observations with the EPG recording were combined in order to relate head movements, muscle pulsation and probe ending to the various waveforms identified. The first downward movement (nod) of the head appeared to coincide with the P waveform in the EPG signal (Fig. 3B). The coinciding nods were well observed in most P waveforms. However, they were not visible in a few of the video images. Subsequently, when the head moved upward the Q waveform was observed. The period after the nod in P or the last nod in S, when the mouth cone was in continuous contact with the leaf without body movements, coincided with the R waveform (Fig. 3C, D). The frequency of the pulses visible in the head during waveform R was significantly correlated with the frequency of the waves in R ($n = 5$, $r = 0.996$, $P < 0.001$, Spearman's Correlation test, SPSS 11.0).

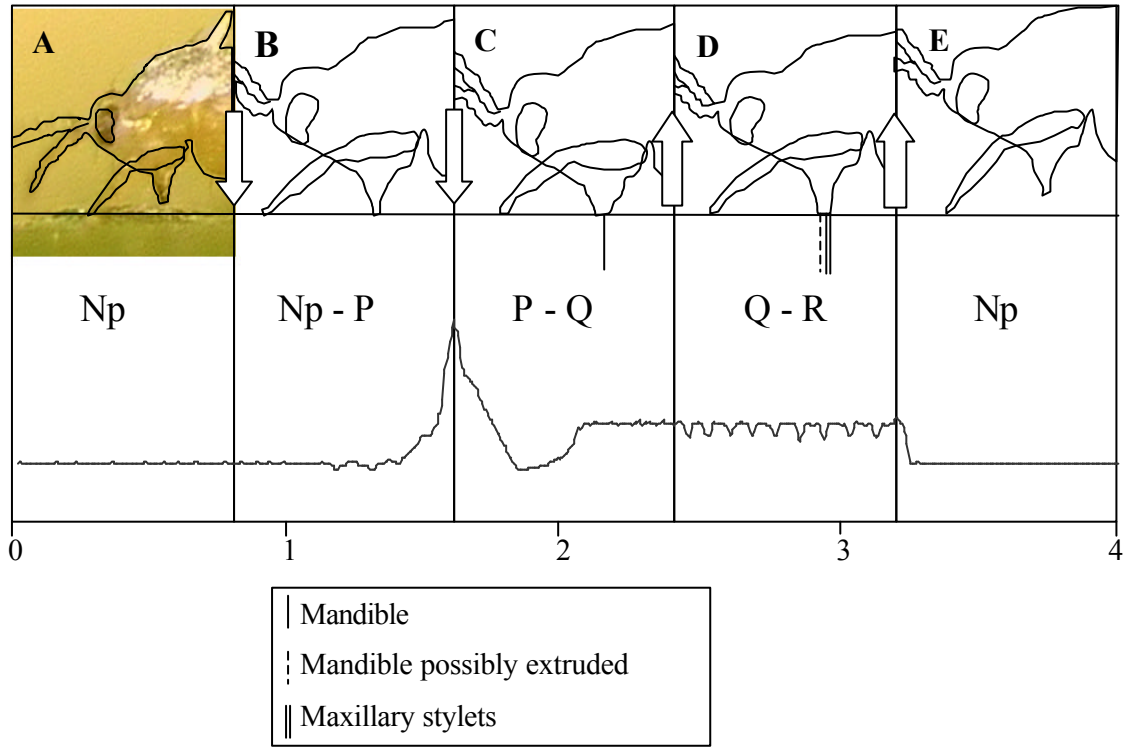

Fig. 3. Feeding behaviour of the Western flower thrips; combination of video and EPG. Part **A**, thrips not penetrating; **B,** thrips before penetrating; **C,** thrips penetrated - insertion of the mandible; **D,** thrips feeding – insertion of maxillary stylets and maybe mandible and **E**, thrips stopped penetration. The lower part of figure shows the corresponding EPG waveforms during the different behavioural elements.

Some pulsation was also visible in the mouth cone during the Q and S waveforms, but this could not be correlated with any waveform details. The S waveform was recorded when the nodding movements of thrips were observed while the mouth cone remained in contact with the leaf surface. The frequency of nodding is significantly correlated with the frequency of the S waves in the EPG signal ($n=5$, $r = 0.999$, $P < 0.001$, Spearman's Correlation test, SPSS 11.0). During the pseudoprobes in the EPG no mouth cone contact with the leaf was observed. Thus, the simultaneous video and EPG recordings allowed us to link the four waveforms with head movements or some pulsation in the head.

3.4. Radioactive tracer ingestion by thrips

The supposed liquid ingestion during waveform R was confirmed by radioactive tracer intake. The amount of ingested ^{32}P-labelled 10% sucrose solution was significantly correlated with the duration of R waveforms for females ($P = 0.002$), males ($P = 0.04$), second instars ($P < 0.001$) and first instars ($P = 0.003$) (Fig. 4).

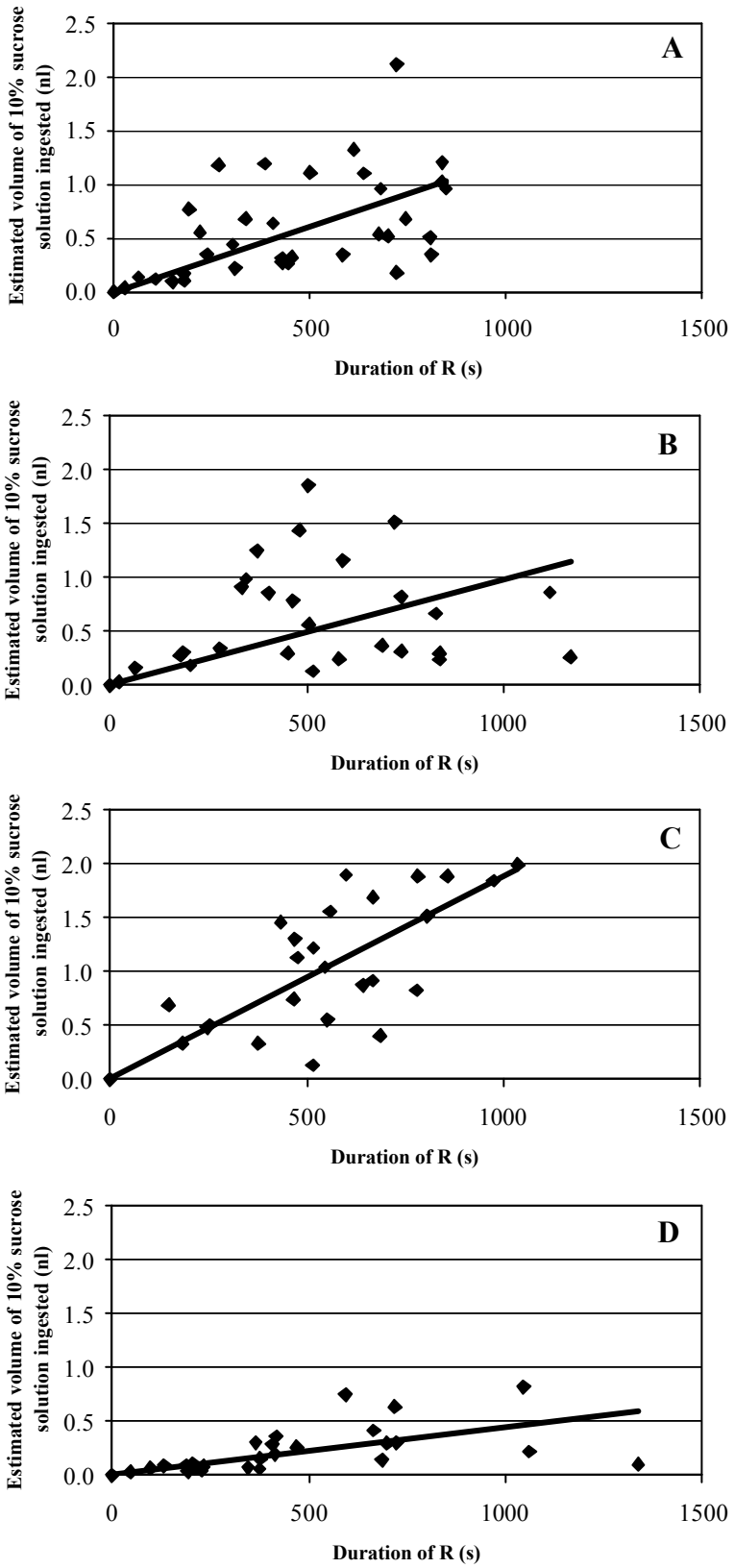

Fig. 4. Regression line of the amount of 10% sucrose solution ingested by thrips during feeding on ^{32}P labelled sucrose solution and the duration of waveform R. Regression coefficient equals the feeding rate and is given in pl (= 10^{-12} litre) / s. **A**, Females 1.21 pl/s (SE ± 0.136) (n=34); **B**, males 1.04 pl/s (SE ± 0.158) (n=28); **C**, second instar larvae 1.87 pl/s (SE ± 0.144) (n=25), and **D**, first instar larvae 0.44 pl/s (SE ± 0.065) (n=26) were tested.

The durations of the P and Q waveforms were not correlated with the amount of sucrose solution ingested. The ingestion rate was determined by linear regression and was highest for the second instar, 1.87 pl/s (SE ± 0.144) (Fig. 4C). For adult males it was 1.04 pl/s (SE ± 0.158), for adult females 1.21 pl/s (SE ± 0.136), and for first instar larvae 0.44 pl/s (SE ± 0.065). The ingested volume can be estimated for any probe using these ingestion rates and the durations of the R waveform.

3.5. Histology of pepper leaves in relation to probing damage

The effect of thrips probing on pepper leaves might be better understood when both the depth of the stylet penetration and the sizes of the penetrated plant cells and cell layers are known. The mean thickness of the adaxial epidermis (single cell layer) of pepper leaves (Fig. 5A) was 17.0 µm (SE ± 0.48, n= 85), the palisade parenchyma layer (distance perpendicular to the leaf surface) was 36.4 µm (SE ± 0.87, n = 85), the spongy mesophyll layer was 59.2 µm (SE ± 1.49, n = 85) and the abaxial epidermis was 11.8 µm (SE ± 0.35, n = 85). The shortest distance to the vascular bundle was approximately 30 µm, measured from the adaxial and 27 µm from the abaxial side. In addition to length (from the thickness mentioned above) we measured an average width of the elongate palisade cells (parallel to the leaf surface) to be 15.5 µm (SE ± 1.39, n = 10) (Fig. 5A). Heavily damaged leaves showed several silver spots that had deteriorated cells to a depth of several cell layers (Fig. 5B), or in which all cell layers were damaged between the adaxial and abaxial sides of the leaf. In moderately damaged spots, multiple cells of the palisade parenchyma or the spongy mesophyll were collapsed depending on the leaf side penetrated. Only a few or even a single cell was collapsed in spots with minor damage. The damage caused by thrips probing seemed to vary, but could affect all cell layers in a leaf.

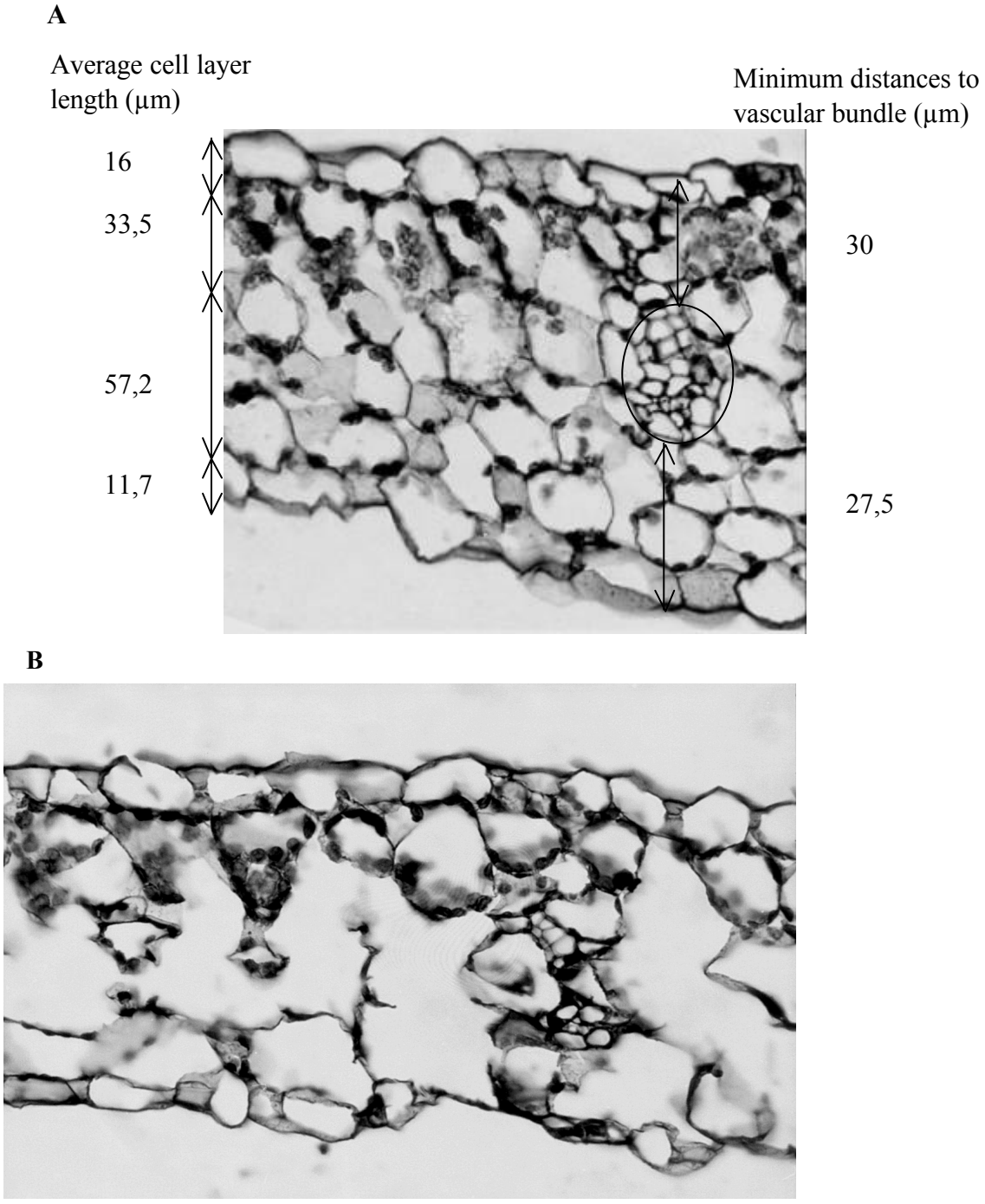

Fig. 5. Leaf morphology of 4 weeks old *Capsicum annuum* leafs; **A**, undamaged by thrips. The average depth of the different cell layers and the minimum distances to the vascular bundle are given in µm; **B,** severely damaged by thrips.

4. Discussion

Feeding behaviour of WFT was studied by integrating different methods: EPG recording, video observation, radioactive tracer ingestion studies and histology of fed-upon and undamaged leaves, which led to a more complete characterisation and biological relevance of the thrips' EPG waveforms, compared with previous studies.

The first waveform, denoted P, has been previously associated with the mandible piercing the epidermis (Harrewijn et al., 1996a). Our study showed that each P waveform corresponded with a downward movement (nod) of the head (Fig. 3B,C). Previous authors (Reijne, 1927; Heming, 1993) have considered the mandible insertion of Terebrantia (suborder to which WFT belong) to be caused by this head movement. Thus the EPG reflects an increased electrical conductivity as caused by the mandible puncturing of the epidermis - or at least the cuticle - during the head nod while waveform P was recorded. A new EPG waveform was added by our study to those distinguished by Harrewijn et al. (1996a). This waveform, called S, was also recorded by Harrewijn et al. (1996a, fig. 1C), but was considered by these authors to be an integral part of waveform P. The S waveform was associated with repeated nodding, as observed by video, which suggests mandibular action during this waveform. The most straightforward function would be the piercing of multiple cells in the leaf. However, the S waveform was not observed in every probe where ingestion of multiple cells was suspected. Hence, the significance of these repeated actions remains uncertain. Waveform S also occurred during a period of R, or at the end of a probe.

The Q waveform seems to be a transition phase between P (or S, when present) and R. Most likely, Q represents insertion of maxillary stylets into the plant, but additional supporting evidence is still needed. The maxillary stylets include the food canal and must, therefore, be involved in the actual food intake (Chisholm and Lewis, 1984). Since the mandible has no canal (Chisholm and Lewis, 1984), its main function is apparently to punch a hole. In contrast, the maxillary food canal will serve as a fluid conductor in which possibly a fast fluid stream may occur during the Q waveform, thus explaining its emf origin (streaming potentials; c.f. Tjallingii, 1985; 2000). Ingestion was found to occur during the R waveform periods as the radioactive tracer experiment clearly demonstrated. Therefore, the regular shape of the waveform suggests the activity of a sucking pump. The simultaneously observed pulsation in the head then may reflect the cibarial pump activity (Chisholm and Lewis, 1984; Heming, 1978).

The maxillary stylets can only be extended for about 30 µm (Hunter *et al.,* 1993) and can only reach the first one or two cell layers below the epidermis but certainly not vascular tissue (Fig. 5A). Nevertheless, the tissue beyond these two layers was also affected in leaves with severe damage (Fig. 5B). No unaffected cells could be discerned in such regions. Similar observations were made on cotton leaves and flower tissues of navel orange (Wardle and Simpson, 1927; Childers and Achor, 1991). Through which mechanism the tissue surrounding the probing site is affected is not known. The damage could be caused by excreted saliva but alternatively, damaged sites may just cause dehydration (Wardle and Simpson, 1927).

Using the average ingestion rates found on liquid diet, the dimensions of plant cells and the duration's of the waveform R-periods, a theoretical estimate could be made of the time needed to empty a cell, neglecting pressure differences inside and outside the cells (if any). The volume of a palisade parenchyma cell was about 8.0 pl (33.5 x 15.5 x 15.5 µm). This means that a second instar larva, showing the highest average ingestion rate of 1.87 pl/s could empty such a cell in 4.2 s, which corresponds with 23 R-waves (4.2 s x 5.5 Hz) in the EPG. A first instar larva with a ingestion rate of 0.47 pl/s could empty a cell in 17 s. Individual R waveform periods on leaves were often found to be much longer. The longest continuous R waveform period observed on a leaf was 5700 s. In this R-period a female could hypothetically ingest a volume of about 8 nl (5700 s x 1.4 pl/s), i.e. the volume of 1000 palisade parenchyma cells or an area on the leaf of 24.5 mm^2 (15.5^2 µm x 1000). Though the calculations were based on ingestion rates derived from the feeding on liquid diets, long probes as mentioned earlier seem to reflect ingestion of multiple cells contents. Presumably the number of emptied plant cells is overestimated in this way because ingestion of cytoplasm with organelles may be more difficult and therefore slower than a sucrose solution. Our ingestion rates are higher than those previously found rates by Wiesenborn and Morse (1985, 1986) on liquid diets. This could be expected because the EPG technique allowed us to correlate the ingested volume with the real ingestion time (waveform R only), excluding the access time during which the thrips is not ingesting. However, higher ingestion rates on liquid diets have also been found by Day and Irzykiewicz (1956) and Chisholm and Lewis (1984). These higher rates may be mainly due to the use of other (i.e. larger) thrips species (Wiesenborn and Morse, 1986). The rate of ingestion might have been negatively affected by the tethering of the thrips and exposing them to CO_2. Tethering might have influenced the duration of ingestion periods, but affecting the ingestion rate seems less likely. We observed that the effect of CO_2 exposure on behaviour was lost after one hour (unpublished data). Therefore the thrips were left for one hour to recover from the CO_2 treatment before the EPG

monitoring started. Notwithstanding, it is possible that tethering effects occur as has been found for aphids (Tjallingii, 1986) and whiteflies (Lei et al, 1996 and 1997). However, for whiteflies it was shown that if the EPG recording started after a wire adaptation period of 2 h the effect decreased. The studies show that the results obtained by EPG recording cannot blindly be extrapolated to the natural situation and indicate the need for further investigations of these aspects in thrips.

The present study gives no information about the minimal time that is needed for viruliferous thrips to eject sufficient Tospovirus with its saliva into the plant for a successful inoculation. Additionally, the moment at which virus containing saliva is excreted remains uncertain. From the morphology of the head it is known that the salivarium, the cavity in which the salivary ducts end, merges with the food canal at the point where the hypopharynx ends. At this position, the maxillary stylets are not yet fused, so the saliva may either flow through the food canal (Hunter and Ullman, 1992), or may leave the mouth cone via the lumen outside the maxillary stylets (and the mandible) and inside the mouth cone (Fig. 26-28 in Heming, 1978; Fig 7. in Mickoleit, 1963). The latter, 'extra-maxillary' option will leave the maxillary canal mainly unused for salivation. An extra-maxillary salivation might avoid a clear salivation waveform in the EPG, as found for saliva injection in aphid EPGs (Prado and Tjallingii, 1994; Tjallingii and Cherqui, 1999; Martin et al., 1997). The parallel electrical circuit outside the maxillary stylets will 'short circuit' any streaming potentials evoked by saliva passing through the maxillary canal. This hypothesis needs to be tested further. Harrewijn et al (1996a) suggested that waveform Q was involved in salivation, but in our results no support has been found for this idea. Moreover, this seems unlikely since the appearance of the Q waveform varies considerably. Although we have made a number of interesting correlations between the probing activities and EPG waveforms of WFT, the role of these activities in the transmission of TSWV remains to be studied further.

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CHAPTER 3

Electrical penetration graphs of thrips revised: combining DC- and AC-EPG signals

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Electrical penetration graphs of thrips revised: combining DC- and AC-EPG signals

Abstract

In DC-EPG monitoring, sequences of four waveforms P, Q, R and S have been earlier distinguished and found to represent mandibular stylet insertion, maxillary stylet insertion, ingestion, and repetitive mandibular piercing, respectively. However, in most cases the exact moment of a transition from one waveform to the next is unclear, which makes duration measurements ambiguous. In order to improve the quantitative reliability of EPG data, AC-EPG signals – providing somewhat different information – were recorded concurrently. The additional AC information did not solve most quantification problems, however. On the basis of the results obtained it is proposed to merge some waveforms instead of trying to analyse all known waveforms separately. Distinguishing only two waveform phases during thrips probing behaviour will provide a more reliable and much less laborious analysis; the two waveforms that can be distinguished are 1) a 'puncture phase' and 2) a 'feeding phase'. The puncture phase (PQ) includes waveforms P, Q, and S, while the feeding phase (R) includes waveforms R, T, and U. The latter, T and U are two novel waveforms identified here by combining DC- and AC-EPG recordings with video recording. Waveform T represents a single mandibular thrust (function unknown) embedded in waveform R and waveform U represents the end of a probe, presumably the retraction of the maxillary stylets.

1. Introduction

Feeding behaviour of piercing-sucking phytophagous insects is difficult to observe visually. Therefore, electrical monitoring, i.e. the use of the electrical penetration graph (EPG) technique forms a good and well-established alternative to visual observation. The EPG technique has been used extensively in studies of aphid feeding behaviour (McLean and Kinsey, 1964; Tjallingii, 1988; Tjallingii and Hogen Esch, 1993; Prado and Tjallingii, 1994) and more recently also in analyses of feeding behaviour of thrips (Thysanoptera) (Hunter et al., 1993; Harrewijn et al., 1996b; Kindt et al., 2003). Thrips feed on the contents of cells in the epidermal, mesophyll and parenchymal cell layers (Chisholm and Lewis, 1984; Kindt et al., 2003). In contrast to aphids, thrips are not able to feed on the phloem and their mouthparts have a completely different structure (Heming, 1978; Chisholm and Lewis, 1984; Hunter and Ullman, 1992). Consequently, the EPG signals are rather different.

Two principally different EPG recording systems are in use, indicated as the AC-system and the DC-system (Walker, 2000). The consequence of the different recording principles is that the AC-system only records the fluctuations of electrical resistance during the plant penetration, whereas the DC-system records fluctuations of resistance and electromotive force (emf) (Tjallingii, 2000). The latter fluctuations include a number of biologically important phenomena during insect probing, such as intracellular punctures (Tjallingii, 2000)

In the DC-EPG recordings of thrips feeding, waveforms P, Q, R and S have been distinguished (Kindt et al., 2003), whereas with the AC-system waveforms for probe initiation, salivation/stylet movement, ingestion, and probe termination have been described (Hunter et al., 1993). The DC-signals have so far been better evaluated, contain more information, and seem to be more distinct than the AC-signals. However, there still remain a few difficulties in distinguishing some DC waveform features and especially in establishing the transitions between the different waveforms. This results in imprecise quantification of waveform duration. Previously, we used simultaneously recorded video images of probing thrips at high magnification combined with EPG and behavioural information to analyse thrips feeding behaviour (Kindt et al., 2003). However, analysis through video recording is very laborious, since only one thrips can be analysed at a time. Moreover, it has not solved the problems in establishing the transitions between waveforms.

The present study aims to improve the analysis and interpretation of the DC-EPG recordings by adding the information of a simultaneously recorded AC-EPG and, in some cases video images as well. For this purpose, an amplifier that records DC- and AC-signals simultaneously was constructed, as had been suggested earlier (Tjallingii, 2000).

First, the variations of known waveforms and the details of their transitions were examined to characterise when one waveform changes into the next. Subsequently, we also examined the differences between EPG signals recorded on several feeding substrates and found some novel waveforms. The main aim, however, was to make the EPG analysis more reliable and less laborious.

2. Material and methods

2.1. Insects and plants

The IS2 population of Western flower thrips (*Frankliniella occidentalis* Pergande) used in our study originated from Israel on mango (van de Wetering et al., 1995). Thrips were reared continuously on bean pods (*Phaseolus vulgaris* L, cv. Prelude) in a climate room at 16/8 hr light/dark and 25 ± 0.5° C. Additionally, some pollen of *Pinus* were supplied for nutrition. In the experiments we used the thrips-susceptible pepper (*Capsicum annuum* L.) accession "Pikante Reuzen" (Maris et al., 2003). The plants were grown in a greenhouse at 16/8 hr light/dark and 24° ± 2° C. In a few cases we also recorded probing on *Datura stramonium* L plants, bean pods of *P. vulgaris* and an artificial diet of only tap water enclosed by stretched Parafilm®.

2.2. Electrical penetration graph recording

In all experiments thrips were attached to a thin gold wire (10 µm in diameter) for EPG recording (Kindt et al., 2003). The two electrical penetration graphs (EPG) methods, AC and DC were incorporated in an 'AC-DC' system (developed at Wageningen University; Tjallingii et al., in prep). This device allows simultaneous recording of both signals (Tjallingii, 2000), in contrast to the earlier AC and DC recordings made by Reese et al. (2000) who alternated recording between an AC- and a DC-system. In thrips DC-EPG monitoring, sequences of four waveforms P, Q, R and S have been distinguished and found to represent

mandibular stylet insertion, maxillary stylet insertion followed maybe by salivation, ingestion, and repetitive mandibular piercing, respectively (Kindt et al., 2003); two new waveforms (T and U) are described here for the first time.

2.3. Video recordings combined with EPG recording

The behaviour of the thrips was observed with a high magnification video set-up in some cases. A colour CCD camera (Panasonic AW-300E with studio card AW-PB305 and remote control WV-RC550) was mounted on a stereomicroscope (Zeiss Stemi, SV 11) and video signals were recorded with an S-VHS recorder (Panasonic AG-7330). This video signal was synchronised with the EPG signals and the analysis of these recordings were made as described in Kindt et al. (2003).

2.4. Experiments

Approximately 55 AC-DC recordings of 1 or 2 hours were made using adult female thrips. During 5 recordings the feeding substrates were altered by successively moving the same insect to different feeding substrates. This provided EPG recordings from pepper and *Datura* plants, as well as from bean pods and water covered by Parafilm®. In 10 recordings AC-DC was combined with video recordings. All experiments were done at 21 ± 3°C.

2.5. Waveform and video analysis

First, AC- and DC-signals from pepper were analysed focusing on transitions of one waveform to the next, especially. The analysis was performed by splitting the probes in the different waveforms in the DC-EPG signal first and then in the AC-signal. Per probe both signals were compared. Then, the pepper EPGs were compared with those from the other feeding substrates by looking if there were differences in the general characteristics of the waveforms. Finally, the combined video/EPG recordings were used to study some hitherto undescribed waveforms as well as pseudo probes of the thrips.

3. Results and Discussion

3.1. EPG waveforms on pepper

DC-signal

Different combinations of waveforms occurred in the sequences per probe and these combinations are shown in the figures. An individual probe is defined as the start of the electrical contact between the mouth cone of the thrips and the leaf till the end of this contact.

Fig. 1A illustrates a probe with a complete sequence, containing waveforms P, S, Q, R and U (U representing a new waveform, described below), successively. In the DC-EPG the probe starts with a distinct sharp peak, which has been defined as waveform P (Harrewijn et al., 1996b; Kindt et al., 2003). After the first peak a period of multiple smaller peaks occurs at a lower voltage level, representing the S waveform (Kindt et al., 2003). When exactly waveform P stops and waveform S starts cannot be marked precisely. During the transition phase a few relatively small peaks occur, which may either belong to waveform P or to waveform S. After waveform S, a quick decrease of the voltage occurs. This decrease has previously been called the 'Q-dip', the distinct beginning of the Q waveform (Kindt et al., 2003). Gradually then, the voltage level increases and square-like waves (top side) are shown, which indicate the start of waveform R (Kindt et al., 2003). The Q-R transition is not clear, because it is not known which of the less clear waves during the voltage increase can be considered as waveform R. At the end of the R period, the waves seem to fade away till they are no longer visible. Waveform R apparently ends then and a new waveform starts, which we will call waveform U (further description follows below). The precise moment of the transition between waveforms R and U is questionable again: do the R-waves really stop? The end of the probe is clear due though to the rapidly decreasing voltage.

A different combination of waveforms can be discerned in the DC-EPG of Fig. 1B (top panel). After a distinct P-peak at the beginning of the probe the voltage decreases gradually but is not followed by waveform S, as very frequently observed on pepper. Also, in the subsequent signal section with a slowly decreasing voltage level (between the two dotted lines) there is no pattern and this phase is designated as waveform Q. This is mainly due to the missing Q-dip, which appears to be much less common in probes than expected on the basis of earlier data (Harrewijn et al., 1996a, b; Kindt et al., 2003). In spite of the missing Q-dip we assume that the main part between the P-peak and the rather clear start of R represents

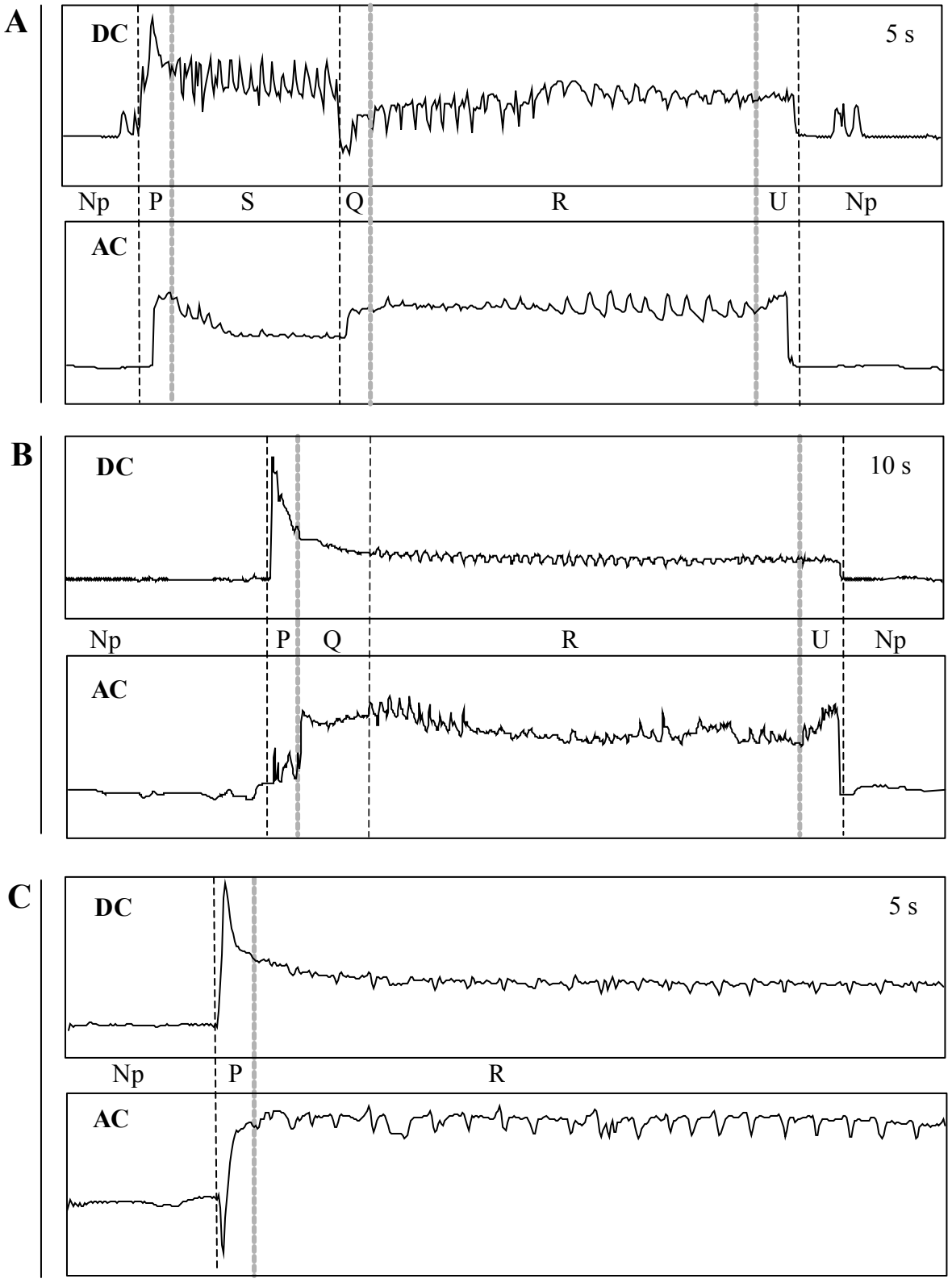

Fig. 1. EPG waveforms related to three probes on pepper recorded with the AC-DC recorder. Upper panels with DC and lower panels with AC signals. Waveforms are indicated by letters between the panels (Np is non-probing). Transitions between the waveforms in the DC signal are unequivocal when indicated by a thin dashed line in black or equivocal when indicated by a thick dotted line in grey. In panels voltage (not calibrated, no units) is plotted against time. **A**, probe of 5 s with waveforms P, S, Q, R. **B**, probe of 10 s with waveforms P, Q, R, U. **C**, beginning of a probe, 5 s with waveforms P and R.

waveform Q, although the exact end of waveform P and start of waveform Q remains unclear (first dotted grey line, Fig. 1B). The square-like waves of waveform R gradually increase in amplitude. the R-waves seem to vanish at the end of the probe, indicating the end of waveform R before the probe has really ended. Again, we would like to indicate the last part as waveform U, although no exact R-U transition point can be indicated. The end of the probe is clear by the abrupt drop of the voltage to the baseline.

Another waveform combination is shown in Fig. 1C but only the beginning of the probe is now considered. The clear waveform P is not followed now by waveform S or Q but directly by waveform R. After the P-peak in the DC-EPG (top panel) when the voltage declines and stabilises, the square-like R-waves appear, with gradually increasing amplitude. Waveform Q may be so brief that it cannot be distinguished or it does not occur at all. As it is assumed that during waveform Q the maxillary stylets are inserted and some saliva is injected into the epidermal wound (Kindt et al., 2003), the lack of Q suggests that ingestion can occur without such a distinct salivation or stylet insertion period. P-R sequences happen frequently when many probes occur quickly after each other (Fig. 4C). The moment of transition from waveform P to R is unclear.

The first three panels in Figure 1 present the most common combinations of waveforms for probes with ingestion (associated with waveform R) (Figs. 1A-C). Probes without ingestion phase account for more than half of the total number of probes (58% probes without ingestion, Chapter 4+5). These probes are very short and usually consist of waveforms P and Q only (Fig. 4B). Most of these PQ probes do not show any Q-dip and the P-Q transition is always unclear then, as in any kind of probe without a Q-dip.

In general, waveform P in DC-EPGs always starts as a clearly recognizable pattern. The P-S transition is not clear but waveform S is rare during probes on pepper leaves and, consequently, so are the S-Q transitions (In 0.3% of the probes occurs waveforms S, unpubl. results). Though the P-Q transitions are clear if a Q-dip occurs, this dip is not common, which makes the P-Q transition cryptic in general. Also, Q-R waveform transitions are not very clear (Fig. 1A, C) in most cases, often the amplitude of the R waveform increases gradually. In EPG recordings with a low voltage, presumably due to a high insect resistance or poor voltage adjustment (amplifier setting) the end of a probe, transition of R (or U) to non-probing (Np) is not obvious.

AC-signal

Analysing the concurrently recorded AC-signals of Fig. 1 (lower panels) might help to understand and interpret the DC-signals and the waveform transitions, as suggested earlier (Tjallingii, 2000). Reese et al. (2000) showed that comparing DC- and AC-signals of some aphid waveforms can be useful.

In the lower panel of Fig. 1A the probe starts with a blunt peak at the time that the P-peak in the DC-signal (upper panel) reaches its highest point. After the blunt peak, a gradual decrease of the voltage level is shown in the AC-signal with some peaks in the beginning. While these peaks diminish, the voltage continues to decrease until it reaches a steady level with some small peak remnants. Although these peak remnants coincide with the S peaks in the DC-signal, their amplitude is much smaller and the whole S waveform is inconspicuous. The AC-signal shows a remarkable decrease of the voltage level during the clear S waves in the DC-signal (see also Fig. 2A). At the end of waveform S, the AC-signal quickly returns to the higher voltage level. This increase coincides with the DC waveform Q, with the Q-dip actually starting a fraction of a second earlier. Similar to the DC-signal the remaining part of the AC waveform Q is not obvious and the Q-R transition is also indistinct. The AC-signal does not provide any better information than the DC-signal since the R-waves are less clear and the typical square-like wave features are missing. In contrast, the upward AC-peaks are sometimes sharp, whereas the downward peaks are blunt but not square-like. Remarkably, the amplitude of the R-waves changes in an opposite way in the two signals. The biological meaning of these opposite changes remains to be resolved. At the end of the probe, during the newly distinguished waveform U, a distinct voltage increase makes the AC-signal of this waveform clearer than the DC-signal. The probe ends somewhat earlier in the AC-signal than in the DC-signal but the voltage level also declines rapidly to the baseline.

In Fig. 1B only some small peaks in the AC-signal coincide with the P-period in the DC-signal. Then a quick voltage increase in the AC-signal is thought to reflect the beginning of Q, similar to the increase in Fig. 1A at the beginning of waveform Q. In Fig. 1B, however, there is no Q-dip in the DC-signal and both signals suggest a much longer Q waveform than in Fig. 1A. Also, the Q-R transition is rather obvious in the AC-signal as well as in the DC-signal. Soon after the start, the R-waves become unclear and irregular in the AC-trace but at the end of the probe a clear voltage increase marks the R-U transition, which is hardly visible in the DC-signal. The probe ends at about the same moment as in the DC-signal.

The AC-signal in Fig. 1C confirms the P-R sequence shown in the DC trace. Only the quick negative P-peak, opposite to the DC-peak, differs from Fig. 1A and B, the origin of

which is unclear. The P-R transition seems somewhat clearer in the AC-signal and the R-waves now have a similar square-like appearance as is common in the DC-signal.

Comparing the DC- and AC-signals in general, the P-peak is clearer in the DC-signal and so is the Q waveform, if present. In the AC-signal the low voltage level of waveform S is a rather clear AC-feature and at its end a quick voltage increase marks the start of waveform Q better if no Q-dip is present in the DC-signal. None of the other Q features are clear in both signals. The R waveform is in general less clear in the AC-signal and there is more variation than in the DC-signal. This variation occurs between, as well within individuals. At the end of probes, waveform U is clearer in AC-signals but the R-U transitions are cryptic in both signals. The transitions from one waveform to the next are in some cases clearer in the AC-signal but this does not help very much in making decisions about the waveform transitions during EPG analysis. Especially, quantification of the duration of waveforms P, Q, and S does not become more reliable when AC-signals are used or when AC information is added to the DC-signals.

3.2. Waveforms on different feeding substrates

AC-DC recording was not only used to study thrips on pepper leaves, but also on *Datura* leaves, bean pods and on water covered by Parafilm®. While on *Datura* and pepper leaves the waveforms are very similar, on the two other feeding substrates remarkable differences were found.

In the bean pod's DC-signal, the P-peak (Fig. 2A) is similar to that on pepper (Fig. 1B). A second P-peak (Fig. 2A, top panel) is more common on bean pods than on pepper. After the subsequent Q-dip, which is similar to pepper, the voltage increases step-wise to a rather constant level, the beginning of waveform R, although its typical square-like waves are not yet obvious, making the Q-R transition somewhat unclear. During the further R period several small dips in the voltage level are visible but in the DC-signal this seems to be nothing special. At the end of the probe some irregular features likely indicate the end of R and the start of waveform U.

In the AC-signal of Fig. 2A a positive P-peak is visible at the beginning of the probe and also, a smaller second P-peak is shown. Then the voltage increases in the AC-signal just when the Q-dip is shown in the DC-signal. Subsequently, there is no clear R waveform in the AC-signal, neither in the first nor in the later parts of R. However, three characteristically low voltage periods are shown in the AC-signal.

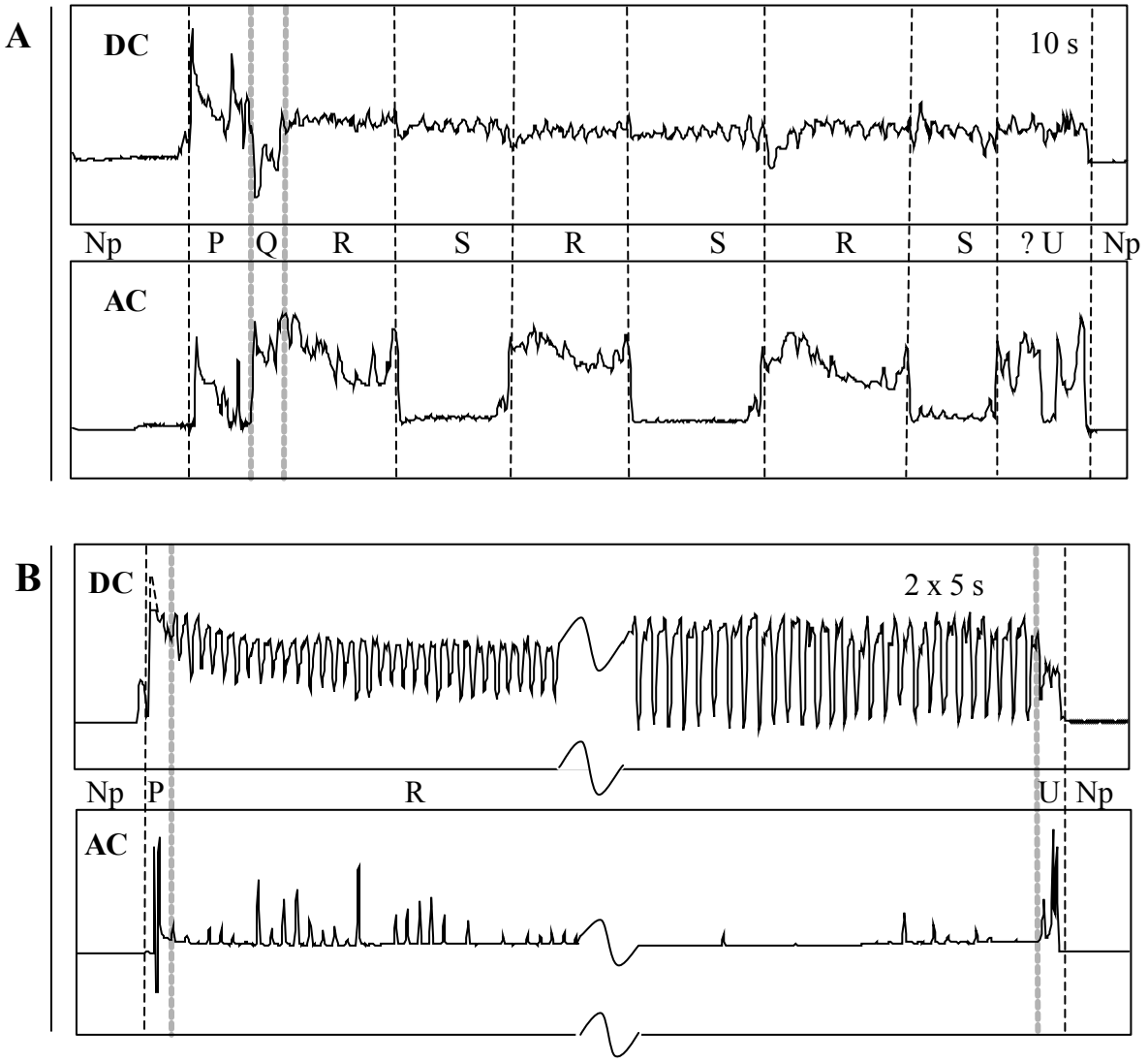

Fig. 2. Two probes on different feeding substrates recorded with the AC-DC recorder. Panels and other conditions as in figure 1. **A,** probe of 5 s recorded on bean pods with waveforms P, S, Q, R and U. **B**, probe of 2 X 5 s recorded on water covered with Parafilm with waveforms P, R, and U. The designation '?U' in panel A indicates that we are uncertain whether only U or also waveform R occurs here.

Some small peaks are visible during these periods (the third period especially), coinciding with the much clearer peaks in the DC-signal, very similar to waveform S (cf. Fig. 1A). Therefore, we infer that this probe has three periods of waveform S, interrupting the R-period. The small dips in the voltage level of the DC-signal coincide with the start or the end of the S-periods in the AC-signal.

On bean pods, thrips show the waveform S much more frequently, in the beginning of a probe as well as during waveform R, intermittently (waveform S occurs in at least 10% of the probes, unpubl. results). This may be due to the bean pod's surface being tougher than that of pepper leaves and hence more mandible activity (correlated to S waveform) to penetrate

the surface may be required. The end of the probe, after the last S-period, shows an irregular AC-signal - possibly an unclear mixture of R and S elements - in which only the final peak seems to indicate waveform U.

Thrips EPG signals from Parafilm® covered water are rather different from the signals on the plant substrates (Fig. 2B). P-peaks are less pronounced or even absent in the DC-signal (Fig. 2B). However in the example of Fig. 2B this cannot be seen because the height of the peak is not known exactly, the signal went out of scale, but in general the peak is less pronounced. Neither Q nor S has been observed in any probe on water covered with Parafilm. This could be due to the fact that Parafilm® is easy to penetrate with the mandible and the maxillary stylets do not have to be extended for sucking up water. In contrast, waveform R is extremely well pronounced (Fig. 2B). At the end of the probe, the vanishing amplitude of the waves indicates the start of waveform U and the probe ends with a 'normal' decline to the baseline voltage. In this example waveform U is relatively clear in the DC-signal.

In the AC-signal even more differences to other substrates are visible. A brief P-peak can be observed (Fig. 2B, bottom panel) but no Q and S waveforms, like in the DC-signal. The R-waves are totally absent and only sometimes short peaks are visible, in contrast to the other feeding substrates (Fig. 1). On the other hand, the voltage increase of waveform U at the end of a probe is clear and is seen in most probes.

In general, all different feeding substrates tested - except Parafilm® covered water - result in the same EPG waveforms and their features are rather similar. Also no differences were found compared to DC-EPG recordings on tomato (Joost, 2003). Parafilm® covered water does not show all the waveforms and the appearance of especially waveform R is rather different. Previously waveform Q was found to occur in probes made on Parafilm® covered water, but these Q waveforms were very unclear (Kindt et al., 2003)

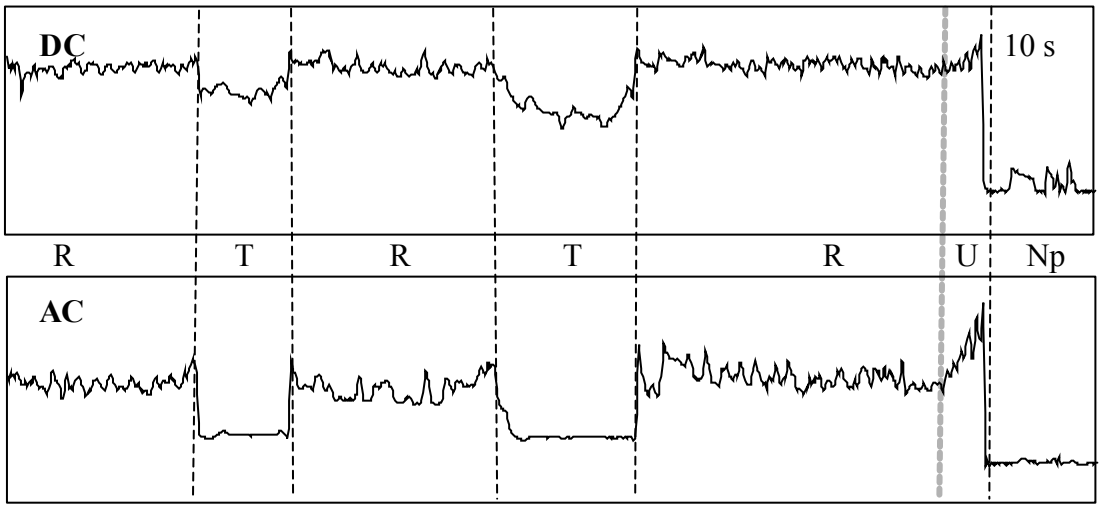

Fig. 3. New waveforms T and U in a probe on pepper, as recorded with the AC-DC system. Panels and other conditions as in figure 1.

3.3. New waveforms

Two novel waveforms are identified. The first is waveform U, already mentioned above. Previously, we called this 'waveform X' (Kindt et al., 2003) or considered it as just an unclear part of waveform R in the DC-EPGs (Fig. 1A, B). Although a voltage increase is occasionally seen in the DC-signal at the end of a probe (Fig. 3), this increase appears to be the main feature in the AC-signals (Figs. 1A, B, 2B and 3), reflecting a separate property: a reduced electrical resistance. The biological activity related to waveform U is presumably the retraction of the stylets from the leaf. Maybe also some salivation occurs to lubricate the retraction of the stylets as suggested by Kirk (1997). This voltage increase has also been described previously (Hunter et al., 1993).

Also new is waveform T (Fig. 3). In the DC-signal it is characterised by a more or less clear, sustained, lowered voltage for about one second, and always interrupts R, as far as our present observations go. In the AC-signal waveform T is more evident (Fig. 3, bottom panel) and looks similar to the AC waveform S but it has no peaks. Waveform T is not very often shown in our recordings. If present a T-period occurs alone; however, occasionally two occur after each other, as shown in Fig. 3. On video the thrips show a single head nod during waveform T, which is similar - though slower - to the repeated nods in the S waveform and the single head nod during the P-peak. These head nods appeared indicative for mandibular insertion (Kindt et al. 2003). It could be argued that waveform T represents piercing of new cells during ingestion, but this seems not very likely because it is shown only in a few long

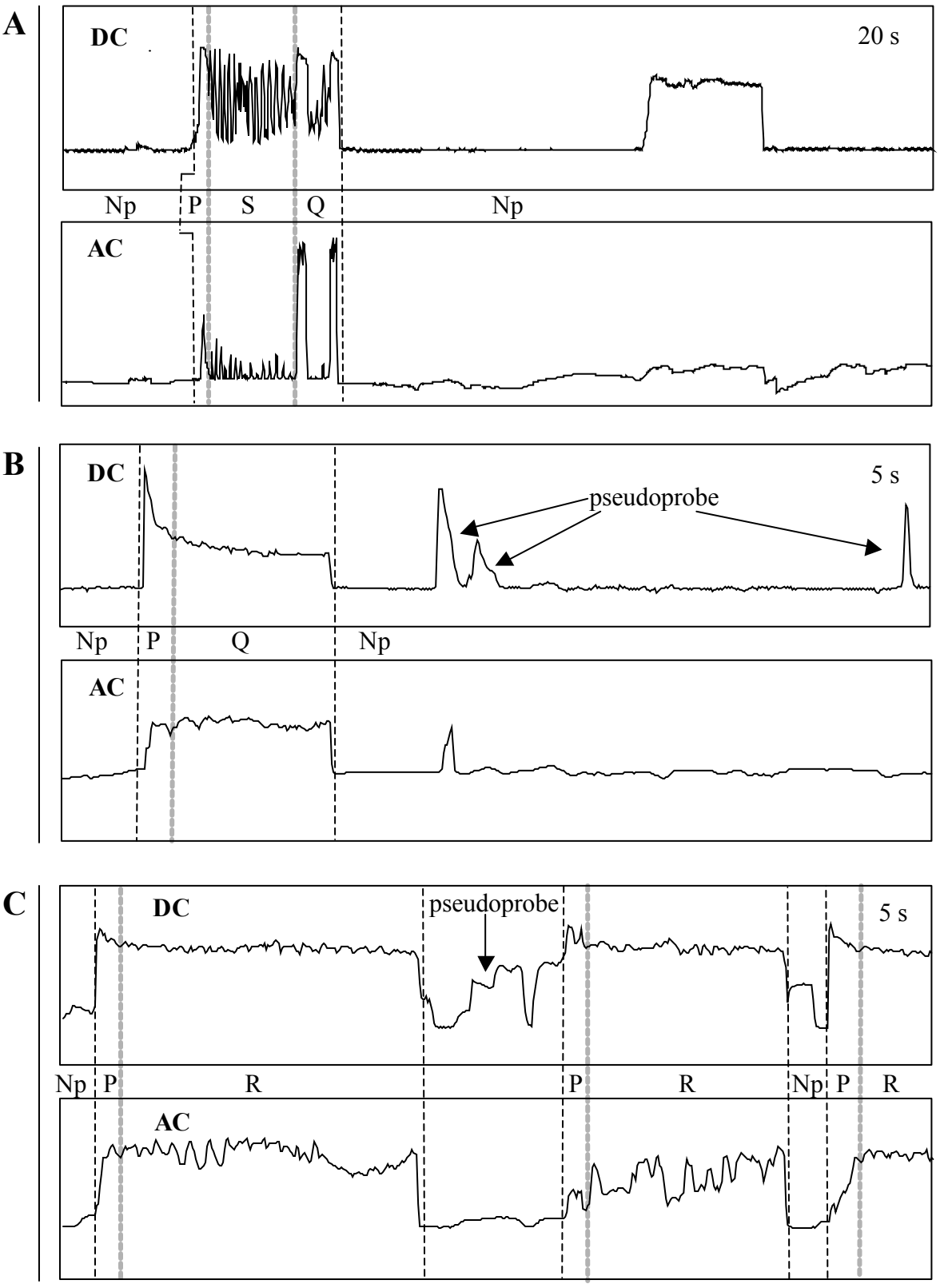

Fig. 4. Probes and pseudo probes on pepper recorded with the AC-DC system. Panels and other conditions as in figure 1. **A**, probe with waveforms P, S, Q and a pseudo probe during 20 s of EPG recording **B**, probe with waveforms P, Q and three pseudo probes during 5 s of an EPG recording. **C**, three probes with waveforms P, R and after the first probe a pseudoprobe during at total of 5 s of EPG recording.

probes and especially at the end of these probes. In relatively shorter probes T is recorded in the beginning of a probe. Possibly the mandible is being used to widen the feeding hole to release the maxillary stylets as suggested previously (Heming, 1978; Chisholm and Lewis, 1984).

The two newly described waveforms are not always easy to distinguish in the DC-signal, whereas they are obvious in the AC-signal. Although the transition from waveform R to waveform U is gradual, the transition of waveform R to T is clear.

3.4. Pseudoprobes

Thrips EPGs regularly show some signals that resemble short probes without any typical waveform features. Concurrently recorded video tracks show no mouthparts contacts with the leaf surface during such signals. Therefore, we have called these events 'pseudoprobes' (Kindt et al., 2003). We aimed to improve the distinction between real and pseudoprobes with AC-DC recording. In the DC-signal of Fig. 4A the second 'probe' looks more or less similar to a real probe, but this is not supported by any parallel AC-signal. Also, on the concurrent video track no mouth cone-leaf contact is shown during this 'probe', thus confirming the pseudoprobe nature.

Other examples of some clear pseudoprobes can be seen in Fig. 4B. Three of such pseudoprobes can be distinguished after the first well-recognisable real probe. In the AC-signal the latter two of these pseudoprobes are not visible and in the video track no mouth cone-leaf contact is seen during any of these three pseudoprobes, thus indicating that there was no probing at all.

A third pseudoprobe example is shown in Fig. 4C. In the DC-signal this is the probe-like signal with a lower voltage than the three normal probes between which it occurs. The AC track suggests a pseudoprobe, because hardly any signals are visible. However, in the corresponding video a mouth cone-leaf contact could not be ruled out, indicating that it is not always easy to distinguish between real and pseudoprobes.

The pseudoprobes can be misleading during EPG analysis, especially when the signal is not very clear because of electrical interference or sub-optimal equipment adjustments. We propose to ignore doubtful probes and consider them as pseudoprobes. Though this may lead to missing a few real probes, it seems preferable to including pseudoprobes in an analysis as real probes.

3.5. Recommendations

The best way to monitor thrips probing behaviour would be to use the AC-DC system with concurrent video recording but the analysis would be very laborious and only one thrips at a time could then be recorded. To save time and to get a reliable analysis we propose here to use only the DC-EPG system that overall provides clearer signals.

For analysis we propose here to merge the waveforms P, Q and S into a single phase, to be indicated as the 'puncture phase' of a probe or as 'waveform PQ'. Although the difficulties in establishing the moment of a waveform transition is a matter of only split seconds, the waveform periods of P, S, and Q are short and they occur at high numbers so that the effect is very serious. The effect of the transition errors on durations of waveform R – the clearest of all thrips waveforms – will be much smaller as R has a much longer duration, in general. Any minor errors at the beginning of an R period (Q-R) or at the end (R-U) will affect the results of the analysis only slightly. Similarly, distinguishing waveform U or not will not dramatically change R totals. Merging R, T and U into waveform R is therefore proposed here. The duration of both waveforms is relatively small compared to waveform R and the waveforms can often not be distinguished in the DC-signal. Harrewijn et al. (1996a) also divided the probing activity in non-ingestion and ingestion when comparing different host plants. As a consequence, waveform analysis will be less detailed but more reliable and less laborious. Such an analysis is very suitable to test what effect resistance has on the probing behaviour.

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CHAPTER 4

Tomato spotted wilt virus is inoculated by thrips in the early phase of a probe

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Tomato spotted wilt virus is inoculated by thrips in the early phase of a probe

Abstract

Tospoviruses are transmitted when viruliferous thrips probe in plants. Probes consist of a cascade of activities leading to stylet penetration into plant tissues, saliva ejection and ingestion of cells contents. Previous studies on the monitoring of thrips probing activities by the (direct current) electrical penetration graph (EPG) technique revealed that the waveform R could be associated with ingestion of cell contents so that *Tomato spotted wilt virus* (TSWV) acquisition could be inferred in this phase of a probe. Here, we demonstrate that viruliferous Western flower thrips, *Frankliniella occidentalis* (Pergande), successfully inoculate TSWV into plants during the first phases of the probing process. The virus is transmitted in the Q phase as shown by EPG analysis of the transmission results obtained in four different inoculation access periods of 15 to 120 min. The virus can be transmitted at a low rate of a single probe per approximately 125 probes (0.8 % of the probes is successful). However, successful virus inoculation does not only depend on the number of probes made, but also on the duration of the waveform Q per probe, as shown by statistical analysis of the EPGs using a generalized linear model. When the Q waveform is followed by waveform R, the transmission probability decreases, indicating a negative effect of ingestion of cell contents on the TSWV inoculation events.

1. Introduction

Eight different thrips (Thysanoptera; Thripidae) species, all belonging to the genera *Thrips* and *Frankliniella*, have so far been identified as vectors of one or more *Tospoviruses* (van Regenmortel et al., 2000; Nagata and Peters, 2001). Thrips feed on the contents of the mesophyll, parenchymal and epidermal plant cells after piercing these cells with their mouthparts. During this feeding *Tospoviruses* are acquired from systemically infected plants by first instar larvae. After ingestion, the virus infects the epithelium of the insect's midgut and subsequently the circular and longitudinal muscles of the mid-gut tissues. The virus particles are then transported to the salivary glands (Ullman et al., 1992; Wijkamp et al., 1993; van de Wetering et al., 1996; Nagata et al., 1999; Nagata and Peters, 2001; Chatzivassiliou et al., 2002; de Assis et al., 2002). Infection of salivary glands might result from temporary contacts between the midgut and the salivary glands during the development of the larvae (Moritz, 2001). After replication in the salivary glands the virus will be transmitted to the plant by second instar larvae and adults with the saliva they excrete (Wijkamp et al., 1993; Nagata and Peters, 2001). Transmission will occur in one of the phases as distinguished during the analysis of the probing behaviour with the direct current (DC) electrical penetration graph (EPG) technique. Previous EPG studies on the probing behaviour of thrips show that probes were initiated by penetration of the leaf with the mandible and this behaviour was associated with waveform P (Harrewijn et al., 1996; Kindt et al., 2003). After this penetration, saliva excretion may occur (Chisholm and Lewis, 1984; Hunter and Ullman, 1989; Hunter et al., 1993) which has been suggested to occur during the Q waveform in the EPG (Harrewijn et al., 1996). Acquisition of *Tomato spotted wilt virus* (TSWV) will most likely occur during ingestion, which has been associated with waveform R in DC-EPG recording (Kindt et al., 2003).

This study aims to identify the probing activities (waveforms) of *Frankliniella occidentalis* during which plants are inoculated with TSWV. To this end inoculation experiments with viruliferous thrips were made on pepper (*Capsicum annuum*) plants, while the probing behaviour was simultaneously monitored with the EPG technique. Single probe experiments were used to confirm whether or not transmission could be achieved during a single probe without ingestion.

2. Materials and methods

2.1. Insects, virus and plants

Western flower thrips (*F. occidentalis*) IS2 population, used in our study, was originally collected in Israel from mango (van de Wetering et al., 1995). The insects were reared continuously on bean pods (*Phaseolus vulgaris* cv. Prelude) supplied with *Pinus* pollen in a climate room at 16/8 h light/dark regime and 25 ± 0.5°C.

For the experiments the pepper (*C. annuum* L.) accession "Pikante Reuzen", susceptible to both *F. occidentalis* and TSWV (Maris et al., 2003), was used. The plants were grown in a greenhouse at 16/8 h light/dark regime and 24° ± 2° C. In the inoculation access period (IAP) experiments, 3-4 weeks old pepper plants were used, which had four true leaves. In the single probe experiments leaf discs (diameter 15 mm) were used from 4-6 weeks old plants, placed on 1.5 % agar (Duchefa, Haarlem, the Netherlands).

In the IAP experiments, the thrips could acquire the virus on leaf disks from *Datura stramonium* L. plants systemically infected with the TSWV isolate BR01 (de Avila et al., 1990). A more virulent isolate S3 was used in the single probe experiments (Maris pers. comm.). To produce viruliferous thrips, 0-12 h old first instar larvae were fed for at least 48 h on 25 mm diameter discs which were placed on 1.5 % agar and covered with cling film in small petri dishes (35 mm in diameter). After 48 h, the larvae were collected and transferred to a petri dish with a healthy *Datura* leaf disc and some pollen. Discs were refreshed when necessary. The potential capacity of these thrips to transmit TSWV was subsequently tested by transferring the emerged adults individually to a *Petunia* leaf disc for an IAP of 24-72 h (Wijkamp and Peters, 1993). In the single probe experiments we tested the second instar larvae for their capacity to transmit just before pupation. The viruliferous individual adults could then be used directly after their emergence. In the single probe experiments, only those thrips were used that had produced 10 or more local virus lesions on the *Petunia* leaf discs. Both experiments were done at 21 $\pm$ 3^{o}C and the probing behaviour was analysed by EPG recording (Kindt et al., 2003).

2.2. IAP experiments

To identify the EPG waveforms involved in virus inoculation, viruliferous adult thrips were placed on the first true leaf of a pepper plant while simultaneously recording the probing behaviour. The thrips were tested in IAPs of 15, 30, 60 and 120 min. Eight thrips were simultaneously tested, each one individually on a separate plant. The thrips were first submitted to a 60 min IAP, then lifted with the wire electrode and then each individual was provided with a new test plant for a 30 min IAP. The same thrips were then successively provided with new plants for an IAP of 15 min and subsequently for the last IAP of 120 min. After these IAPs the test plants were placed in a greenhouse and analysed for infection two weeks later using two leaf discs (5 mm in diameter); one disc from a top leaf and the other disc from the inoculated leaf. They were ground in a Polähne press in phosphate-buffered saline with 0.05% Tween 20 (Maris et al., 2003) and the extracts were analysed by an enzyme-linked immunosorbent assay (ELISA). ELISA values higher than the average value of six negative control discs plus three times the standard deviation were considered to be positive. This experiment was performed with in total 66 adult thrips.

2.3. Single probe experiments

For single probe inoculations, viruliferous adult thrips were allowed to make only one probe on a small pepper leaf disc (15 mm diameter) on agar in a petri dish (35 mm diameter). After ending the probe each tethered insect was transferred to a new disc. These transfers were repeated until each thrips had made as many as 120 probes, when possible. After the transfers all discs were floated on 2-3 ml water in the wells of 24-wells plates. The discs were incubated in a climate room at 16/8 h light/dark regime and 25 ± 0.5° C for 3-4 days for the development of the infection and assayed by ELISA after being stored for some days at -20° C. EPG recordings of the probes that resulted in an infected leaf disc were analysed.

2.4. EPG recording and analysis

A Giga-8 model DC-EPG amplifier was used in the IAP experiments, allowing concurrent recording of probing by eight insects. In the single probe experiments a Giga-1 model (1 channel) amplifier also equipped with a 7 Hz pulse marker was used.

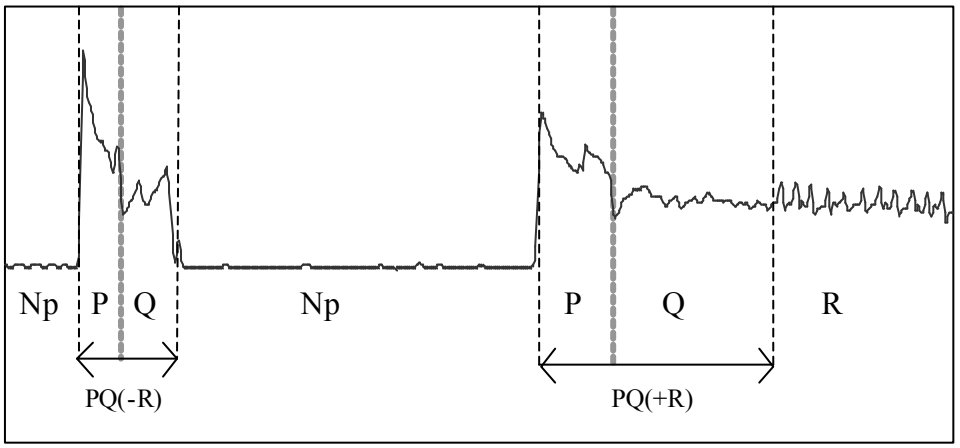

Fig. 1. Probing parameters in an EPG recording. The duration of one PQ(-R) and one PQ(+R) are indicated (double headed arrows). The dotted grey line between waveform P and Q indicates the transition between the two waveforms, which is often difficult to determine.

The EPGs were recorded continuously and the important signal periods were marked by a short 7 Hz signal to the EPG signal at the beginning and at the end of the leaf disc access. Analogous output signals were digitised at about 100 Hz sample frequency (conversion rate) by an RS232 connected AD device and then stored on PC hard disk using *Stylet 3.7* software. All peripheral instruments and software were developed and constructed at Wageningen University (WU) (Tjallingii, 1988; Tjallingii, 2000).

To analyse the EPG recordings, we merged the waveforms P, Q and S (S occurred only occasionally on pepper, (Kindt et al., 2003)) into 'PQ' (Fig. 1 and 3) and the waveforms R, T and U into R, thus avoiding uncertain decisions about the time of waveform transitions (Chapter 3). The average number of probes made per thrips, the total duration of all probes per thrips, the total duration of probes with R or without R per thrips, and the average duration of the probes per thrips enabled us to select fifteen probing parameters to be analysed (Table 1). Since the virus might be transmitted during the merged PQ phase, three types of PQ waveforms were distinguished. One was PQ waveform followed by waveform R: (PQ(+R)), one was PQ waveform not followed by waveform R: (PQ(–R)), and PQ waveforms followed or not followed by waveform R: (PQ(±R)).

2.5. Statistical methods

The effect of the IAP duration on the inoculation success was analysed by logistic regression analysis with a binominal distribution in a generalized linear model (GLM) using qualitative parameters for the different IAP durations. The statistical model tested was:

$$\text{Logit}\,(p_t) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3$$

in which logit (p_t) is the logit of the probability of a successful transmission, β_0 is a constant based on the shortest IAP of 15 min, $\beta_1, \ldots\beta_3$ are constants of the different dummy variables $X_{1,\ldots}X_3$. X_1 =1 for the IAP of 30 min, and the respective other X values (IAPs) are then 0. In the subsequent tests of the model X_2 =1 (IAP 60 min) and X3 = 1 (IAP 120 min), whereas the other X values were 0. The data analysed were obtained from 66 thrips used in the four IAPs of the experiment.

The 15 EPG parameters that were expected (Table 1) to potentially play a role in the virus inoculation were analysed by a quantitative GLM analysis to detect the probing phases in which TSWV was transmitted. From every set of four EPG's (four IAP's) obtained with the each of the 66 thrips, only one EPG (= one IAP) per thrips was used to reduce the immense

Table 1. Parameters of the analysed electrical penetration graph (EPG) recordings of the probing behaviour of 33 thrips, which transmitted *Tomato spotted wilt virus* in inoculation access periods of 15, 30, 60 or 120 min, and 33 thrips, which failed to transmit in these periods. The differences between thrips that did and thrips that did not transmit TSWV for each parameter, which were analysed by logistic regression analysis in a generalised linear model.

Parameter	**Description of the parameter**	**Parameter data ± SE from EPG recordings resulting in**	
		no transmission	transmission
1. N Prb	Number of probes recorded (#)	124 ±30	205 ± 46
2. N PQ(–R)	Number of probes without R (#)	87 ±21	105 ±20
3. N PQ(+R)	Number of probes with R (#)	37 ±8.9	101 ±30
4. Sum Prb	Total duration of all probes (s)	629 ±110	915 ± 155
5. Sum Prb(+R)	Total duration of probes with R (s)	520 ±93	778 ±138
6. Sum PQ(–R)	Total duration of PQ of probes without R (s)	110 ±30	139 ±25
7. Sum PQ(+R)	Total duration of PQ of probes with R (s)	20 ±3.5	48 ±16
8. Sum PQ(±R)	Total duration of PQ of all probes (s)	130 ± 32	187 ±36
9. Sum R	Total duration of R of all probes (s)	499 ± 92	728 ±127
10. Avg Prb	Average duration of all probes (s)	34 ±26	8.3 ±2.0
11. Avg Prb(+R)	Average duration of all probes with R (s)	51 ±27	21 ±4.1
12. Avg PQ(–R)	Average duration of PQ of probes without R s)	1.2 ±0.08	1.5 ±0.08
13. Avg PQ(+R)	Average duration of PQ of probes with R (s)	0.7 ± 0.08	0.5 ±0.03
14. Avg PQ(±R)	Average duration of PQ of all probes (s)	1.1 ±0.07	1.1 ±0.07
15. Avg R	Average duration of R of probes with R (s)	48 ±26	20 ±4.0

EPG analysis task; this was done while taking care to get similar numbers of thrips for each IAP and for probes with and without successful inoculation. In this way a balanced data set with 66 independent replicates was obtained. These data were used in the GLM analysis. The duration of the IAPs was neglected in this analysis, as we were only interested in the role of the specific probing activities with respect to TSWV transmission. The statistical model in which we tested each parameter or combination of parameters was:

$$\text{Logit}\,(p_t) = \beta_0 + C_1V_1 + C_2V_2 + \ldots\ldots + C_nV_n$$

In which logit (p_t) is the logit of the probability of a successful TSWV transmission, β_0 is a constant, C_1, C_2, ….C_n are constant factors for the different probing parameters V_1, V_2, … V_n. The statistical analyses were made with Genstat (6th edition for Windows).

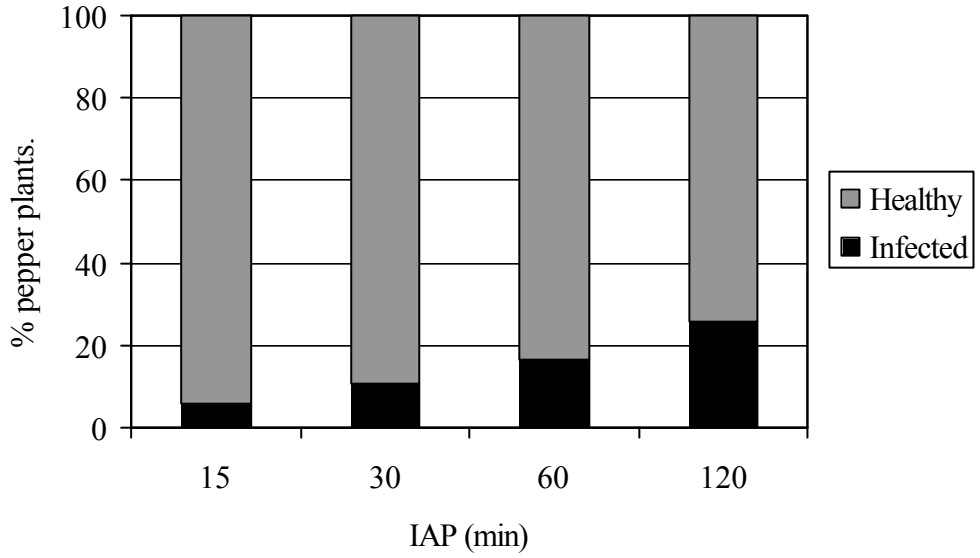

Fig. 2. Effect of inoculation access period (IAP) on transmission of TSWV by adult female thrips. Virus infection was assessed by ELISA. The percentage infested plants increases significantly with the IAP duration (logistic regression analysis, P = 0.009).

3. Results

3.1. Effects of inoculation access period (IAP) and probing activities on virus transmission

To generate EPG data for determining the specific probing activities involved in TSWV transmission, the probing behaviour of 66 thrips was recorded during four IAPs of different lengths. Extension of IAP (from 15 to 120 min) resulted in significant increase of the number of infected plants (from about 5 to 25%, n=66, P = 0.009, Fig. 2).

Data of EPG waveform analyses were processed and described in 15 parameters (Table 1). The association of these parameters with TSWV transmission was analysed by a quantitative GLM analysis. Only three parameters (Table 1) showed a statistically significant ($P < 0.05$) association with transmission, when individually tested (Table 2). These parameters were: 1) the number of probes with ingestion (N PQ(+R), $P = 0.026$), 2) the average PQ duration of probes without ingestion (Avg PQ(–R), $P = 0.019$), and 3) the average PQ duration of probes with ingestion (Avg PQ(+R) , $P = 0.015$). Subsequently, all possible combinations of two or more of these 15 parameters were also tested in the model. Some of these combinations explained the virus transmission much better than any of the single parameters.

Table 2. The EPG parameters or combinations of parameters (for description of parameters see Table 1) which had a significantly positive or negative effect ($P < 0.05$) on the probability that thrips transmitted *Tomato spotted wilt virus* (logistic regression model). See Table 3 for method to determine the effect on the probability (p_t).

Parameter(s)	**Effect on p_t**		**P-value**
N PQ(+R)	+		0.026
Avg PQ(–R)	+		0.019
Avg PQ(+R)	–		0.015
Avg PQ(–R) + Avg PQ(±R)	+	–	< 0.001
Avg PQ(–R) + Avg PQ(+R)	+	–	0.002
Avg PQ(–R) + N PQ(+R)	+	+	0.003

The best fitting model was found when the average PQ duration of probes without R (Avg PQ(–R)) was combined with average PQ duration of all probes (Avg PQ(±R)) (Table 2, $P < 0.001$). Two other good fitting models consisted of the combinations of the parameter Avg PQ(–R) with Avg PQ(+R) ($P = 0.003$) and with N PQ(+R) ($P = 0.002$), respectively. These results indicate that the average duration of the PQ phase plays a crucial role in probes with successful inoculation. The empirical values in the best fitting model of logistic regression are:

$$\text{Logit}(p_t) = -1.75 + 6.23 * \text{Avg PQ}(–R) – 5.77 * \text{Avg PQ}(\pm R)$$

To evaluate the effect of the two parameters Avg PQ(–R) and Avg PQ(±R), we used this model to predict the transmission probability (p_t) by assigning realistic values of 0.5, 1.0 and 1.5 s to each of the two parameters (Table 3). The probabilities found show that the average duration of waveform PQ of the probes without ingestion (Avg PQ(–R)) has a positive influence, whereas the average duration of PQ as measured over all probes (Avg PQ±R) has a

negative influence (Table 3), as reflected in the opposite signs of their respective constants in the model (Table 2). The other models and the significant single parameters listed in Table 2 were evaluated similarly, only the negative or positive effects of the respective parameters are shown.

Table 3. Predicted probability of TSWV transmission on the basis of the model Logit (p_i)= –1.75 + 6.23 * Avg PQ(–R) – 5.77 * Avg PQ(±R) in which three realistic averages for both parameters are used.

	Avg PQ(±R) (s)		
Avg PQ(-R) (s)	**0.5**	**1.0**	**1.5**
0.5	0.18	0.01	<0.01
1.0	0.83	0.22	0.02
1.5	0.99	0.86	0.86

Table 4. Duration of waveforms of EPG recordings of all 9 replicates, that resulted in successfully inoculated pepper leaf discs.

Waveform	Avg duration (s)	SE	Avg number	SE
PQ	1.85	0.78	1.22	0.22
R	13.77	7.42	0.78	0.17

3.2. Transmission by single probes.

Two experiments were performed to investigate whether TSWV could be transmitted in a single probe without ingestion. The first single probe experiment demonstrates that TSWV can be transmitted by viruliferous thrips in a single probe. Only 1.7 % of the leaf discs (9 out of 533) became infected. If we consider only the thrips that made one or more successful inoculations, 9 out of 268 (3.4 %) leaf discs became infected. The values of the parameters Avg PQ(±R) and Avg R (Table 4) did not substantially differ from those obtained in the IAP experiment (Table 1). No S waveform occurred in any of the successful probes and no clear Q dip could be observed. The duration of the probes was in the same range as found in the IAP experiment. In two successful inoculations it could not be prevented that the thrips made more than one probe resulting in an average number of probes higher than 1 (Table 4). Waveforms PQ occurred in all successful inoculations. However, in one of them, waveform R did not occur (Fig. 3A).

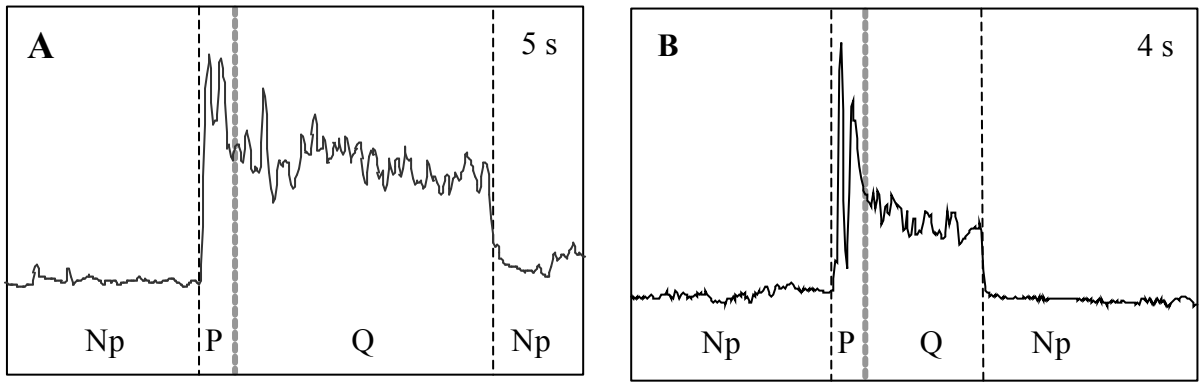

Fig. 3. Two single probes that resulted in successful inoculation and in which only waveform P and Q occurred. The dotted grey line between waveform P and Q indicates that the precise border between these waveforms is difficult to determine. **A,** a probe from the first single probe experiment. **B,** a probe from the second single probe experiment.

In the second single probe experiment the transmission ratio was 0.25 % (2 out of 807 leaf discs). The two thrips that made these successful inoculations probed on 134 leaf discs, hence 1.5 % of the 134 became infected. In these two successful inoculations, one of the single probes showed PQ without waveform R (Fig. 3B), whereas in the other case two short probes were made each with a PQ phase and a clear period of waveform R. The occurrence of the two probes without an R waveform show that TSWV can be transmitted in probes with only a PQ phase.

4. Discussion

This study shows that the P and Q waveforms, which were merged into PQ in this study (Fig. 1), as recorded by the DC-EPG technique, play a role in TSWV inoculation on pepper by thrips. Quantitative GLM analysis shows that the transmission is significantly enhanced when the average duration of waveform PQ is longer, especially if it is not followed by ingestion of cell contents (waveform R). Waveform P has been associated mainly with a single short head nod as observed by video recordings (Kindt et al., 2003). These nods reflect short mandible thrusts (Harrewijn et al., 1996; Kindt et al., 2003). Since these thrusts are very short events, the most important contribution to the increase of the PQ duration has to be attributed to the waveform Q (Figs. 2 and 3). Most likely, saliva is excreted during waveform Q when the stylets penetrate the plant tissue. Saliva excretion has been observed in water after stylet penetration of the Parafilm® membrane covered water (Chisholm and Lewis, 1984). Since the

saliva of viruliferous thrips may contain TSWV particles, inoculation will take place during cell penetration (Harrewijn et al., 1996; Kirk, 1997; Nagata and Peters, 2001). This observation and our results suggest that the virus will be inoculated during salivation coinciding with waveform Q.

Using the best fitting generalised linear model to infer the role of PQ, it appears that the transmission probability increases with the Avg PQ if this waveform is not followed by ingestion (waveform R; Table 3). The parameter Avg PQ(–R) is the quotient of the total duration of all probes without ingestion (Sum PQ(–R)) and the number of probes without ingestion (N PQ(–R)) for each thrips. However, these two parameters have no significant effect on the transmission, when they are separately tested in the logistic regression model.

The transmission probability is the highest for those individuals that combine a high Avg PQ(–R) with a low Avg PQ(±R). The first parameter has a positive effect on the transmission probability and the latter has a negative effect (Table 3). The parameter Avg PQ(±R) in this best fitting model has no significant effect when tested alone in the logistic regression model (therefore not included in Table 2). The parameter Avg PQ(±R) is the sum of the parameters Avg PQ(–R) and Avg PQ(+R). When they are tested alone in the model, Avg PQ(–R) shows a positive effect (Table 2, line 2), whereas Avg PQ(+R) has a negative effect (Table 2, line 3). The negative effect of the Avg PQ(±R) in the best fitting model should most likely be attributed to the Avg PQ(+R) and not to the Avg PQ(–R) component in the parameter Avg PQ(±R).

A longer PQ duration, in which Q is usually much longer than P as mentioned above, may not only result in longer periods of salivation, but also in the ejection of more virus in the longer salivation period. These longer Q's may also be associated with a greater maxillary stylet activity and the penetration of more cells. The negative effect of the Avg PQ(±R) suggests that the virus particles excreted during the PQ phase might be ingested during the R phase. Alternatively, sustained ingestion (waveform R) following salivation (waveform Q) may also result in a larger damage to the cells and thus strongly reducing the inoculation success. A successful inoculation (transmission) may also be the result of an occasional, but minor penetration into a cell by which virus particles overcome the barrier to pass the plasmalemma. If this cell damage cannot be repaired after the penetration, and thus inoculation, it is doubtful whether the virus might replicate in the penetrated cells.

The single probe experiments confirm that a successful inoculation can be achieved when this probe consists of the waveform P and Q activities only (Fig. 3). The transmission probability of single probes is rather low, only 0.8 % of all single probes (11 out of 1340

tested leaf discs were positive). A success ratio of 0.8% would mean that a viruliferous thrips would make one successful inoculation in every 125 probes. With an average number of about 160 probes per hour (data not shown), every viruliferous thrips could make a successful inoculation within approximately one hour. This is similar to what was found in the experimental procedure used to select the viruliferous thrips. Twenty to 30 local lesions were often found in a *Petunia* leaf disc after an IAP of 24 h; hence, they made at least one infective puncture every hour. Similar results were also obtained in other inoculation access studies showing that 1 to 2 h were required to infect 50% *Petunia* and *Datura* plants exposed to viruliferous thrips (Wijkamp et al., 1996). Lower transmission efficiencies were obtained in our IAP experiments (18% during IAP of 60 min; Fig. 2). This lower efficiency might be an effect of the tethering of the thrips in this study.

The results presented here have been obtained with the Western flower thrips. It is likely that the results can be extrapolated to other vector species of *Tospoviruses*, as it is expected that these will have a similar feeding behaviour and, hence will exhibit a similar way of transmission.

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CHAPTER 5

Evaluation of host plant resistance to Western flower thrips using electrical penetration graphs

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Abstract

Thrips cause damage in two ways to their host plant, i.e. directly by probing resulting in cosmetic damage, and indirectly by transmitting *Tospoviruses* (e.g. *Tomato spotted wilt virus*, TSWV) during probing. Thrips resistance is a promising component of crop protection when it also functions as vector resistance for TSWV. Here we analysed probing behaviour of Western flower thrips, *Frankliniella occidentalis*, on four pepper accessions differing in thrips and virus resistances. Probing behaviour was investigated with the DC electrical penetration graph (EPG) technique, monitoring individual thrips for a duration of one hour. In general, the differences found in probing behaviour were rather small and most pronounced for female thrips. Males were hardly affected by the plant phenotypes tested while the females performed less on one of the thrips resistant accessions. The differences in probing activity indicated that TSWV transmission might be reduced, and certainly not enhanced, on the thrips resistant accessions.

1. Introduction

Thrips cause serious economic damage to a broad range of ornamentals and vegetable crops. Direct damage is caused by feeding, often resulting in cosmetic damage reducing marketable yields. Indirectly, thrips may even cause more damage by vectoring *Tospoviruses* (Goldbach and Peters, 1994; Riley, 2000). Currently, the western flower thrips (*Frankliniella occidentalis* Pergande) is regarded to be the most detrimental and most wide spread vector of *Tospoviruses* (Nagata and Peters, 2001). Damage in plants is caused by thrips feeding in a piercing-sucking manner (Heming, 1978; Chisholm and Lewis, 1984; Hunter and Ullman, 1989; Kindt et al., 2003). The emptied cells of the epidermis, parenchyma and mesophyll result in 'silver' scars and growth distortions. During saliva excretion, virus is transmitted to cells that survive penetration (Chapter 4). Resistance to thrips in crop plants may potentially serve a dual aim; it can reduce the cosmetic damage and it can restrict virus transmission. In both cases this is caused by the qualitative and quantitative impact on the insect's feeding behaviour. In most studies on thrips behaviour the feeding scar damage is used to quantify thrips feeding (Broadbent et al., 1990; van Dijken et al., 1994; de Jager et al., 1995). However, this damage is an unreliable parameter to determine thrips resistance because accessions may respond differently and quantification of feeding damage only assesses the amount of ingestion and not the number of probes made (Maris et al., 2003c). Resistance to thrips, i.e. vector resistance, may have opposite effects on the virus transmission, reducing or enhancing the transmission, depending on the actual effect on thrips behaviour (Amin, 1985; van de Wetering et al., 1996; van de Wetering, 1999). These effects could be due to different mechanisms but also, they may differ between females and males by their different feeding strategies (van de Wetering et al., 1998; Joost, 2003). Vector resistance of host plants is an indirect way to control virus spread, which is sometimes the only available method of breeding plants for resistance against virus damage, because not in all plant species virus resistance is available. For pepper (*Capsicum* spp.), a single dominant resistant gene has been described which provides a good level of resistance against *Tomato spotted wilt virus* (TSWV), the most important tospovirus (Black et al., 1991). This gene has been introduced in several pepper hybrids (Boiteux et al., 1993). Because this TSWV resistance is based on a single dominant gene there is a considerable risk for resistance breakdown, certainly after long-term exposure to high vector pressure (Kumar et al., 1995). Combining vector and virus resistance could give a better and especially, more sustainable protection (Kumar et al., 1995).

To study the effects of both kinds of resistance, i.e. vector and virus resistance, four different pepper accessions have extensively been investigated recently (Maris et al., 2004). These accessions represented all possible four combinations of virus and vector resistance: double susceptible (S), only thrips (vector) resistant (TR), only virus resistant (VR) and double resistant (TVR) (Maris et al., 2003c). Electrical monitoring of probing behaviour of *F. occidentalis* using the DC electrical penetration graph technique (Kindt et al., 2003) has shown earlier that some waveform parameters, reflecting the probing activities are correlated with TSWV transmission (Chapter 4). Therefore, an increased or decreased value of such parameters on a specific accession will indicate a potential effect on TSWV transmission.

This study aims at a behavioural approach to establish the effects of thrips resistance on probing and the potential impact on virus transmission, using the same four pepper accessions as in Maris' studies (Maris et al. 2004). Probing behaviour of adult female and male thrips (*F. occidentalis*) was electronically monitored for this purpose on each pepper accession.

2. Material and methods

2.1. Insects and plants

In this study, cohorts of 2-3 days old adult females and males of Western flower thrips, *F. occidentalis* Pergande, population IS2 (van de Wetering et al., 1995), were used. Thrips were reared continuously on bean pods (*Phaseolus vulgaris*, cv. Prelude) in a climate room at 16/8 h of light/dark regime and 25 $\pm$ 2°C. To obtain the cohorts, newly hatched larvae were transferred from bean pods to leaf discs of *Datura stramonium* every day. The larvae were maintained until the adult stage on these discs on 1.5% agar in small petri dishes (3.5 cm in idamtere) covered by cling film. *Datura* leaf discs were regularly refreshed.

Table 1. Four selected *Capsicum* accessions, indicated as S, TR, VR, TVR, – indicates that the accession is susceptible for respectively thrips or TSWV, and + indicates that the accession is resistance against respectively thrips or TSWV.

Accession	Abbreviation	Thrips resistance	TSWV resistance
Pikante Reuzen	S	–	–
CPRO-1	TR	+	–
PI 159236	VR	–	+
PI 152225	TVR	+	+

The four *Capsicum* accessions (Table 1) we used were, 1) Pikante Reuzen (S), susceptible to thrips and TSWV, 2) CPRO-1 (TR), resistant to thrips and susceptible to TSWV, 3) PI 159236 (VR), susceptible to thrips and resistant to TSWV, and 4) PI 152225 (TVR), resistant to thrips and TSWV. The thrips resistances of CPRO-1 and PI 152225 come from different sources and presumably do not have the same genetic background. These accessions were selected on basis of the study by Maris et al. (2003c). Plants were grown in a greenhouse at 16/8 h light/dark regime and 24° ± 2° C. In the experiments, 4.5-5 weeks old plants with 4 true leaves were used but the slower growing CPRO-1 plants were one week older. All experiments were done at 21 ± 3 ^{o}C.

Table 2. Parameters of probing behaviour which has been calculated from the electro penetration graph (EPG) analysis of the recording of the probing behaviour. In brackets the dimension of the parameters are given, # = number, and s = seconds.

Parameter	Explanation
1. N Prb	Number of probes recorded per thrips (#)
2. N PQ(-R)	Number of probes without ingestion per thrips (#)
3. N PQ(+R)	Number of probes with ingestion per thrips (#)
4. Sum Prb	Total duration of all probes per thrips (s)
5. Sum Prb(+R)	Total duration of probes with ingestion per thrips (s)
6. Sum PQ(-R)	Total duration of puncture phases without ingestion per thrips (s)
7. Sum PQ(+R)	Total duration of puncture phases with ingestion per thrips (s)
8. Sum PQ(±R)	Total duration of all puncture phases per thrips (s)
9. Sum R	Total duration of feeding phase per thrips (s)
10. Avg Prb	Average duration of all probes per thrips (s)
11. Avg Prb(+R)	Average duration of probes with ingestion per thrips (s)
12. Avg PQ(-R)	Average duration of puncture phases without ingestion per thrips (s)
13. Avg PQ(+R)	Average duration of puncture phases with ingestion per thrips (s)
14. Avg PQ(±R)	Average duration of all puncture phases of all probes per thrips (s)
15. Avg R	Average duration of all feeding phases per thrips per thrips (s)

2.2. EPG recording and signal analysis

In each 1h-EPG recording session the first true leaf was used of each of 8 plants, 2 of each accession. Plants were randomly placed in a row and thrips, 4 adult females and 4 adult males were put on the adaxial side of the leaves, one insect per leaf and per plant. Each thrips was attached to a thin gold wire electrode and connected to one of the 8 channels of the GIGA-8 DC-EPG recorder (Kindt et al., 2003). Prior to EPG recording, the thrips were allowed to adapt to the tether for at least one hour on a 10% sucrose solution covered by stretched Parafilm® membrane. Insects that appeared to be not properly tethered, i.e. those that escaped

from the wire or showed less than 10 probes during recording, were discarded. EPGs were analysed using the Stylet 3.7 (DOS) software (developed at Wageningen University). As in Chapter 3, we distinguished events (periods) of only waveform PQ, into which P, Q and S were merged – referred to as the puncture phase of probing – and waveform R, into which R, T and U were merged – referred to as ingestion phase (Chapter 3). Probes (Prb) were divided in PQ periods followed by ingestion (PQ(+R)) or not followed by ingestion (PQ(-R)). Per individual, numbers (N), and total durations (Sum) of events were calculated, whereas average durations (Avg) were calculated per event and successively, means of these averages per individual. Differences in EPG parameters (Table 2) between pepper accessions were tested pair-wise with the Mann Whitney-U test ($P < 0.05$) (SPSS for Windows 10.1).

Table 3. Probing of thrips on four pepper accessions, S = thrips and TSWV susceptible, TR = thrips resistant and TSWV susceptible, VR = TSWV resistant and thrips susceptible, and TVR = thrips and TSWV resistant. Parameters of probing behaviour of thrips without distinction between sexes of which only those parameters are shown which are indicative for the probing behaviour and are important in virus transmission (indicated by *, see Chapter 4 for explanation). Mean values per insect ± standard error of the mean. Significant differences ($P < 0.05$) between accessions are indicated by different letters. *N* = number of replicates.

Accession (*N*) **Parameter**	**S (50)**		**TR (56)**		**VR (62)**		**TVR (50)**	
N Prb	84 ±10.0	a	70 ±6.2	a	80 ±8.6	a	88 ±11.9	a
N PQ(-R)	48 ±5.3	a	53 ±5.0	a	55 ±6.6	a	54 ±7.1	a
* N PQ(+R)	36 ±5.5	a	17 ±2.1	b	25 ±4.6	ab	34 ±7.0	ab
Sum R	364 ±53	a	189 ±28	b	268 ±46	ab	337 ±70	ab
Avg R	20 ±6.0	a	14 ±3.1	a	25 ±12.1	a	16 ±5.3	a
* Avg PQ(±R)	0.86 ±0.05	a	0.91 ±0.04	a	0.90 ±0.04	a	0.90 ±0.06	a
* Avg PQ(-R)	1.0 ±0.05	a	1.01 ±0.04	a	1.02 ±0.05	a	1.0 ±0.06	a
* Avg PQ(+R)	0.72 ±0.06	a	0.65 ±0.06	ab	0.55 ±0.04	b	0.71 ±0.08	ab

3. Results

3.1. Differences between pepper accessions without thrips' sex distinction

Potentially different effects of four pepper accessions (Table 1) on the probing behaviour were evaluated using 15 parameters (Table 2), without distinction between males and females. Between the accessions S and TR the most obvious differences measured (Table 3) were a decreased number of probes with ingestion (N PQ(+R)) on the TR plants ($P = 0.039$) and a shorter total duration of ingestion (Sum R) on this accession ($P = 0.010$). No such differences

were observed between both virus resistant accessions VR and TVR on which the same total duration of ingestion (Sum R) was recorded and which was similar to the level on S plants. No significant differences were found in Avg R (the average duration of ingestion per probe with ingestion) between the accessions (Table 3). Neither the total number of probes (N Prb) nor the numbers of probes without ingestion (N PQ(–R)) differed between the accessions compared.

3.2. Different responses of males and females

When male and female EPG data were separately analysed, some different behavioural responses to the accessions were noted. Among females larger differences were shown than among males (Table 4). Females made more probes with ingestion (N PQ(+R)) on S than on TR ($P = 0.030$) or VR ($P = 0.043$). Also, the total duration of ingestion (Sum R) differed significantly between S and TR ($P = 0.021$) while also the total duration of PQ periods of probes with ingestion (Sum PQ(+R)) significantly differed between S and VR ($P = 0.011$). Between the virus resistant accessions (VR and TVR) none of the parameters considered differed significantly. With respect to their effect on thrips probing behaviour, the virus resistant accessions VR and TVR performed much more similar than S and TR. The trend in the differences between VR and TVR – Sum R, Avg R, and Avg PG(+R) – were even opposite to the S/TR differences (Table 4).

For males, no differences at all between S and TR accessions or between VR and TVR were noted. The only significant difference found was between the accessions S and VR with respect to the average duration of per probe (Avg Prb) ($P = 0.030$).

3.3. Potential impact of the observed differences between the pepper accessions on virus transmission

Four of the parameters in Table 3 (* marked) have previously been related to virus inoculation (Chapter 4). Two of these parameters showed significant differences in the current EPG studies when male and female data were not separately analysed (Table 3). The number of probes with ingestion (N PQ(+R)) was significantly higher on S than on TR. Since the magnitude of this parameter has a positive relation to TSWV inoculation (Chapter 4), this difference may be indicative for a better inoculation on accession S. No such differences were recorded between VR and TVR accessions, neither did the two VR accessions differ from S

or TR, with respect to this parameter. The average duration of puncture phases (PQ periods) in probes with ingestion (Avg PQ(+R)) was significantly lower on accession VR than on S (P = 0.020) (Table 3). The magnitude of this parameter showed a negative relation to TSWV inoculation (Chapter 4) and therefore, thrips will potentially inoculate the virus better on accession VR than on S plants.

For females, N PQ(+R) was higher on S than on TR, as was found for the combined data for both sexes, indicating a potentially better TSWV inoculation on S than on TR. In males, no significant differences important for virus transmission were revealed (Table 4). There was only a trend for a shorter Avg PQ(+R) on VR than on TVR (P = 0.085), similar to the non-separated data. This could indicate that males may inoculate TSWV slightly better on VR than on TVR.

Table 4. Differences in EPG parameters between the accessions for both sexes separately, S = thrips and TSWV susceptible, TR = thrips resistant and TSWV susceptible, VR = TSWV resistant and thrips susceptible, and TVR = thrips and TSWV resistant. Mean values per insect with standard errors of the mean. Significant differences (P < 0.05) between accessions indicated by different letters. * = probing parameters important for virus inoculation (see Chapter 4). *N* = number of replicates.

Parameters	**Accessions (*N*)**							
Females	**S (25)**		**TR (29)**		**VR (31)**		**TVR (25)**	
N Prb	106 ±16	a	82 ±10	a	92 ±13	a	105 ±17	a
* N PQ(+R)	49 ±9.2	a	21 ±3.3	b	31 ±8.5	b	47 ±12.8	ab
Sum PQ(+R)	31.5 ±6.8	a	13.7 ±2.5	ab	13.0 ±3.1	b	26.7 ±7.2	ab
Sum Prb	521 ±89	a	277 ±42	a	421 ±91	a	518 ±129	a
Sum R	433 ±77	a	198 ±38	b	338 ±83	ab	430 ±124	ab
Avg R	9.3 ±1.5	a	10.7 ±2.3	a	16.5 ±4.4	a	11.7 ±4.8	a
Avg Prb	5.3 ±0.9	a	4.3 ±1.0	a	7.2 ±3.2	a	6.7 ±3.4	a
* Avg PQ(±R)	0.89 ±0.08	a	0.91 ±0.07	a	0.90 ±0.06	a	0.94 ±0.09	a
* Avg PQ(-R)	0.98 ±0.05	a	1.02 ±0.05	a	1.02 ±0.07	a	0.98 ±0.09	a
* Avg PQ(+R)	0.67 ±0.05	a	0.64 ±0.06	a	0.54 ±0.05	a	0.61 ±0.06	a
Males	**S (25)**		**TR (27)**		**VR (31)**		**TVR (25)**	
N Prb	62 ±9.7	a	57 ±6.6	a	68 ±10.4	a	71 ±15.7	a
* N PQ(+R)	22 ±4.7	a	13 ±2.4	a	19 ±3.2	a	21 ±4.8	a
Sum PQ(+R)	11.0 ±2.0	a	8.5 ±1.9	a	11.2 ±2.6	a	12.6 ±2.9	a
Sum Prb	341 ±71	a	238 ±43	a	266 ±44	a	306 ±64	a
Sum R	294 ±70	a	179 ±43	a	198 ±38	a	243 ±60	a
Avg R	31.1 ±11.7	a	17.9 ±5.9	a	32.9 ±24.1	a	19.4 ±9.5	a
Avg Prb	6.6 ±1.2	a	5.5± 1.3	ab	5.2 ±1.3	b	7.2 ±2.9	ab
* Avg PQ(±R)	0.89 ±0.08	a	0.91 ±0.07	a	0.90 ±0.06	a	0.94 ±0.09	a
* Avg PQ(-R)	1.03 ±0.09	a	1.00 ±0.06	a	1.02 ±0.07	a	1.01 ±0.08	a
* Avg PQ(+R)	0.76 ± 0.11	a	0.66 ±0.11	a	0.57 ±0.07	a	0.80 ±0.16	a

Within a given accession, hardly any significant differences were observed for females and males (Fig. 1). Only on accession S the number of probes with ingestion (N PQ(+R)) was higher for females (P = 0.024) than for males while on accession TVR the total number of probes (N Prb) was also higher for females (P = 0.028). Additionally, a general trend was noted that females probed more frequently than males on all accessions (Fig 1).

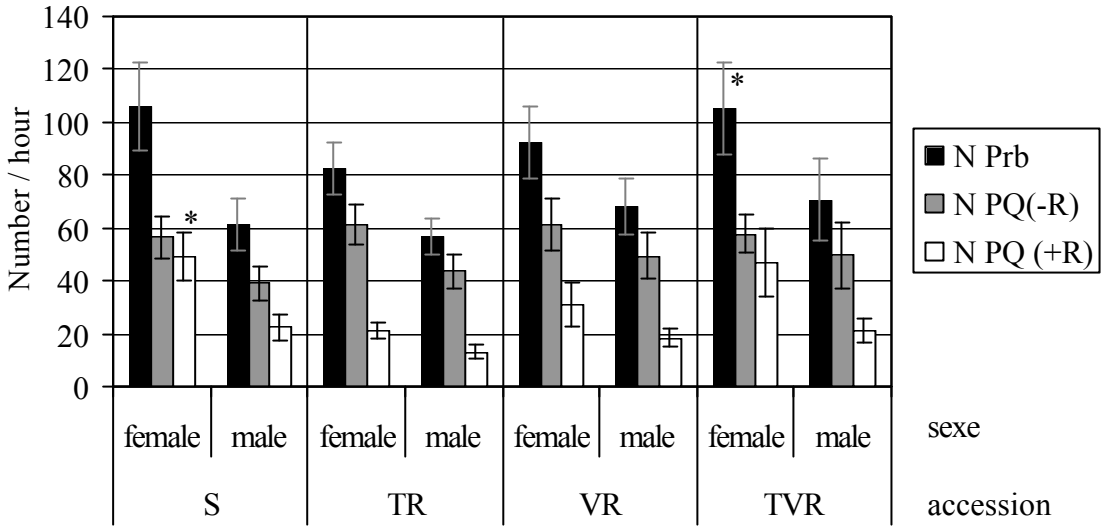

Fig. 1. Differences between sexes for numbers of different probes. N Prb = number of all probes, NPQ(-R) = number of probes without ingestion, and N PQ(+R) = number of probes with ingestion. The abbreviations of the different accessions are: S = thrips and TSWV susceptible, TR = thrips resistant and TSWV susceptible, VR = TSWV resistant and thrips susceptible, and TVR = thrips and TSWV resistant. * indicates significant differences between males and females (P < 0.05).

4. Discussion

Using detailed EPG analysis only small differences were recorded in the probing behaviour of adult females and males of *F. occidentalis* on the different pepper accessions tested. The results indicate that the thrips and virus susceptible accession (S) is indeed more susceptible than the thrips resistant accession (TR) in terms of the number of probes with ingestion and the total ingestion by probing thrips. Between VR and TVR, though, no significant difference was found. Also between these both accessions at one hand and S on the other, hardly any difference was scored. In contrast, Maris et al. (2003c) showed in a non-choice test that thrips reproduction was much better on accession S than on any of the other accessions tested here, indicating that the thrips resistant phenotype is based on other parameters than those which can be analysed during an one-hour EPG monitoring. In most parameters, the probing on TVR does not differ from that on the S and VR accessions (Table 3), and with respect to the

parameters Sum R and Avg R the double resistant TVR accession even displays a tendency to be more susceptible than accession VR.

Despite that the main effects of the thrips resistance traits have been related to decreased preference and impeded reproduction (Maris, 2004), several parameters may indicate that – notwithstanding the short duration of the experiments – there is also an additional effect of thrips resistance on probing behaviour, at least when comparing the TR and TVR accessions. This effect may be based on different mechanisms of thrips resistance in TR and TVR plants as suggested by the data recorded for several probing parameters. In TR plants, apparently the ingestion is affected more directly than in TVR plants, indicating a certain degree of deterrence, whereas on TVR plants probing differs only slightly and thrips performance is affected later (Maris et al., 2003c). Also the other genetic properties of the plant may influence the phenotypic resistance effects. For example, such properties may be the nutritional value, the presence of protease inhibitors of which is known that they affect oviposition but not the mortality (Annadana et al., 2002) or some other properties. Though the resistance in TR shows a direct effect on probing behaviour, it is questionable whether only this effect can explain the strong long-term impact on performance found by Maris (2004).

A potential reduction of TSWV transmission is suggested by our data for one of the four relevant parameters (Table 3) in the combined female and male data on the thrips resistant (TR) versus the thrips susceptible (S) accession, i.e. the number of probes with ingestion, N PQ(+R). The effect is apparently due to an effect on females since it does not hold for males (Table 4). No other effects of any of the resistant accessions was recorded for males and in general, there was a trend that females show more probes than males (Table 4 and Fig. 1). These data are in accordance with published results (van de Wetering et al., 1998; Joost, 2003) and suggest that females would transmit TSWV better than males.

Though our data may indicate a potential reduction on virus transmission, at least in the TR accession, the few differences remain small and somewhat unclear. This is congruent with the results of Maris et al. (2003c), reporting that only inoculation access periods of 16 h and longer resulted in reduced TSWV transmission efficiency in TR as compared to accession S. Whether the effects on probing found with EPG monitoring increases with time or are too small to act within a 16 h period remains unclear. Monitoring during longer periods seems no feasible option. The one-hour long observations here reported and analysed certainly do not indicate that vector resistance would enhance TSWV transmission. Therefore, the use of vector and virus resistance in combination seems in favour of a more sustainable crop protection in pepper.

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CHAPTER 6

Summarising discussion and concluding remarks

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Summarising discussion and concluding remarks

Thrips belong to the most damaging pest insects in agriculture and horticulture. In particular, the Western flower thrips (*Frankliniella occidentalis),* after its recent emergence since the 1980's, represents a worldwide pest in many ornamental and vegetable crops. The direct damage caused by thrips is mainly cosmetic while indirect damage is caused by their property to transmit *Tospoviruses* of which *Tomato spotted wilt virus* (TSWV) is the type species. Both types of damage are the result of the feeding of thrips on plants. Thrips can feed on different parts of a plant; leaves, flowers, stems, and fruits. On leaves, thrips feed from the epidermis, mesophyll and parenchyma. They can empty multiple cells per probe, but the mouthparts cannot reach the vascular tissue (Chapter 2). Feeding behaviour, referred to as probing, includes stylet penetration, salivation and ingestion, but ingestion does not occur in every probe.

In this thesis quantification and detailed observation of probing behaviour by *F. occidentalis* has been done using the electrical penetration graph (EPG) technique. Thrips' probing can be divided into several behavioural phases by different waveforms recorded with an EPG system. Two electrically different EPG systems exist, the DC- and the AC-system. The DC-EPG system has the advantage of overall clearer and more detailed signals and containing more biological information. In Chapter 3 the AC and the DC systems were simultaneously used, recorded from the same insect this has resulted in a more detailed characterisation of the probing behaviour of thrips. Six waveforms (P, Q, R, S, T and U) have been characterised, which represent different probing activities of thrips (Chapter 2 and 3). The activities during the previously described waveforms P, Q and R were confirmed in this study. Waveform P represents the insertion of the mandible in the leaf, waveform Q the insertion of the maxillary stylets and salivation into the plant, and waveform R represents the ingestion of the cell contents. The activities reflected by waveforms S and T are mandibular actions, the biological function remains a subject for further investigations (Chapter 2 and 3). Waveform U represents probably the retraction of stylets from the leaf (Chapter 3).

Transitions from one waveform to another are often unclear (Chapter 3). This can lead to rather ambiguous results of the duration measurements since the thrips' activities (waveform periods) are very short and occur in a fast sequence. Therefore, it is suggested in Chapter 3 to distinguish only two probing phases: 1) the 'puncturing' phase consisting of waveforms P, Q, and S, and 2) the 'feeding' phase consisting of the waveforms R, U, and T. This simplification leaves the separation of the most relevant probing activities intact, improves the reliability of analysis, and is time saving (Chapter 4 and 5 data) because thrips make relatively large numbers of probes, which all have to be analysed.

The indirect effect of thrips probing on plants is the transmission of TSWV. Since the salivary glands of the viruliferous thrips contain the virus, it must be transmitted during salivation. This study provides evidence that this occurs during waveform Q (Chapter 4). Also evidence is obtained, that the number of probes made during inoculation access periods is positively correlated with the probability of virus transmission, whereas sustained ingestion has a negative effect on this probability. Still the question remains how saliva excretion during Q contributes to the EPG waveform. The features of waveform Q are not very pronounced - except for the Q dip - and consistent (Chapter 2 and 3). This can presumably be explained by the fact that the saliva excretion does not only occur via the food canal (formed by the two maxillary stylets) but also follows a second 'extra-maxillary' route via the mouth cone (Chapter 2 and 4). This extra-maxillary route of the saliva may short-circuit the waveform caused by the maxillary canal passage of saliva and therewith, obscure the EPG signal.

Another important objective of this study was to investigate how the probing behaviour is affected by host plant resistance to thrips. To provide a more sustainable protection against TSWV, combining thrips and virus resistance in a crop can be very suitable. A prerequisite for an added value of thrips (vector) resistance though is that this trait should not induce behavioural changes resulting in enhanced transmission of TSWV. This could occur, for example, when thrips resistance would lead to higher probe frequencies and higher mobility of the insects, in search of a better feeding host. In this Ph D study no such adverse effects in probing behaviour were found on thrips resistant pepper accessions. In contrast, some EPG parameters showed a weak indication for a reverse effect: the thrips resistant (TR) accession CPRO-1 showed a potentially reduced probability of TSWV transmission, as compared to the susceptible (S) accession Pikante Reuzen (Chapter 5). It should be noted here that the one-hour duration of these experiments does not allow much extrapolation to field conditions.

The EPG recordings as presented in this thesis have elucidated many details of probing behaviour by thrips, especially, by combining this approach with other techniques such as video recording, microscopy, radioactive tracer assays and virus transmission experiments. On the other hand, the effect of wiring, i.e. the attachment of the insect to an electrode may have influenced the studied behaviour. Although such effects can be expected in probing on susceptible as well as on resistant plants, it is recommendable to establish such effects in future studies. Also, some remaining questions on thrips probing behaviour need further investigations. In conclusion, it can be stated that EPG recording is a too complicated and laborious technique to become a routine procedure in screening programs for thrips resistance, but the experimental data compiled in this thesis demonstrate that the technique is of great value to understand the probing behaviour of non-phloem feeding piercing-sucking insects as thrips.

Samenvatting

Deze samenvatting is bedoeld voor mensen die niet thuis zijn in de wereld van het wetenschappelijk onderzoek. Allereerst zal ik de insecten introduceren waar ik vier jaar aan heb gewerkt. Tripsen behoren tot de meest schadelijke insectensoorten in de landbouw. Ze veroorzaken direct en indirect schade door zich te voeden op vele verschillende gewassen op alle bovengrondse delen van planten. De directe schade is voornamelijk cosmetische schade, lelijke plekken, waardoor de marktprijs daalt. Indirecte schade wordt veroorzaakt door enkele tripsensoorten die plantenvirussen kunnen overdragen met als gevolg een verminderde opbrengst of een totale mislukking van de oogst. De soort virussen die ze overbrengen zijn *Tospovirussen*. De meest schadelijke tripsensoort is de Californische trips, *Frankliniella occidentalis*. Deze soort komt wereldwijd voor, vooral dankzij het vervoer van planten en vooral snijbloemen met vliegtuigen over de hele wereld.

Ik heb onderzoek uitgevoerd naar het voedingsgedrag van de Californische trips en de wijze van virusoverdracht, zodat we kunnen bepalen welke invloed resistentie tegen de Californische trips heeft op virusoverdracht. Waarom dit van belang is wordt later besproken.

Wat al bekend was over het voedingsgedrag van tripsen is dat ze op een blad van een plant eerst een gat prikken en daarmee een of meerdere cellen doorboren waarna ze de inhoud van die cellen opzuigen. Daarbij wordt ook speeksel in het blad gebracht waarin virusdeeltjes kunnen zitten die de plant kunnen infecteren. Om dit proces in detail te onderzoeken heb ik gebruik gemaakt van video-opnames en een techniek die gedrag elektronisch kan registeren, de elektropenetratie-gram (EPG) techniek.

De EPG techniek registreert het voedingsgedrag door de verschillen in voltage te meten. Deze verschillen ontstaan wanneer een trips die via een elektrode (draadje op de rug) deel uitmaakt van een elektrisch circuit begint te voeden (Fig. 1). De verschillen in gemeten voltages vormen in de tijd een grafiek waardoor verschillende fases tijdens het voeden herkend kunnen worden. In mijn onderzoek heb ik zes fases kunnen onderscheiden in het voedingsgedrag van tripsen. Van sommige fases heb ik de functie kunnen achterhalen en van andere is die nog niet bekend. Zo wordt eerst de mandibel, een soort naald, in de plant geprikt door middel van een knik met de kop van de trips. Daarna steekt de trips zijn maxillaire stiletten in het gat dat is gevormd door de mandibel. De maxillaire stiletten vormen samen een buis waardoor de celinhoud naar binnen kan worden gezogen. Ongeveer tegelijkertijd wordt speeksel in de plant gebracht. Daarna kan één cel of kunnen meerdere cellen worden

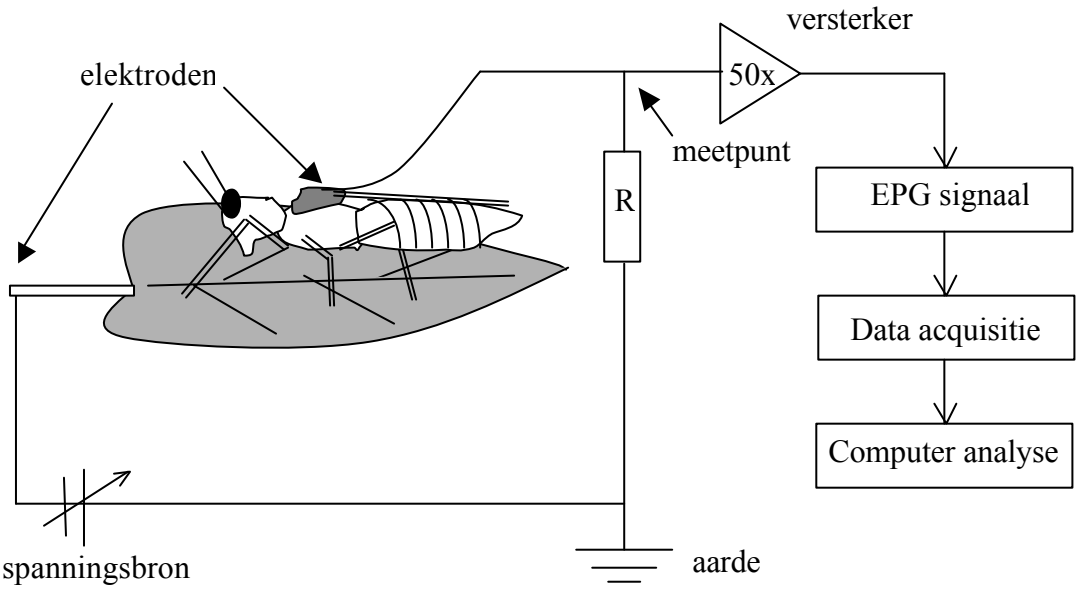

Fig. 1. Schematische weergave van de elektropenetratie gram (EPG) techniek waarbij een trips en een los blad in het elektrische circuit zijn opgenomen.

leeggezogen. Dit opzuigen is dus het echte voeden en de duur kan variëren van minder dan één seconde tot langer dan een uur. Dan zijn er nog twee fases waarin ook mandibel-activiteit plaatsvindt. De functie van deze activiteit is niet bekend. Aan het eind van een penetratie worden de maxillaire stiletten uit het gat getrokken.

Zoals eerder vermeld kunnen tripsen *Tospovirussen* overdragen. Tijdens dit onderzoek heb ik naar één soort virus gekeken en wel het tomatenbronsvlekken virus, dat in het Engels *'Tomato spotted wilt virus'* (TSWV) heet. Ik heb bestudeerd hoe de tripsen tijdens het voeden op een blad het virus overdragen. Zoals gezegd, wordt het virus via het speeksel overgedragen. Het vermoeden bestond al dat het in de tweede fase gebeurde in het voedingsgedrag en in dit onderzoek heb ik daar het bewijs voor geleverd. Een aantal factoren speelt een rol bij de al dan niet succesvolle overdracht van het virus door de tripsen. Ten eerste moeten de speekselklieren van de tripsen het virus bevatten. Ten tweede heeft het aantal penetraties dat een trips maakt in een plant een positieve invloed op de kans dat de plant wordt geïnfecteerd. Ten derde is het van belang hoe lang en hoeveel speeksel per penetratie in de plant wordt gebracht. Ten slotte is het van belang wat er na de speekseluitscheiding plaatsvindt. Het leegzuigen van de cellen kan er namelijk voor zorgen dat de cellen niet meer blijven leven waardoor het virus zich niet meer kan vermeerderen en de plant niet kan infecteren.

Een heel andere factor bij de virusoverdracht is de plant zelf. Planten kunnen namelijk virus-resistent en trips-resistent zijn. Wanneer de planten virus-resistent zijn, dan kunnen ze

niet geïnfecteerd worden met TSWV. Deze resistentie is direct gericht op het virus; de trips brengt dus wel TSWV over, maar de plant zorgt ervoor dat het virus zich niet kan verspreiden door de plant. Deze resistentie werkt in principe goed, maar kan worden doorbroken. Het virus past zich dan aan en kan de planten daardoor wel infecteren. Om de kans van de doorbreking van de virus-resistentie kleiner te maken zou eventueel ook een trips-resistentie kunnen worden gebruikt. Hiermee zou het aantal penetraties waarbij virus wordt overgedragen minder kunnen worden. Of dit inderdaad gebeurt, heb ik onderzocht door het voedingsgedrag van tripsen te vergelijken op trips-resistente en trips-vatbare paprika planten. Het bleek dat er niet veel verschil was in het voedingsgedrag van tripsen op resistente en vatbare paprikaplanten en dat waarschijnlijk daardoor ook de virusoverdracht niet drastisch wordt beïnvloed. Dit betekent dat het gebruik van trips-resistentie niet *direct* leidt tot een verminderde virusoverdracht, maar wel *indirect* omdat op de trips-resistente paprikaplanten niet zoveel tripsen voorkomen en er dus minder TSWV wordt overgedragen. Dus kan de combinatie van trips en virus resistentie inderdaad leiden tot een duurzamere bescherming van paprikaplanten tegen schade veroorzaakt door tripsen en TSWV.

Door mijn onderzoek is nu meer bekend over hoe tripsen zich voeden en weten we hoe en wanneer TSWV wordt overgedragen. Dit laatste vind ik ook het belangrijkste resultaat, want tot nu toe werd er alleen over gespeculeerd, terwijl virusoverdracht in belangrijke mate bijdraagt aan de pest status van de Californische trips.

Cirruculum Vitae

Ik ben op 19 maart 1976 geboren in Alphen aan de Rijn na de lagere school, toen heette dat nog zo, ben ik naar de middelbare school, ALBANIANAE, gegaan en heb daar in 1994 het VWO afgerond. Aansluitend ben ik Biologie gaan studeren in Utrecht, iets wat ik als kind altijd al wilde. In eerste instantie had ik geen idee welke richting van de biologie ik het leukst vond, maar gaandeweg bleek dat ik de gewasbescherming wel erg interessant en leuk vond, daarom heb ik ook twee onderzoeksstages in die richting gedaan. Bij de projectgroep Fytopathologie van de Universiteit Utrecht heb ik mijn eerste onderzoeksstage van 9 maanden gedaan. Ik heb daar onderzoek gedaan aan verschillende bacterie stammen van *Pseudomonas putida* die voorkomen in de bodem en die radijsplantjes helpen tegen een bodemschimmel die verwelkingsziekte veroorzaakt, *Fusarium oxysporum*. Daarna heb ik een onderzoeksstage van 6 maanden gedaan op het voormalige IPO-DLO in Wageningen. Tijdens deze stage heb ik voor het eerst kennis gemaakt met tripsen, want hier heb ik onderzocht of een tripsensoort, *Frankliniella occidentalis*, met een plantensoort, *Arabidopsis thaliana*, gebruikt konden worden als modelsysteem voor wetenschappelijk onderzoek. Uiteindelijk ben ik in november 1999 afgestudeerd als bioloog, terwijl ik al in oktober 1999 was begonnen met mijn promotie onderzoek op het laboratorium voor Entomologie. Het uiteindelijke resultaat van mijn promotie onderzoek heb je nu in je handen.

List of publications

Kindt, F., Joosten, N.N., Peters, D., Tjallingii, W.F., 2003. Characterisation of the feeding behaviour of the western flower thrips in terms of electrical penetration graph (EPG) waveforms. Journal of Insect Physiology 49, 183-191.

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